Mild Equine Asthma: Effects of Commonly Used Treatments on the Respiratory Microbiota, Inflammatory Gene Expression, and Aerobic Performance during High-Intensity Exercise

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doctoral thesis

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Mild Equine Asthma: Effects of Commonly Used Treatments on the Respiratory Microbiota, Inflammatory Gene Expression, and Aerobic Performance during High-Intensity Exercise

by

Stephanie Laura Bond

A THESIS

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Abstract

Mild equine asthma (MEA) is an inflammatory airway disease of the horse which affects a large proportion of the equine population. The pathogenesis of MEA is not fully elucidated, however, it is widely assumed to be a multifactorial disease, with expression of clinical signs largely influenced by environment. There is very little information available on the effects of treatment, which is typically focused on reducing airway inflammation through corticosteroid administration. Investigations into the equine respiratory environment in states of health and MEA were performed using: i) High-throughput sequencing techniques (16S and ITS2) to report the respiratory microbiota and mycobiota respectively, and ii) changes in relative inflammatory mRNA cytokine expression in bronchoalveolar lavage fluid (BALF). Changes in expression of inflammatory cytokine mRNA, equine herpesvirus (EHV)-1,2,4,5 glycoprotein B gene expression and changes in respiratory bacterial and fungal communities following dexamethasone treatment of healthy horses and those with MEA are explored. A portable equine ergospirometry system was used to determine the efficacy of treatment designed to reduce lung inflammation on aerobic (\( \dot{V}O_2 \text{peak} \)) and anaerobic performance in horses with MEA.

There were clear differences between the lower respiratory tract environment in healthy horses versus MEA. There was a clear separation in both the microbiota - *Streptococcus* was increased in horses with MEA – and relative inflammatory cytokine expression. Horses with MEA had a lower concentration of IL-10 in BALF than healthy controls, consistent with human asthmatics. There was significant up-regulation of IL-17 in horses with MEA, suggesting these horses exhibit “allergic” airway inflammation in response to environmental antigens. The single most important factor in the prevention and treatment of MEA appears to be environmental improvement, manifest by reduced inhaled particulate matter.

Treatment with injected dexamethasone in horses with MEA was associated with down-regulation of IL-5, indicating a shift away from a dysregulated Th-2 response. Treatment with dexamethasone significantly affected the microbiota diversity, but not the mycobiota, which was overwhelmed by the effect of a sustained dusty environment. There is strong evidence that without environmental modifications, corticosteroid therapy alone fails to normalize airway neutrophilia. Treatment significantly increased \( \dot{V}O_2 \text{peak} \) by an average 11.7% (saline) to 14.6% (dexamethasone).
Preface

Chapter 1. Portions of the introduction are used with permission from: Bond, S; Léguillette, R; Richard, E; et al. (2018). “Equine asthma” – integrative biologic relevance of a recently proposed nomenclature. *Journal of Veterinary Internal Medicine, 32*(6): 2088-2098.

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Letters of permission signed by co-authors are provided in Appendix D.
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Dedication

For Mel, who was one in a lifetime
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List of Abbreviations

MEA – Mild Equine Asthma

IAD – Inflammatory Airway Disease

RAO – Recurrent Airway Obstruction

BAL – Bronchoalveolar Lavage

BALF – Bronchoalveolar Lavage Fluid

IL – Interleukin

Ig - Immunoglobulin

IL4Rα - IL-4 receptor α-chain

TNF – Tumor Necrosis Factor

IFN – Interferon

NF-κB - Nuclear Factor-κB

AP-1 - Activator Protein-1

ASM - Airway Smooth Muscle

ECM - Extracellular Matrix

CXCL2 - Chemokine (C-X-C motif) Ligand 2

LT – Leukotriene

REST – relative expression software tool

NMDS – Nonmetric Multidimensional Scaling

OTU – Operational Taxonomic Unit

TTW – Transtracheal Wash
NPS – Nasopharyngeal Swab
URT – Upper Respiratory Tract
LRT – Lower Respiratory Tract
MAOD - Maximum Accumulated Oxygen Deficit
HR – Heart Rate
\( V_{200} \) – Velocity at which heart rate reaches 200bpm
\( \dot{V}O_{2\text{max}} \) – Maximal oxygen consumption; aerobic capacity
\( \dot{V}O_{2\text{peak}} \) – Peak oxygen consumption
\( VLa_4 \) – Velocity at which blood lactate reaches 4mmol/L
\( VLa_{10} \) – Velocity at which blood lactate reaches 10mmol/L
AHR – Airway Hyperresponsiveness
Epigraph

Believe in yourself and in your dream

Though impossible things may seem

Someday, somehow you’ll get through

To the goal you have in view

Mountains fall and seas divide

Before the one who in his stride

Takes a hard road day by day

Sweeping obstacles away

Believe in yourself and in your plan

Say not – I cannot – but, I can

The prizes of life we fail to win

When we doubt the power within

Author Unknown
Chapter 1 - Introduction

Imagination is more important than knowledge. For knowledge is limited, whereas imagination embraces the entire world, stimulating progress, giving birth to evolution. It is, strictly speaking, a real factor in scientific research.

Albert Einstein, Cosmic Religion and Other Opinions and Aphorisms

Rationale

Non-infectious inflammatory airway disease in the horse consists of mild equine asthma (inflammatory airway disease), severe equine asthma (recurrent airway obstruction) and summer pasture-associated recurrent airway obstruction (Couetil, Cardwell et al., 2016). Reports suggest that mild equine asthma affects up to 66% (Wasko, Barkema et al., 2011) of the equine population, however, the domestication of the horse has resulted in exposure to environments such as stables and overgrazed paddocks where exposure to dust and other respirable antigens are increased (Holcombe, Jackson et al., 2001). Depending on environmental control measures employed, it is possible that up to 100% of horses that live in dusty environments have some degree of airway inflammation. By imposing these environmental conditions, it is likely that we are artificially increasing the prevalence of both equine and human asthma; studies investigating biomarkers involved in the development of airway disease have shown that some employees involved in the care and training of horses have signs of bronchial obstruction, which might be provoked by working in a stable environment (Elfman, Riihimäki et al., 2009). Furthermore, individuals exposed to stable environments have a significantly higher prevalence of self-reported respiratory symptoms than a non-exposed group (Mazan, Svatek et al., 2009).

The term “equine asthma” has been proposed to replace inflammatory airway disease (IAD), recurrent airway obstruction (RAO), and summer pasture-associated obstructive airway disease (Lavoie, 2015; Couetil, Cardwell et al., 2016; Lavoie, 2017). Whilst the term will increase comprehensibility for both
the lay and scientific communities, its biological relevance must be compared and contrasted to its current utilization in human medicine. While it is of interest to examine the similarities and differences between comparable equine and human conditions, it is important to recognize the limited availability of peer-reviewed equine-derived data, which are largely restricted to clinical signs, measures of airway obstruction and inflammation and response to therapy. Such limitations have an inevitable impact on the extent to which meaningful comparisons with human asthma phenotypes can be made.

The majority of research regarding non-infectious inflammatory airway disease in the horse has been limited to severe equine asthma. While mild and severe equine asthma are considered as separate diseases, it is presently unclear whether this distinction reflects a dissimilar pathogenesis, or simply a difference in the clinical severity (Couetil, Cardwell et al., 2016). Currently further differentiation of the term “equine asthma” is limited to clinical severity (Lavoie, 2017); it is hoped that future subclassification efforts might consider additional criteria such as pathogenetic pathways and immunological characteristics. There is evidence that Th-1, Th-2 and Th-17 immune responses are involved in the pathogenesis of mild and severe equine asthma (Hughes, Nicolson et al., 2011; Lavoie, Cesarini et al., 2011; Beekman, Tohver et al., 2012; Richard, Depecker et al., 2014). Evidence of a Th-1 response in the lower respiratory tract, characterized by upregulation of IFN-γ mRNA in bronchoalveolar lavage fluid (BALF)-derived cells, has repeatedly been reported in association with a generalized increase in BAL inflammatory cells, both in the presence and absence of clinical signs (Hughes, Nicolson et al., 2011; Lavoie, Cesarini et al., 2011; Richard, Depecker et al., 2014). A Th-2 cytokine signature has been detected in BAL cells derived from mastocytic forms of mild equine asthma, characterized by increased expression of IL-4 and IL-5 mRNA (Lavoie, Cesarini et al., 2011; Beekman, Tohver et al., 2012). Additionally, a Th-17 response has been implicated in neutrophilic mild equine asthma, with an association between the BALF neutrophil ratio and increased IL-17 and IL-23 mRNA expression (Hughes, Nicolson et al., 2011; Beekman, Tohver et al., 2012). Further clarification of the cytokine responses in the lower respiratory tract of horses with chronic airway inflammation would facilitate a greater understanding of the possible etiopathological pathways involved in mild equine asthma; this is explored in Chapters 3 and 4.
There is evidence in humans which suggests that lower airway bacterial communities play a role in the pathogenesis of asthma (Huang, Nelson et al., 2011), and that viral respiratory tract infections have a profound effect on the expression of asthma, as well as disease exacerbation (Busse, Lemanske Jr et al., 2010). The involvement of infectious agents in the pathogenesis of mild equine asthma is suspected; there is increasing evidence emerging within the peer-reviewed literature which supports a relationship between bacterial, fungal and viral infections and mild equine asthma (Burrell, Wood et al., 1996b; Christley, Hodgson et al., 2001b; Wood, Newton et al., 2005b; Dauvillier, ter Woort et al., 2018). An increase in tracheal mucus has been associated with isolation of bacteria (particularly *Streptococcus equi var. zooepidemicus* and *Actinobacillus/Pasturella* species) from tracheal washes in racehorses, both in the presence and absence of clinical signs (Christley, Hodgson et al., 2001a; Wood, Newton et al., 2005a; Wood, Newton et al., 2005b; Cardwell, Smith et al., 2014). Furthermore, a recent, culture-based study investigated the prevalence of fungi in respiratory samples of horses diagnosed with mild equine asthma, and assessed risk factors associated with the presence of fungi in the airways (Dauvillier, ter Woort et al., 2018), and found that horses with fungi present in tracheal wash cytology are 2 times more likely to have mild equine asthma than those without (Dauvillier, ter Woort et al., 2018). Further investigation of the relationship between infectious agents, lower respiratory tract microbiota and the development of mild equine asthma is warranted, and has been identified as a priority for future research (Couetil, Cardwell et al., 2016); this is explored in Chapters 2 and 4.

Whilst a deeper understanding of the pathogenesis of mild equine asthma is undoubtedly beneficial from both clinical and research perspectives, and can help in the application of more selective and effective treatments, perhaps of greater interest to the wider equine community are the functional implications of lower airway inflammation: how much does it affect performance, and how effective is treatment? It has recently been shown that mild equine asthma decreases racing performance in a population of Thoroughbred racehorses (Ivester K, 2017). Furthermore, in studies performed in a controlled environment on a high-speed treadmill, gas exchange is impaired after exercise in horses with mild equine asthma (Couetil and Denicola, 1999; Courouce-Malblanc, Pronost et al., 2002;
Sanchez, Couetil et al., 2005). Therefore, given that one of the presenting complaints of mild equine asthma is poor performance (Couetil, Cardwell et al., 2016), and that horses are typically treated empirically, with very little evidence regarding the corrective efficacy of treatment on respiratory performance, statistical evidence would be invaluable; this concept is explored in Chapter 6. The concept of “performance” is explored in the introductory chapter; Chapter 5 describes a study which was performed to determine a viable means to determine aerobic and anaerobic energy contributions in the field. Appendix A describes a study which, while not central to the topic of this thesis, was performed to determine the sensitivity of the ergospirometry mask (i.e. is it able to detect a change in aerobic capacity in response to training?), which would indicate whether the equipment was suitable for addressing the hypothesis central to this thesis: treatment targeting lung inflammation improves aerobic performance in horses with mild equine asthma.

**History of Nomenclature: Equine Asthma**

Numerous terms have been used to describe chronic inflammatory lower airway disease in the horse, including heaves, RAO, equine chronic obstructive pulmonary disease, IAD, tracheal IAD, bronchial IAD, small airway disease, chronic bronchitis, summer pasture associated chronic obstructive pulmonary disease, summer pasture-associated obstructive pulmonary disease, summer pasture-associated obstructive airway disease, summer heaves and summer RAO. Progressive awareness of various clinical and pathological features of equine inflammatory lower airway disease precipitated the evolution of the above nomenclature; however, this has become unsustainable, resulting in confusion within both the veterinary and lay communities. It has recently been proposed that chronic non-infectious inflammatory lower airway disease in the horse be re-assigned the designation “equine asthma” (Lavoie, 2015; Couetil, Cardwell et al., 2016; Lavoie, 2017). As highlighted during the 6th World Equine Airway Symposium (2017), the biological appropriateness of applying the term “equine asthma” must be considered in light of its current utilization in human medicine prior to its widespread adoption in the veterinary literature (Pirie, 2017). Increasing comprehensibility amongst the horse-
owning public and the veterinary profession would constitute a clear benefit of the newly proposed terminology; however, the validity and limitations of the proposed change in nomenclature must first be considered and described (Bond, Léguillette et al., 2018). Prior to the proposed utilization of the term “equine asthma”, RAO/Heaves and IAD have been widely used and accepted due to their accurate descriptions of the two disease processes to which they refer. While a distinction between these two phenotypes was initially proposed for research purposes to facilitate comparison between study results (Robinson, 2001; Robinson, 2003), it was not the intent of the workshop participants to suggest that they were two separate conditions. However, different names lead clinicians to subsequently consider them to be distinct and both have individually been the subject of expert panels’ workshops (Robinson, 2003; Marti, Gerber et al., 2008; Richard and Robinson, 2016) and publications (Robinson, 2001; Couëtil, Hoffman et al., 2007; Couëtil, Cardwell et al., 2016). In contrast to, and distinct from IAD, horses with RAO exhibit increased respiratory effort at rest (Robinson, 2001). This distinguishing feature is attributable to the magnitude of bronchoconstriction, increased mucus production and bronchiolar inflammation associated with this disorder (Robinson, Berney et al., 2003; Couëtil, Hoffman et al., 2007). While IAD and RAO are considered as separate diseases, it is presently unclear whether this distinction reflects a dissimilar pathogenesis, or simply a difference in the clinical severity.

There are many factors which potentially differ amongst the spectrum of diseases which fall within the proposed new “equine asthma” classification, including severity of clinical signs, pathogenetic pathways and rates of recurrence. Therefore, further differentiation of the term into mild, (moderate) and severe equine asthma has been advocated (Lavoie, 2017). Although application of these qualifying terms is currently limited to clinical severity, with mild/moderate and severe equine asthma being analogous to IAD and RAO respectively (Lavoie, 2017), it is hoped that future sub-classification efforts might consider additional criteria such as pathogenetic pathways and immunological characteristics.

The aims of this introduction are to: i) propose minimum inclusion criteria supporting utilization of the term “equine asthma”, ii) compare and contrast features of equine asthma with the most common human asthma phenotypes, iii) propose typical features for sub-categories of equine asthma, and iv) provide context for the research chapters (2-6). Upon consideration of the shared factors between human asthma, IAD and RAO outlined in this introduction, we conclude that adoption of the term equine asthma is...
appropriate, whilst acknowledging that important significant heterogeneity exists within this broad disease category (Bond, Léguillette et al., 2018). We therefore support the proposal that the term mild/moderate equine asthma replace IAD and severe equine asthma replace RAO in the literature, from this point onwards, whilst recognizing the need to preserve the spectrum of diseases which fall within the proposed new “equine asthma” classification (Bond, Léguillette et al., 2018). From this point onwards in the thesis, IAD will be referred to as mild equine asthma (MEA), with the exception of Chapter 2, which was accepted prior to the adoption of this terminology.

**Inclusion criteria: Equine Asthma**

The biological appropriateness of the term “equine asthma” must be considered relative to its current utilization in human medicine. It is important to consider both a minimum set of criteria shared by all human and equine asthma phenotypes, as well as additional criteria shared between specific human and equine asthma phenotypes (Pirie, 2017).

**Minimum inclusion criteria for application of the term “asthma”: Equine and Human**

Asthma in humans is a heterogeneous disease characterized by non-septic chronic airway inflammation (GINA, 2017). Patients have a history of respiratory symptoms (coughing, wheezing, shortness of breath and tightness of the chest) which vary in intensity and over time, combined with airway hyperresponsiveness and expiratory airflow limitation of fluctuating severity (GINA, 2017). The pathology accompanying this phenotype includes bronchoconstriction, airway wall thickening, increased mucus secretion and airway remodeling (GINA, 2017). With the exception of shortness of breath and chest tightness, which, as subjective descriptors of a perceived sensation, are not feasibly applicable to the horse, this phenotype is largely shared by both mild and severe equine asthma. Horses with severe equine asthma exhibit the same pathologic features as human asthma; namely bronchoconstriction, airway wall thickening, increased mucus production and airway remodeling (Gerber, Lindberg et al., 2004; Bullone, 2017). This pathology is associated with the increased
respiratory effort observed at rest in horses with severe equine asthma (Robinson, Olszewski et al., 2000; Robinson, 2001). Horses with mild equine asthma exhibit inflammation of the trachea and bronchi, with an excessive accumulation of mucus in the airways (Cardwell, Wood et al., 2011; Koblinger, Nicol et al., 2011), resulting in a mild increased resistance to airflow during exercise (Couetil, Rosenthal et al., 2001; Bedenice, Mazan et al., 2008a; Richard, Fortier et al., 2009a). It has been recently shown that mild equine asthma decreases racing performance in a population of Thoroughbred racehorses (Ivester K, 2017). The pathology exhibited by horses with mild asthma typically manifests in clinical signs that are subtle at rest, with horses exhibiting chronic (>3 weeks) occasional coughing and normal respiratory effort (Couetil, Cardwell et al., 2016); at work, coughing, increased nasal discharge and poor performance are observed (Couetil, Cardwell et al., 2016).

Objectively, impaired pulmonary gas exchange limits performance, and several studies performed on high-speed treadmills have reported that intensely exercising horses with mild asthma exhibit worsening of exercise-induced hypoxemia (Couetil and Denicola, 1999; Sanchez, Couetil et al., 2005; Courouce-Malblanc, Deniau et al., 2010). However, the bronchoconstriction in horses with mild asthma is sufficiently mild to evade clinical detection via the appreciation of increased respiratory effort at rest without bronchoprovocation. Airway remodeling – and it’s reversibility in response to treatment with corticosteroids, antigen avoidance and bronchodilator therapy - has been well described in severe equine asthma (Leclere, Lavoie-Lamoureux et al., 2011; Leclere, Lavoie-Lamoureux et al., 2012; Bullone, 2017; Bullone, Vargas et al., 2017). Whilst airway remodeling has not yet been studied in horses with mild equine asthma, a recently published study investigating histopathologic changes in the small airways of actively racing horses – a subset of the equine population with very high incidence of mild asthma - reported that peribronchiolar infiltration of inflammatory cells (82/95 horses) and bronchiolar smooth muscle hyperplasia (93/95 horses) are common (ter Woort, Caswell et al., 2018). Although eosinophils or mast cells (or both) were present in the bronchiolar wall of some horses, it was not possible to determine if these findings would have corresponded to a clinical diagnosis of mild equine asthma (ter Woort, Caswell et al., 2018). Notably absent from this list of minimum inclusion criteria is the predominant airway inflammatory cell; this notable omission is discussed below under “Phenotypes”.

8
**Additional inclusion criteria between specific human and equine asthma phenotypes**

Any efforts to advocate equine asthma as an appropriate disease model for the study of human asthma must take into consideration the fact that multiple human asthma phenotypes exist, not all of which will share attributes with mild and severe equine asthma. Similar considerations also relate to the translational application of human asthma derived scientific findings to the horse, and *vice versa*. Therefore, the appropriateness of any such cross-species comparisons necessitates the application of additional criteria which specifically distinguish certain human asthma and mild/severe equine asthma phenotypes based on disease-specific key features. It has previously been proposed that severe equine asthma is an ideal equine model for the study of non-allergic, late-onset and severe asthma phenotypes (Bullone and Lavoie, 2015), however, the biologic appropriateness of mild equine asthma for the study of specific human asthma phenotypes has not yet been investigated and is a focus of this introduction.

**Phenotype vs Severity**

An ‘asthma phenotype’ is a recognizable cluster of demographic, clinical and/or pathophysiological characteristics (Bel, 2004; Moore, Meyers et al., 2010; Wenzel, 2012); however, these do not always have a strong correlation with specific pathologic processes, or even treatment responses (GINA, 2017). In humans, various asthma management guidelines have described methods to categorize asthma severity, however, there are substantial theoretical and practical differences between recommendations (Colice, 2004). Asthma severity is differentiated into mild, moderate and severe categories (GINA, 2017) and is predominantly based upon the level of treatment required to control symptoms and exacerbation; it is not a static feature of the disease and changes over time. In some instances, it is also used to describe the intensity of symptoms or the magnitude of airflow limitation. However, these approaches do not focus on quantifying markers of airway inflammation, which would assess the severity of the disease process itself. For practical reasons asthma is only classified following institution
of effective treatment and therefore assessment is always subject to treatment effect. To date no treatment-naive predictors of disease severity have been reported.

It has been proposed that mild/moderate equine asthma replace IAD, and severe equine asthma replace RAO (Couëtil, Cardwell et al., 2016; Lavoie, 2017). Certain criteria have recently been proposed for the sub-categorization of equine asthma based on severity. Specific cut-off values or recommendations were proposed for the following methods: clinical presentation, airway endoscopy, airway cytology and pulmonary function tests (Robinson, 2001; Gerber, 2017). However, the objective applicability of these criteria to mild and severe equine asthma subcategorization remains somewhat arbitrary. A meta-analysis of published studies based on client-owned horses with mild equine asthma and severe equine asthma would likely offer valuable information on the relative contributions of each of the above criteria to the overall equine asthma sub-categorization exercise. Moreover, a poor correlation exists between specific pathology (i.e. severe inflammatory bronchoalveolar lavage profile) and clinical signs (i.e. increased respiratory effort at rest). Although the inclusion of severity of clinical signs as a key criterion in the sub-categorization of equine asthma is easy to comprehend (particularly amongst the horse owning public), it should not be applied exclusively; particularly in light of the inconsistent correlation between severity of airway inflammation and clinical signs in both human (GINA, 2017) and equine asthma (Lapointe, Lavoie et al., 1993; Leguillette, Desevaux et al., 2002; Couëtil, Chilcoat et al., 2005). Despite the challenges facing any effort to further sub-categorize equine asthma, such an exercise can clearly be justified by its potential to reveal more specific therapeutic and/or prophylactic targets.

Clinical presentation of mild equine asthma

Mild equine asthma can affect horses of all ages, although it is more commonly reported in young to middle age horses. This may partly be due to a study bias; the disease has been identified as the second most common cause of veterinary care in young racing Thoroughbreds (Wilsher, Allen et al., 2006), which provides significant economic incentive to study this subset of the equine population. Horses with mild equine asthma exhibit inflammation of the trachea and bronchi, with an excessive accumulation of mucus in the airways (Cardwell, Wood et al. 2011, Koblinger, Nicol et al. 2011),
resulting in a mild increased resistance to airflow (Couëtil, Rosenthal et al. 2001, Bedenice, Mazan et al. 2008, Richard, Fortier et al. 2009). This typically manifests in clinical signs that are subtle at rest, with horses exhibiting chronic (>3 weeks) occasional coughing and normal respiratory effort (Couëtil, Cardwell et al. 2016); at work, coughing, increased nasal discharge and poor performance are observed (Couëtil, Cardwell et al. 2016). If poor performance is the only complaint, other causes must be ruled out (e.g. upper airway obstructive disorder, viral infection, cardiac disease, musculoskeletal disease) (Couëtil, Cardwell et al. 2016). Poor performance is typically very subjective, and is based on the experience of the rider or trainer of a horse. Objectively, impaired pulmonary gas exchange limits performance, and several studies performed on high-speed treadmills have reported that intensely exercising horses with mild equine asthma exhibit worsening of exercise-induced hypoxemia (Couëtil and Denicola 1999, Sanchez, Couëtil et al. 2005, Courouce-Malblanc, Deniau et al. 2010). However, there is no data available that quantifies the impact that mild equine asthma has on respiratory performance in the field; this has been acknowledged as a research priority, essential to improving our understanding of mild equine asthma (Couëtil, Cardwell et al. 2016); this is studied in Chapter 6. As clinical signs of coughing, excess tracheal mucus and poor performance are not very specific, diagnosis must be confirmed.

**Diagnosis**

International guidelines emphasize the diagnostic importance of spirometry in the diagnosis of asthma in human patients with respiratory symptoms (GINA, 2017). A presumptive diagnosis can initially be based on a detailed clinical history, physical examination (which may be normal at the time of presentation) and confirmed through spirometry. Diagnostic tests include the forced expiratory volume in 1 s (FEV₁) and mean forced expiratory flow at 25% to 75% of the forced vital capacity (FEF₂₅₋₇₅); the latter is regarded as a more sensitive measure of small airway obstruction (Chung, Wenzel et al., 2014).Whilst radiography and context-specific screening questionnaires (Thiadens, De Bock et al., 1998; Tinkelman, Price et al., 2006) can be of value in positively screening for high risk chronic airway
disease patients, they are seldom weighted in usual clinical diagnosis. That airflow tests are central to definitive diagnosis is especially pertinent considering the shared features common to both asthma and chronic obstructive pulmonary disease. Likewise, a presumptive diagnosis of mild or severe equine asthma is generally based on patient history and clinical presentation, the latter of which has been incorporated into both the independently validated risk-screening questionnaire (RSQ) and horse owner assessed respiratory signs index (HOARSI) (Hotchkiss, Reid et al., 2006; Ramseyer, Gaillard et al., 2007). Whilst these clinical-sign based screening tools have both excellent sensitivity and negative predictive values for detecting severe lower airway inflammation (severe equine asthma), they fail to differentiate between healthy horses and those with mild airway inflammation (mild equine asthma) (Laumen, Doherr et al., 2010; Wasko, Barkema et al., 2011; Rettmer, Hoffman et al., 2015). Furthermore, in light of the poor diagnostic sensitivity of coughing, mucoid nasal discharge and poor performance, reliance is placed on additional tests, such as tracheal endoscopy, bronchoalveolar lavage (BAL) cytology and lung function evaluation (Couetil, Cardwell et al., 2016), in an attempt to maximize diagnostic accuracy of both severe and mild equine asthma.

**Airway endoscopy**

Airway endoscopy can be used to determine the presence of tracheobronchial mucus. A scoring system for tracheal mucus has been validated, where Grade 0 = no visible mucus, Grade 1 = single/multiple small blobs of mucus, Grade 2 = larger but nonconfluent blobs, Grade 3 = streams of mucus, Grade 4 = pool forming mucus, and Grade 5 = profuse amounts of mucus (Gerber, Lindberg et al. 2004). Scores ≥2/5 (racehorses) and ≥3/5 (sports/pleasure horses) have been previously associated with coughing in racehorses in training and older pleasure horses (Christley, Hodgson et al. 2001, Cardwell, Smith et al. 2014), and with poor performance in racehorses (≥2/5) (Holcombe, Robinson et al. 2006), sports horses and dressage horses (≥3/5) (Widmer, Doherr et al. 2009). Tracheobronchial mucus score is significantly correlated with neutrophilia in bronchoalveolar lavage fluid (Gerber, Straub et al., 2004; Wasko, Barkema et al., 2011). While the consensus statement (Couëtil, Cardwell et al. 2016) only requires documentation of lower airway inflammation through excess tracheobronchial mucus to meet the
proposed definition of mild equine asthma, it would be inconsistent with a chronic inflammatory process without abnormal bronchoalveolar lavage fluid (BALF) cytology.

**Bronchoalveolar lavage**

Mild equine asthma is characterized by a mild to moderate increase in neutrophils (>5%), eosinophils (>1%), and/or metachromatic (>2%) cells (Couëtil, Cardwell et al. 2016). However, several studies have documented BALF mast cell percentages >2% and <5% in clinically healthy horses (Sweeney, Rossier et al. 1992, Sweeney, Rossier et al. 1994, Gerber, Robinson et al. 2003), and it is not uncommon to find BALF neutrophil percentages >5% and <10% in asymptomatic horses (Couetil, 2014). Variation in inflammatory cell percentages in BALF between studies could be due to differences in sampling technique, with the consensus publication recommending that a minimum of 250ml of saline be instilled (Couëtil, Cardwell et al. 2016). Variation could also be due to differences in environment, climate, inter-horse variability, lung-side sampled, staining method (for the detection of mast cells), and differential counting method used (Leclere, Desnoyers et al., 2006; Fernandez, Hecker et al., 2013; Depecker, Richard et al., 2014).

**Tracheal wash cytology**

Historically, research investigating airway inflammation in racehorses focussed on tracheal wash cytology (Wood, Burrell et al., 1993a; Burrell, Wood et al., 1996a; Chapman, Green et al., 2000). This is because in the UK, it can be difficult to get permission to perform a BAL on racehorses. Whilst tracheal wash neutrophilia has been associated with coughing (Christley, Hodgson et al., 2001a); there is a lack of association between tracheal cytology and poor performance (Holcombe, Robinson et al., 2006). Furthermore, the correlation between tracheal wash and BALF cytology is weak, possibly because the trachea is more susceptible to the influence of the external environment than the terminal equine airways (Derksen, Brown et al., 1989; Malikides, Hughes et al., 2003; Fraipont, Van Erck et al., 2011). For these reasons tracheal wash cytology is not considered an appropriate alternative to BALF cytology for diagnostic confirmation of mild equine asthma (Couetil, Cardwell et al., 2016).
**Lung function evaluation**

Unlike the cardiac and musculoskeletal systems, the respiratory system does not adapt to training (Art and Lekeux, 1993; Roberts, Marlin et al., 1999). Therefore, the ability of the respiratory system to compensate for any minor disease inducing a decrease in capacity, such as mild equine asthma, is limited, particularly during high intensity exercise where obligatory locomotory-respiratory coupling imposes a mechanical constraint on respiration (Lafortuna, Reinach et al., 1996). Mild equine asthma has been shown to decrease racing performance in a population of Thoroughbred racehorses (Ivester K, 2017). While it is normal for racehorses experience arterial hypoxemia and hypercapnia at very high exercise intensities (discussed further below, under Gas exchange) (Bayly, Hodgson et al., 1989), exercise-induced hypoxemia and hypercapnia is exacerbated in horses with mild equine asthma (Couetil and Denicola, 1999; Courouce-Malblanc, Pronost et al., 2002; Sanchez, Couetil et al., 2005). Lung function tests can be useful in determining the functional significance of respiratory signs, providing an objective means of detecting disease before more obvious clinical signs develop (Hoffman, Mazan et al., 1998). Conventional lung mechanics which integrate pleural pressure measurements and flow to calculate lung resistance lack the sensitivity to detect the low level of airway function impairment present in mild equine asthma. Therefore, for increased diagnostic sensitivity, a variety of different techniques and technologies are used, including forced oscillometry, flowmetric plethysmography, forced expiratory maneuvers and bronchoprovocative challenges (see below). Whilst standard lung mechanics measured at rest are usually within reference values in racehorses with mild equine asthma (Couetil, Rosenthal et al., 2001), changes consistent with airway obstruction can be found through the use of a rebreathing technique (Pirrone, Albertini et al., 2007; Haltmayer, Reiser et al., 2013), or forced expiration (Couetil, Rosenthal et al., 2001). Currently, there is no “standard” way to measure equine lung mechanics, as no single lung function test meets all the needs of the clinician. A clinician requires a test to be: i) sensitive, ii) repeatable, iii) precise (when compared to a reference technique), iv) able to localize disease (i.e. upper versus lower airways, central versus peripheral), v) able to detect and quantify lung dysfunction, vi) able to analyze the breathing pattern, and vii) portable for use in the field (Hoffman, 2002). Historically, lung function tests are typically only used in a research context, although
A comprehensive review of the application of available techniques in clinical patients is available (Hoffman, 2002). A notable exception is the Equine Hospital at Tufts University, a specialized referral center, where forced oscillometry is used in a clinical diagnostic context (Mazan and Hoffman, 2003).

Conventional pulmonary testing: oesophageal balloon catheter technique

The conventional method of lung function testing is often considered to be the reference technique for the diagnosis of airway obstruction (Hoffman, 2002), although its’ sensitivity for low-grade airway obstruction is poor; values at rest are usually within the normal reference range in racehorses with mild equine asthma (Couetil, Rosenthal et al., 2001). This method is technically demanding, and not suited for field-testing. It describes the mechanical properties of the lung through the measurement of esophageal pressure (which is used as an estimate of pleural pressure (Mead and Whittenberger, 1953; Petit and Milic-Emili, 1958; Milic-Emili, Mead et al., 1964)) and respiratory air flow (using a pneumotachograph) together. This technique enables the pulmonary resistance, dynamic compliance, and work of breathing to be calculated (Hoffman, 2002).

Forced oscillometry

Forced oscillatory mechanics (oscillometry) is the study of dynamic lung function through the measurement of the response of the respiratory system to external forces (pressure or flow) (Hoffman, 2002). Briefly, low frequency pulses of air (oscillations) are forced into the respiratory system via a facemask. This produces a measurable pressure and flow exceeding that produced by the horse’s own respiratory system (Mazan and Hoffman, 2003). Baseline respiratory signals are filtered out during analysis (Young, Tesarowski et al., 1997). The concepts underlying oscillometry are applied from electrical models which describe impedance. These models are based on the assumption that the impedance to airflow is the sum of three forces across the respiratory system: resistance, elastance, and inertance (Hoffman, 2002). Resistance refers to the pressure required to overcome the frictional forces of the airways and tissue resistance. Compliance is the inverse of elastance; it refers to the pressure necessary to fill the lungs with zero airflow. In the horse dynamic compliance is measured as a breath.
hold cannot be requested of the patient. Inertance denotes the pressure needed to accelerate a column of air within the respiratory system. Whilst likely insignificant at rest, it becomes quite important in the exercising horse (Mazan and Hoffman, 2003).

In a clinical context, the primary disturbance observed in horses with mild equine asthma is low-grade airway obstruction. It is not expected that the baseline respiratory system resistances for different frequencies be abnormal; they can be mildly elevated. However, in horses with mild asthma the shape of the resistance-frequency curve is reflective of heterogeneous, peripheral airway disease; oscillometry is a sensitive technique for the detection of mild equine asthma (Hoffman, Mazan et al., 1998; Mazan, Hoffman et al., 1999; Hoffman, 2002; Mazan and Hoffman, 2003; Richard, Fortier et al., 2009a).

**Flowmetric plethysmography (flowmetrics)**

Flowmetrics is equally sensitive to conventional pulmonary testing, although not as sensitive as oscillometry (Miller, Hoffman et al., 2000). It can be combined with bronchoprovocative challenge (see below) to provide a sensitive technique for the detection of mild equine asthma in the field (Mazan and Hoffman, 2003).

**Forced expiratory maneuvers**

Forced expiratory maneuvers are very useful in the diagnosis of human asthma. The most commonly used maneuver, FEV1 – the maximum volume that can be exhaled in 1s - is used to measure flow limitation, and is easy to administer in humans. In healthy individuals, there exists an intrathoracic pressure beyond which airflow ceases. When the patient has airway obstruction, airflow ceases earlier, at a lower intrathoracic pressure (Lumb, 2016). However, as the measurement of FEV1 requires cooperation from the patient, the technique was required to be refined for use in the awake horse (Couetil, Rosenthal et al., 2000). Whilst it can differentiate between healthy horses and those with MEA and severe equine asthma (Couetil, Rosenthal et al., 2000), the huge technical challenges associated with the technique have meant that its use, even in a research context, has been limited.
Airway hyperresponsiveness

Airway hyperresponsiveness can be reliably – and objectively – demonstrated using a histamine bronchoprovocative challenge (Klein and Deegen, 1986; Doucet, Vrins et al., 1991; Nolen-Walston, Kuehn et al., 2009). This enables the measurement of both airway sensitivity (the threshold of the bronchoconstriction response) and reactivity (the magnitude of the bronchoconstriction response). Measurable parameters include the histamine provocative concentration which causes a 20% drop in FEV1 (forced expiratory volume in 1s); in patients with symptomatic asthma, a positive cutoff PC20≤ 8mg/ml is 100% sensitive and 93% specific (Cockcroft, Murdock et al., 1992). Airway hyperresponsiveness is a valuable tool for both research and clinical practice; it can be detected prior to the development of more obvious clinical signs (Hoffman, Mazan et al., 1998). Airway hyperresponsiveness has been associated with BAL mastocytosis (Hare and Viel, 1998; Hoffman, Mazan et al., 1998; Bedenice, Mazan et al., 2008a), respiratory clinical signs, and exercise intolerance (Klein and Deegen, 1986; Hare, Viel et al., 1994). Histamine bronchoprovocation can be performed in the field and shows satisfactory reproducibility (Nolen-Walston, Kuehn et al., 2009).

Hay challenge trial

A practical way of discriminating severe from mild equine asthma is to perform a hay challenge. Horses susceptible to mild equine asthma exposed to moldy hay can exhibit increased coughing and pulmonary neutrophilia; in contrast, horses with severe equine asthma develop increased respiratory effort or lung dysfunction at rest (Couëtil, Rosenthal et al., 2001). Whilst useful for the characterization of research subjects, a challenge trial is not recommended for clinical diagnosis.

Exclusion criteria

Exclusion criteria for horses to be diagnosed with mild equine asthma include systemic signs of infection (fever, anorexia, lethargy, abnormalities on bloodwork), or increased respiratory effort at rest (which is indicative of severe equine asthma) (Couëtil, Cardwell et al. 2016).
**Timeline**

Clinical signs (e.g. excess mucus, poor performance, coughing) can last for 3-9 weeks in Thoroughbred racehorses (Wood, Newton et al. 2005), and can persist for months to years in nonracing horses (Couetil, Rosenthal et al. 2001).

**Phenotypes**

There is a need to identify and apply criteria to further sub-categorize equine asthma, and it has been suggested that a new classification based on immunological signature data may have greater relevance (Pirie, 2017), particularly in the context of novel, targeted biologic therapeutic approaches (Klier, Lehmann et al., 2015). In humans, it is recognized that asthma is a heterogeneous disease, with the underlying pathogenesis differing among phenotypes (GINA, 2017). There is evidence that severe equine asthma has a genetic background with possible locus heterogeneity (Jost, Klukowska-Rötzler et al., 2007) (discussed below under *Allergic asthma*). In comparison, while genetic susceptibility is suspected in mild equine asthma, it has not yet been investigated. In light of the biologic characteristics common to both equine and human asthma and the marked disease heterogeneity in both, endeavoring to apply currently defined human asthma phenotypes to the horse seems to represent a logical starting point in the process of equine asthma sub-categorization. There are multiple human asthma phenotypes, the most common of which are allergic asthma, non-allergic asthma, late-onset asthma, asthma with fixed airflow limitation, and asthma in obese patients (GINA, 2017). While mild and severe equine asthma do not necessarily share attributes with all phenotypes, similarities and differences between these equine diseases and human asthma are discussed below, and summarized in Table 1.1. Furthermore, Table 1.1 also identifies the equine diseases which, at this time, we (Bond, Léguillette et al., 2018) propose to be biologically appropriate models for each human asthma phenotype, acknowledging the requirement for further research to better support these preliminary proposals. The introductory chapter of the thesis aims to focus on the biologic relevance of the proposed nomenclature;
for an extensive discussion of the advantages and disadvantages of the equine asthma model, the reader is referred to the excellent review article (Bul lone and Lavoie, 2015).
Table 1.1 - Features of asthma phenotypes in humans and mild/severe equine asthma in horses, appropriateness of equine asthma model, and areas identified for future research

Abbreviations: ICS, inhaled corticosteroid; BALF, bronchoalveolar lavage fluid; FEV1 – forced expiratory volume in 1 second.

<table>
<thead>
<tr>
<th>Asthma phenotype</th>
<th>Features in humans</th>
<th>Features supporting phenotype model in horses</th>
<th>Equine model appropriate?</th>
</tr>
</thead>
</table>
| Allergic asthma  | • Allergenic trigger associated with respiratory symptoms/expiratory airflow limitation  
• Often commences in childhood  
• Past/family history of allergic disease (eczema/allergic rhinitis/food or drug allergy)  
• Sputum often reveals eosinophilic airway inflammation  
• Usually respond well to ICS treatment  
• Th-2 CD4+ lymphocyte response - IL-5 mediated eosinophil recruitment  
• IL4Rα gene associated with the development of asthma, skin allergies and parasite defense | Mild equine asthma  
• Antigenic triggers central to development of lower airway inflammation  
• Stabling exposes horses to high levels of airborne particulates (e.g. dust, endotoxin, fungi, molds, ultrafine particles, noxious gases), and is a risk factor for mild equine asthma  
• Antigenic triggers (e.g. dust, mold spores) associated with increased neutrophil/mast cell% in BALF  
• Antigenic triggers associated with clinical signs (e.g. coughing, poor performance)  
• Often occurs in young horses  
• Eosinophilic phenotype associated with dust exposure in young horses  
• Usually respond well to ICS treatment  
• Th-2 response – increase in IL-4 and IL-5 in BALF linked with mastocytic phenotype | Yes |
| Severe equine asthma | • Allergenic trigger (molds +/-LPS) associated with clinical signs and pathology (Increased neutrophil % in BALF, increased respiratory effort at rest)  
• Associated with multiple hypersensitivities in some families of horses (insect bite hypersensitivity, urticaria, increased parasite resistance)  
• Good response to ICS  
• Association between IL4Rα and severe equine asthma | Yes |
<table>
<thead>
<tr>
<th></th>
<th>Non-Allergic asthma</th>
<th>Mild equine asthma</th>
<th>Severe equine asthma</th>
<th>Yes</th>
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<tbody>
<tr>
<td></td>
<td>• Not associated with allergy</td>
<td>• BALF can reveal neutrophilia and/or eosinophilia and/or mast cells accumulation</td>
<td>• BALF can be neutrophilic or paucigranulocytic (in severe cases where BALF return is low)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Sputum can be neutrophilic eosinophilic or paucigranulocytic</td>
<td>• Th-1 response – mRNA encoding TNF-α, IL-1β and IFN-γ in BALF</td>
<td>• Chronic innate immune activation - chronic activation of peripheral neutrophils</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Often respond less well to ICS</td>
<td>• Th-17 response – increase in IL-17 and IL-23 linked with increased neutrophil % in BALF</td>
<td>• Often respond less well to ICS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Chronically activated mast cells in bronchial mucosa (can be associated with non-allergenic stimulus)</td>
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<tr>
<td></td>
<td>• Th-1 response - cell-mediated immunity and phagocyte-dependent inflammation</td>
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<tr>
<th></th>
<th>Late-onset Asthma</th>
<th>Mild equine asthma</th>
<th>Severe equine asthma</th>
<th>Yes</th>
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<tbody>
<tr>
<td></td>
<td>• Initial presentation as adult (particularly women)</td>
<td>• Insufficient evidence</td>
<td>• Decreased baseline pulmonary function during disease exacerbation</td>
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<tr>
<td></td>
<td>• Less likely to be atopic</td>
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<td>• Mature/older animals</td>
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<td></td>
<td>• Decreased baseline pulmonary function</td>
<td></td>
<td>• Can require higher doses for control</td>
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<tr>
<td></td>
<td>• Often refractory to ICS/require higher doses for control</td>
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<p>|                  |                      |                                                                                   |                                                                                     | Yes |
| Late-onset Asthma |                      |                                                                                   |                                                                                     |     |
|                  |                      |                                                                                   |                                                                                     |     |</p>
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<tr>
<th>Asthma with fixed airflow limitation</th>
<th>Chronic asthma patients with fixed airflow limitation; thought to be due to airway wall remodeling</th>
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<tbody>
<tr>
<td></td>
<td>Increased airway smooth muscle mass and extracellular matrix at all levels of bronchial tree</td>
</tr>
<tr>
<td></td>
<td>Postbronchodilator FEV1 &lt; 70% (predicted)</td>
</tr>
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</table>

| Mild equine asthma | Insufficient evidence |

| Severe equine asthma | Tissue remodeling is reversible - long-term antigen avoidance strategies and corticosteroid therapy decrease airway smooth muscle mass and subepithelial collagen area |

| Severe equine asthma | Insufficient evidence |

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<tr>
<th>Asthma in obese patients</th>
<th>Dyspnea on exertion</th>
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<tbody>
<tr>
<td></td>
<td>Requires objective measurement of variable airflow limitation – obesity-associated respiratory symptoms can mimic asthma</td>
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<tr>
<td></td>
<td>Little eosinophilic airway inflammation</td>
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</table>

| Asthma in obese patients | Correlation between body condition score and body fat (%) and increased expression of IL-1 and TNF-α in plasma |

| Asthma in obese patients | Insufficient evidence |
Allergic asthma

One of the most common human asthma phenotypes is ‘allergic asthma’, a term which reflects the triggering role of antigens in this particular sub-group. Allergic asthma is generally associated with a past/family history of allergic disease (e.g. eczema, food allergy) and cytological analysis of pre-treatment induced sputum derived from affected patients often reveals eosinophilic airway inflammation (Moore, Meyers et al., 2010); the response to inhaled corticosteroid treatment is generally favorable. Currently, mild equine asthma can be further sub-categorized based on the predominant inflammatory cell in bronchoalveolar lavage fluid (BALF); namely, neutrophilic, eosinophilic, mastocytic or mixed granulocytic. Whilst the pathogenesis of mild equine asthma is incompletely defined, it is widely understood to be a multifactorial disease with the relative contribution of etiological influences varying with environment, husbandry, location, season and preventive medicine strategies (Rosenthal, Gruntman et al., 2006; Riihimaki, Raine et al., 2008). Antigenic triggers are central to the development of lower airway inflammation. Horses kept in conventional stables with poor ventilation are exposed to high levels of airborne particulates (Ivester, Couetil et al., 2014a) including dust, endotoxin, fungi, molds, ultrafine particles and noxious gases, and there is strong evidence that stabling of horses is a risk factor for mild equine asthma (Holcombe, Jackson et al., 2001; Gerber, Robinson et al., 2003; Millerick-May, Karnaus et al., 2013; Ivester, Couetil et al., 2014b). However, the level of respirable particulates in the overall stall air does not necessarily reflect the level of challenge a horse experiences, as the majority of dust exposure occurs in the breathing zone during feeding (Ivester, Couetil et al., 2014b). Exposure to hay and its accompanying mold spores, such as Aspergillus fumigatus, Saccharopolyspora rectivirgula, and Thermoactinomyces vulgaris, has been shown to be a risk factor in the development of lower airway inflammation (Robinson, Karnaus et al., 2006; Wasko, Barkema et al., 2011; Pirie, 2014). Furthermore, compared to feeding hay from the ground, feeding hay in a net has been shown to deliver a 4-fold increase in breathing zone respirable particle concentration (Ivester, Couetil et al., 2014b). There is very little information in the literature regarding an association between antigenic triggers (i.e. dust, mold spores) and specific mild equine asthma phenotypes. A prospective, cross-over study did reveal an association between stabling of young horses and a mild
equine asthma phenotype characterized by increased airway neutrophils (Holcombe, Jackson et al., 2001). This phenotype has been associated with coughing and poor performance (discussed above in minimum inclusion criteria for application of the term “asthma”), both of which form the basis for the diagnosis of mild equine asthma. In contrast with the human allergic asthma phenotype, eosinophils are less commonly detected in equine BALF; the eosinophilic sub-type of mild equine asthma has an overall prevalence lower than other cytological sub-types (McGorum and Dixon, 1994; Hughes, Malikides et al., 2003; Hughes, Nicolson et al., 2011; Wasko, Barkema et al., 2011). In young horses, the recruitment of airway eosinophils appears to be associated with dust exposure (Riihimaki, Raine et al., 2008; Ivester, Couetil et al., 2014a) and increased BALF eosinophil ratios have been associated with pulmonary hyperresponsiveness (Hare and Viel, 1998). Further studies are clearly warranted to more fully clarify the role of eosinophils in mild equine asthma pathogenesis and their effect on respiratory function (Couetil, Cardwell et al., 2016). Nevertheless, regardless of the BALF cytologic profile, it appears that antigenic triggers are associated with both the clinical signs and pathology of lower airway inflammation observed in horses with mild equine asthma. Similarly, yet more widely reported in the literature, antigenic triggers are strongly associated with both clinical exacerbations and pathologic changes (e.g. airway remodeling) in horses with severe equine asthma (Bullone and Lavoie, 2015). Of note, however, eosinophils are often absent from the airway wall of horses with severe asthma (Dubuc and Lavoie, 2014).

In humans, an “atopic march” has been described, whereby the first clinical manifestation of allergic disease, atopic dermatitis, is followed by the subsequent development of food allergy, rhinitis and asthma (Barnetson and Rogers, 2002). Evidence suggests that 75% of young children that experience severe atopic dermatitis will develop allergic rhinitis, and 50% will develop asthma (Kulig, Bergmann et al., 1999). In horses, while data supporting the existence of an “atopic march” are lacking, there is genetic, epidemiological and clinical evidence of multiple co-existing manifestations of allergic disease within a single individual. There is a genetic association between severe equine asthma and microsatellite markers syntenic with the IL-4 receptor α-chain (IL4Rα) gene on equine chromosome 13 (Jost, Klukowska-Rötzer et al., 2007). Significantly, the IL4Rα gene is associated with the
development of asthma, skin allergies and parasite defense in humans (Hershey, Friedrich et al., 1997; Ober, Leavitt et al., 2000; Scales, Ierna et al., 2007). Severe equine asthma can be associated with multiple hypersensitivities, including insect bite hypersensitivity (Lanz, Brunner et al., 2017) and urticaria (Kehrli, Jandova et al., 2015), as well as increased parasite resistance (Neuhaus, Bründler et al., 2010). Specifically, members of a half-sibling family with a high-incidence of severe equine asthma shed fewer strongylid eggs compared to genetically unrelated pasture mates unaffected with severe equine asthma (Neuhaus, Bründler et al., 2010). Furthermore, affected offspring within the high-prevalence family had lower strongylid egg counts than unaffected descendants (Neuhaus, Bründler et al., 2010). In this instance, the severe equine asthma-phenotype was associated with the expression of microsatellite markers near the IL4Rα gene, resulting in an upregulation of IL-4 during disease exacerbation (Lavoie, Maghni et al., 2001). However, the association between IL4Rα and severe equine asthma is neither absolute nor universal. The fact that it is not observed in every high-prevalence severe equine asthma family supports the existence of genetic heterogeneity within the currently defined phenotype. Although IL-4 promotes isotype switching from IgM to IgE (Grünig, Warnock et al., 1998), there is inconclusive evidence within the veterinary literature regarding the role of IgE in severe equine asthma; one study reported an elevation in mold-specific serum IgE in horses with severe asthma compared with control horses (Derksen, Scott et al., 1985), whilst several studies failed to generate similar findings (Schmallenbach, Rahman et al., 1998; Tahon, Baselgia et al., 2009). An increase in BALF IgE levels in horses with severe asthma has been reported (Schmallenbach, Rahman et al., 1998). However skin testing and the evaluation of immediate IgE-mediated reactions is not useful in horses with severe equine asthma, suggesting that systemic IgE antibodies to mold or fungal antigens are not developed in horses with this disease (McGorum, Dixon et al., 1993; Jose-Cunilleras, Kohn et al., 2001; Lorch, Hillier et al., 2001; Wong, Buechner-Maxwell et al., 2005; Tahon, Baselgia et al., 2009). Whilst there are presently no reports on the role of IgE in MEA, a Th-2 cytokine signature has been detected in BAL cells derived from mastocytic forms of MEA, characterized by increased expression of IL-4 and IL-5 mRNA (Lavoie, Cesarini et al., 2011; Beekman, Tohver et al., 2012). Whilst further data, derived from longitudinal studies, are required to support the existence of an “atopic march” in the horse, an “allergic equine asthma” phenotype currently appears biologically appropriate.
Non-allergic asthma

A common asthma phenotype in human adults is ‘non-allergic asthma’, where there is no apparent association with allergy. Analysis of pre-treatment patient-derived sputum reveals neutrophilic, eosinophilic or paucigranulocytic inflammation. Human asthma, particularly the allergic phenotype, displays an IL-5 mediated eosinophil recruitment predominantly driven by a Th-2 CD4+ lymphocyte response. However, the role of a Th-1 immune response and its ability to evoke cell-mediated immunity and phagocyte-dependent inflammation is exhibited both in chronic severe asthma and acute asthma exacerbations, the latter being associated with airway neutrophil recruitment as early as four hours following allergen exposure. Furthermore, in chronic asthma in humans, multiple studies have found persistently activated mast cells in the bronchial mucosa, evidenced through persistently elevated cytokine expression and synthesis (Beasley, Roche et al., 1989; Broide, Gleich et al., 1991; Ying, Humbert et al., 1997). Although mast cell activation is often assumed to be allergen induced, there are multiple non-allergenic stimuli which can cause this activation, including proteases (Machado, Horton et al., 1996), cytokines (Okumura, Kashiwakura et al., 2003) and Toll-like receptor ligands (Okumura, Kashiwakura et al., 2003). These and other mechanistic pathways are described in detail in a review article (Bradding, 2008). In addition to the varied mechanisms (both allergenic and non-allergic) which may underpin mast cell degranulation, differences also exist with respect to the kinetics of degranulation. In contrast to the rapid mast cell degranulation observed following allergen challenge, the ultrastructural appearance of some asthmatic airway mast cells appears consistent with a slower degranulation process (Beasley, Roche et al., 1989). Whilst mast cells are well known for their role in allergic and anaphylactic reactions (where rapid degranulation is observed as part of a Th-2-biased response), increasing evidence supports an alternative role of mast cells in inflammation, whereby they exhibit “differential” or “selective” secretion of mediators without degranulation (Theoharides, Kempuraj et al., 2007). Similarly, there is evidence that both the Th-1 and Th-2 immune responses are involved in the pathogenesis of mild and severe equine asthma. However, when interpreting the gene expression data derived from horses with mild equine asthma, it is important to consider whether the diagnosis was based on a generalized increase in airway inflammatory cells (i.e. mixed cytologic
inflammation) or an increase in a specific inflammatory cell (neutrophilic, mastocytic, eosinophilic). Evidence of a Th-1 response in the lower respiratory tract, characterized by upregulation of IFN-γ mRNA in BALF-derived cells, has repeatedly been reported in association with a generalized increase in BAL inflammatory cells, both in the presence and absence of clinical signs (Hughes, Nicolson et al., 2011; Lavoie, Cesarini et al., 2011; Richard, Depecker et al., 2014). Additionally, a Th-17 response has been implicated in neutrophilic mild equine asthma, with an association between the BALF neutrophil ratio and increased IL-17 and IL-23 mRNA expression (Hughes, Nicolson et al., 2011; Beekman, Tohver et al., 2012). It is important to consider that these responses might reflect sequential phases of the chronic inflammatory process in the respiratory tract; consequently, it may not be appropriate to consider them as mutually exclusive (Lavoie-Lamoureux, Moran et al., 2010). Such considerations remain speculative, particularly in naturally occurring cases, and additional studies are required for clarification.

Chronic innate immune activation is a feature of both neutrophilic human asthma, as well as severe equine asthma, which persists during disease remission (Lavoie-Lamoureux, Beauchamp et al., 2012; Wood, Baines et al., 2012). The chronic activation of peripheral blood neutrophils reported in severe equine asthma (Lavoie-Lamoureux, Beauchamp et al., 2012) may, in part, contribute to the greater disease severity compared with mild equine asthma, whereby exposure to an inhaled stimulus (e.g. dust, mold spores) may result in an exaggerated and inappropriate inflammatory response. Although such exposures may induce mild neutrophilic pulmonary inflammation in both healthy horses and humans, the degree of cellular activation decreases in hours/days, even if the inciting stimulus is maintained (Nocker, Out et al., 1999; Leclere, Lavoie-Lamoureux et al., 2011). In contrast, if exposure to an antigenic stimulus is maintained in horses with mild equine asthma, pulmonary inflammation has been reported to persist for up to three months (Holcombe, Jackson et al., 2001). Whilst further research into the innate immune response in mild and severe equine asthma is required to fully understand the role of neutrophil activation in the development of lower airway inflammation, given that a non-Th-2 immune response has also been associated with both mild and severe equine asthma, the proposed existence of a “non-allergic equine asthma” phenotype currently appears biologically appropriate.
Late-onset asthma

Some patients (particularly women) present with asthma for the first time as adults. These patients are less likely to be atopic, as “age of onset” is significantly lower in patients with allergic asthma, compared with those with non-allergic asthma (Romanet-Manent, Charpin et al., 2002). They also have decreased baseline pulmonary function and are either refractory to inhaled corticosteroid therapy or require higher doses of inhaled corticosteroids to achieve asthma control (Moore, Meyers et al., 2010). Horses with severe equine asthma exhibit decreased baseline pulmonary function during disease exacerbation, and tend to be mature to older animals (Couëtil, Cardwell et al., 2016). “Inflamm-ageing” describes a reduction in the capacity of the aging body to cope with a variety of stressors together with a progressively increasing chronic low-grade inflammatory status, associated with aging and provoked by a continuous antigenic load (Franceschi, Bonafè et al., 2000). Age-related increases in pro-inflammatory cytokines have been reported in both humans and horses, with aged healthy horses reported to have increased expression of IL-6, IL-8, IFN-γ and peripheral blood mononuclear cell derived TNF-α mRNA concentration in plasma (McFarlane and Holbrook, 2008). Furthermore, T-cells of geriatric horses (>20 years) exhibit a lower proliferative response than those of younger animals (Adams, Breathnach et al., 2008), and peripheral blood lymphocytes and monocytes derived from this cohort exhibit an increased basal expression of IFN-γ and TNF-α mRNA, respectively (Hansen, Sun et al., 2013). However, age related changes appear to be more tightly regulated in the lungs than in the systemic circulation (Hansen, Sun et al., 2013). Inflammatory cell populations in the lung represent a balance between cellular recruitment, via airway epithelial cell and macrophage-derived chemotactic cytokines, and removal, via apoptosis and phagocyte-mediated clearance. Lung granulocytes (neutrophils and macrophages) in horses with severe equine asthma exhibit altered apoptosis (Niedzwiedz, Jaworski et al., 2014), which together with increased activity of transcription factors such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) (Barnes and Adcock, 1998) may contribute to the maintenance of neutrophilic inflammation in horses treated with glucocorticoids and maintained in an allergenic environment (Couëtil, Art et al., 2006). Whilst no age-related trends in BALF cytological profiles in horses with mild or severe equine asthma have been reported, (Hansen,
Sun et al., 2013) did report an age-associated increase in mRNA expression of IFN-γ producing lymphocytes in stimulated BAL cells. Whilst there is a paucity of definitive data on the progression of mild to severe equine asthma over time, there is anecdotal evidence suggesting the progression from MEA in younger age to severe equine asthma in some horses (Viel, 1997). Although potentially influenced by the high prevalence of MEA, such a phenomenon of disease progression does warrant further study. There is no current correlation between inflamm-aging and the development of chronic inflammatory airway diseases. However, based on the human phenotype, we believe it is biologically appropriate to use severe equine asthma as an equine model for late-onset asthma, as recently reviewed (Bullone and Lavoie, 2017).

**Asthma with fixed airflow limitation**

Patients with fixed airflow obstruction are often grouped under the heading of chronic obstructive pulmonary disease (COPD), with distinct pathological and functional characteristics compared to those with a history of asthma (Fabbri, Romagnoli et al., 2003); for example, asthmatic patients do not exhibit a loss of airway integrity as observed in COPD (McDonough, Yuan et al., 2011). It is thought that fixed airflow limitation in asthmatic patients is due to airway wall remodeling, with both airway smooth muscle (ASM) mass and extracellular matrix (ECM) deposition being increased at all levels of the bronchial tree (Lambert, Wiggs et al., 1993), with the increased ASM mass being the functionally dominant alteration (Oliver, Fabry et al., 2007). Consequently, in addition to the clinical similarities between human and severe equine asthma, both diseases also share certain structural features. The structural alterations seen in human patients with fixed airflow limitation are currently thought to be irreversible; however, appropriate studies are lacking to verify if indeed this is correct. In contrast, tissue remodeling in severe equine asthma is partially reversible under certain circumstances (Bullone, 2017). In horses with severe equine asthma, long-term corticosteroid therapy (fluticasone) and antigen avoidance strategies have been shown to significantly decrease both smooth muscle mass (30% decrease over 3 months, but remained twice that of healthy controls) and subepithelial collagen area (Leclere, Lavoie-Lamoureux et al., 2012; Bullone, Vargas et al., 2017). Corticosteroid administration
increased the rate of decline in smooth muscle mass, although antigen avoidance was better at controlling airway inflammation (Leclere, Lavoie-Lamoureux et al., 2012). Airway remodeling in horses with MEA has not yet been investigated. In light of the paucity of studies investigating peripheral airway remodeling and its reversibility in human asthma and the limited data derived from horses with severe equine asthma, there is currently insufficient evidence to determine the suitability of equine asthma as a model for asthma with fixed airflow limitation.

Asthma with obesity

In humans, obese patients with asthma can have moderate to severe respiratory symptoms, with little eosinophilic airway inflammation; there is no evidence for an increase in sputum inflammatory cells. Whilst it is unknown whether obesity per se contributes to asthma, there are marked alterations to respiratory physiology including an increased demand for ventilation and work of breathing. Breathing at low lung volumes enhances airway responsiveness which improves following bariatric surgery (Dixon, Pratley et al., 2011; Boulet, Turcotte et al., 2012). The altered mechanics of breathing that favor airway narrowing and airway hyperresponsiveness can result in a more severe clinical presentation than that predicted upon consideration of the underlying inflammatory pathology. However, it is likely that obesity-related alterations to ventilation mechanics are not the same in quadrupeds compared to bipeds.

Whilst there is evidence that obesity increases the risk of developing asthma in people, some studies suggest that insulin resistance, systemic IL-6 inflammation and clinical features of metabolic dysfunction have a stronger association with more severe asthma than BMI or body mass (Peters, McGrath et al., 2016). Whilst there is a positive correlation between both body condition score and body fat (%) and IL-1 and TNF-α in equine plasma (Vick, Adams et al., 2007), there is currently no report in the literature of increased expression of inflammatory cytokines in BAL fluid or increased pulmonary resistance in horses with obesity. Furthermore, to the authors’ knowledge there are no reports of a link between equine metabolic syndrome and the presence of chronic lower airway inflammation in horses. Therefore, there is currently insufficient evidence to consider equine asthma a suitable model for human asthma associated with obesity.
Pathogenesis

The pathogenesis of mild equine asthma is incompletely defined, although it is widely understood to be a multifactorial disease with the relative contribution of etiological influences varying with environment, husbandry, location, season and preventative medicine strategies (Rosenthal, Gruntman et al., 2006; Riihimaki, Raine et al., 2008).

Role of environment

Non-infectious agents are central to the development of lower airway inflammation. Horses kept in conventional stables with poor ventilation have been shown to be exposed to high levels of respirable (≤4μm) airborne particulates, with barn environments containing high concentrations of dust, endotoxin, fungi, molds, ultrafine particles and noxious gases (Clarke, Madelin et al., 1987; Woods, Robinson et al., 1993; McGorum, Ellison et al., 1998; Samadi, Wouters et al., 2009; Ivester, Couetil et al., 2014b). There is strong evidence that stabling of horses is a risk factor for MEA (Holcombe, Jackson et al., 2001; Gerber, Robinson et al., 2003; Millerick-May, Karmaus et al., 2013; Ivester, Couetil et al., 2014a). However, the level of respirable particulates in the overall stall air does not necessarily reflect the level of challenge a horse experiences, as the majority of dust exposure occurs in the breathing zone during feeding (Ivester, Couetil et al., 2014a). Even healthy mature horses develop airway neutrophilia when exposed to straw bedding and moldy hay (organic dust challenge), which appears to be dose-dependent (Tremblay, Ferland et al., 1993; Pirie, Dixon et al., 2001; Desjardins, Theoret et al., 2004; Gerber, Lindberg et al., 2004; Leclere, Lavoie-Lamoureux et al., 2011). Exposure to hay and its accompanying mold spores, such as Aspergillus fumigatus, Faenia rectivirgula, and Thermoactinomyces vulgaris (Derksen, Robinson et al., 1988; Pirie, 2014), has been shown to be a risk factor in the development of lower airway inflammation (Robinson, Karmaus et al., 2006; Wasko, Barkema et al., 2011). Furthermore, the hay net has been shown to deliver a 4-fold increase in exposure to respirable particles in the breathing zone, compared to feeding the same hay on the ground (Ivester, Couetil et al., 2014a).
Temperature

Cold, dry environments could contribute to the pathogenesis of airway inflammation, with upregulation of Th-2 cytokines (IL-4 and IL-5) in BALF being observed in horses exercising at submaximal intensity in subfreezing temperatures (Davis, Malayer et al., 2005). Exercise in cold conditions (-5°C) is also associated with higher respiratory impedance and resistance 48 hours after exercise (Davis, Royer et al., 2006). Another study in which Standardbred horses conducted a standardized incremental treadmill test at -25°C found no changes to heart rate, lactate, PaO₂ or lung tissue morphology, however, they found that the respiratory rate was decreased during early stages of exercise, and two and five minutes after exercise (Dahl, Gillespie et al., 1986). PaCO₂ was also elevated two minutes after exercise at -25°C, compared to two minutes after the same test conducted at 17°C (Dahl, Gillespie et al., 1986).

However, the effects on inflammatory BAL cytology are mixed, with one study finding that submaximal exercise at subfreezing temperature induced a transient airway neutrophilia which persisted for 24 hours (Davis, Williams et al., 2007), and another finding no significant change in total or differential cell concentration (Davis, Malayer et al., 2005); the contribution of cold environments to the pathogenesis of mild equine asthma is likely to be limited.

Genetic susceptibility

A genetic contribution to the pathogenesis of mild equine asthma is suspected but has not yet been investigated. However, there is evidence that severe equine asthma has a genetic background with possible locus heterogeneity (Jost, Klukowska-Rötzler et al., 2007; Neuhaus, Bründler et al., 2010; Kehrli, Jandova et al., 2015), similar to asthma in humans (discussed in detail above; Allergic asthma).

Phenotype vs Endotype

Our inability to identify consistent genetic correlations with mild and severe equine asthma can potentially be attributed to our limited understanding of the various pathophysiologic mechanisms underlying these diseases. In human medicine, “asthma endotypes” are disease subtypes defined by their distinct, underlying pathophysiologies (Lötvall, Akdis et al., 2011). The broad syndrome of asthma
can therefore be divided into distinct disease entities, or subtypes, on the basis of seven variables including: i) clinical characteristics, ii) biomarkers, iii) lung physiology, iv) genetics, v) histopathology, vi) epidemiology, and vii) response to treatment (Lötvall, Akdis et al., 2011). Recently, several groups have used transcriptomic data derived from stimulated peripheral blood mononuclear cells (ex vivo) (Pacholewska, Jagannathan et al., 2015) and bronchial epithelium (in vivo) (Tessier, Côté et al., 2017) to identify differentially expressed genes and pathways between horses with and without severe equine asthma. Stimulation with hay dust extract resulted in the greatest differential gene expression (Pacholewska, Jagannathan et al., 2015), the most dominant amongst the up-regulated genes being those involved in immune cell trafficking, neutrophil chemotaxis, immune and inflammatory responses, and cell cycle regulation and apoptosis (Pacholewska, Jagannathan et al., 2015; Tessier, Côté et al., 2017). The most significantly up-regulated hay dust extract-induced chemokine was CXCL13 (Pacholewska, Jagannathan et al., 2015; Pacholewska, Kraft et al., 2017), a B cell chemoattractant predominantly produced by Th17, but not Th1 or Th2, cells (Takagi, Higashi et al., 2008). Rather than indicating a primary gene dysregulation, this might represent an abnormal response to antigens in horses with severe equine asthma. Interestingly, levels of CXCL13 have been shown to be up-regulated eight-fold in BALF from human asthmatics compared to controls (Baay-Guzman, Huerta-Yepez et al., 2012). Furthermore, treatment of a sensitized murine asthma model with an anti-CXCL13 antibody reduces inflammatory cell recruitment, bronchial-associated lymphoid tissue formation, and airway inflammation, potentially supporting CXCL13 as a novel treatment target (Baay-Guzman, Huerta-Yepez et al., 2012). Another potential mechanistic pathway which could underpin the inflammatory cascade in severe equine asthma is the activation of neutrophils by the bronchial epithelium, leading to epithelial injury and impaired repair and differentiation (Tessier, Côté et al., 2017). With the development of new biologic treatments in human asthma and the application of more targeted therapeutic approaches in the horse, it is appropriate to further investigate and clarify the clinical characteristics, biomarkers, lung physiology, genetics, histopathology, epidemiology, and response to treatment to better elucidate the pathophysiologic mechanisms of mild and severe equine asthma, thus enabling the description of the allergic (Th-2), non-allergic (non-Th-2) and late-onset endotypes of equine asthma.
Infectious agents

Although mild equine asthma is primarily an inflammatory process, the involvement of infectious agents in the pathogenesis of mild equine asthma is highly suspected; there is increasing evidence emerging within the peer-reviewed literature which supports a relationship between bacterial, fungal and viral infections and mild equine asthma (Burrell, Wood et al., 1996b; Christley, Hodgson et al., 2001b; Wood, Newton et al., 2005b; Dauvillier, ter Woort et al., 2018).

Role of bacteria

An increase in tracheal mucus has been associated with isolation of bacteria (particularly Streptococcus equi var zooepidemicus and Actinobacillus/Pasturella species) from tracheal washes and aspirates in racehorses, both in the presence and absence of clinical signs (Christley, Hodgson et al., 2001a; Wood, Newton et al., 2005a; Wood, Newton et al., 2005b; Cardwell, Smith et al., 2014). Additionally, the presence of Streptococcus zooepidemicus, Streptococcus pneumoniae, Actinobacillus spp., and Mycoplasma equihinis in tracheal samples has been associated with mild equine asthma, indicating that composition of the lower respiratory tract microbiota could contribute to the pathogenesis (Wood, Newton et al., 2005b). Microbial composition and diversity of the bronchial airways in humans with sub-optimally controlled asthma has been associated with the degree of bronchial hyperresponsiveness, suggesting that lower airway bacterial communities play a role in the pathogenesis of asthma (Huang, Nelson et al., 2011). However, as there is limited evidence and controversy on associating mild equine asthma with bacterial populations in the trachea, further research is required to confirm this association in horses. There is also a need to comprehensively describe the bacterial communities present in states of health and disease in the horse, as it is the overall composition of the bacterial communities, rather than the presence of individual species, which is important in defining health and disease (Hilty, Burke et al., 2010; Klepac-Ceraj, Lemon et al., 2010; Huang, Nelson et al., 2011). Further investigation of the relationship between infectious agents, lower respiratory tract microbiota and the development of mild equine asthma is warranted, and has been identified as a research priority (Couetil, Cardwell et al., 2016); these knowledge gaps are explored in Chapters 2 and 4.
Role of fungi

Historically, the role of fungi has been explored in relation to clinical exacerbation of severe equine asthma in ponies. Administration of aerosolized *Micropolyspora faeni* antigen was associated with an increase in respiratory frequency, minute ventilation and pulmonary resistance, as well as decreased arterial oxygen concentration, when given to severe equine asthma-affected ponies in remission (Derksen, Robinson et al., 1988). A recent, culture-based study investigated the prevalence of fungi in respiratory samples of horses diagnosed with mild equine asthma, and assessed risk factors associated with the presence of fungi in the airways, and found that horses with fungi present in tracheal wash cytology are 2 times more likely to have mild equine asthma than those without (Dauvillier, ter Woort et al., 2018). A positive fungal culture was obtained in 55% (402/731) horses referred for signs of poor performance or respiratory disease (Dauvillier, ter Woort et al., 2018). Risk factors associated with mild equine asthma and with the presence of fungi in tracheal wash cytology included straw bedding and being fed dry hay (Dauvillier, ter Woort et al., 2018). The most commonly isolated fungi were Penicillium (53%), Aspergillus (34%), Rhizomucor (5%), and Candida (5%) (Dauvillier, ter Woort et al., 2018). Given that the overall bacterial composition of the respiratory tract contributes to disease states (see above), it is logical to question the role of the respiratory fungal communities in expression of mild equine asthma. To address this, the respiratory mycobiota is characterized, and the effects of nebulized dexamethasone on respiratory fungal communities in an equine model of asthma are explored in Chapter 4.

Viral infection

Viral respiratory tract infections have a profound effect on the expression of asthma, as well as disease exacerbation, and are a major cause of morbidity (Busse, Lemanske Jr et al., 2010). In school age children, upper respiratory viral infections are associated with 80-85% of asthma exacerbations (Johnston, Pattemore et al., 1995), and there is evidence that viral infections can interact with atopy in infancy to promote later childhood asthma (Kusel, de Klerk et al., 2007).
Although viral infection is a common cause of transient lower airway inflammation in horses, the role of viral infection in the pathogenesis of mild equine asthma is still controversial (Newton, Wood et al., 2003; Wood, Newton et al., 2005a; Fortier, Van Erck et al., 2009). Acute viral respiratory tract infections are frequently a differential diagnosis when horses present with respiratory signs consistent with mild equine asthma, however, the severity, duration and presence of systemic clinical signs often differs, particularly with equine influenza infection (Couetil, Cardwell et al., 2016). While acute infection with viruses including equine herpesvirus (EHV)-1, EHV-4, equine rhinitis A and B or equine adenovirus can be subclinical or present with mild clinical signs consistent with mild equine asthma; infections are usually self-limiting (Diaz-Mendez, Viel et al., 2010). Whilst EHV-2 and EHV-5 are ubiquitous in healthy horses (Fortier, Richard et al., 2013; Hartley, Dynon et al., 2013; Hue, Fortier et al., 2014) they are frequently identified in respiratory secretions of horses with respiratory disease (Fortier, Van Erck et al., 2009; Hue, Fortier et al., 2014), and there is some evidence that infection with EHV-2 is associated with poor performance and airway inflammation (Fortier, Van Erck et al., 2009). Chronic EHV-5 infection can lead to equine multinodular pulmonary fibrosis (Williams, Maes et al., 2007), and can be experimentally induced with EHV-5 infection (Williams, Robinson et al., 2013).

A frequent observation made after cessation of treatment for MEA - corticosteroids are frequently used to treat lower airway inflammation (see Treatment and prevention below) – is the reemergence of clinical signs. Given the ubiquitous nature of EHV, as well as their ability to induce latent infection and recrudesce in times of immunosuppression or stress, it is logical to question whether the reemergence of clinical signs is due to an insufficient improvement in exposure to environmental antigens, or whether immunosuppression caused by corticosteroid treatment results in recrudesce of latent EHV infection? The research question is investigated in Chapter 4.

**Immunology of the equine respiratory tract**

The respiratory system of the horse has an extraordinary capacity, with a minute ventilation at rest of roughly 50-70L/min which increases to >2200L when racing; at the upper end of the spectrum this equates to ~100,000 liters of air in a 24-hour period. It is therefore unsurprising that both inert and
infectious agents are able to reach the lung, and that the respiratory tract must have the ability to defend itself.

**Mechanical defense**

The primary line of defense is mechanical. Particle size determines both the level of penetration into the respiratory tract and the mechanism of removal (Horohov, 2004). Environmental control measures are often based on particle-size selective sampling of particulate matter (PM). The inhalable fraction is the mass fraction of total airborne particles inhaled through the nose and mouth; the extra-thoracic fraction is the mass fraction of inhaled particles unable to penetrate beyond the larynx; the thoracic fraction is the mass fraction of inhaled particles penetrating beyond the larynx; and the respirable fraction is the mass fraction of inhaled particles penetrating to the unciliated airways (EPA, 1997; Brown, Gordon et al., 2013). Relative to total airborne particles, the particle size achieving 50% penetration to the thorax is 10μm; the particle size which achieves 50% penetration to the unciliated airways (i.e. respirable particulate matter) is 4.0μm (EPA, 1997; Brown, Gordon et al., 2013).

**Mucociliary transport apparatus**

After anatomic barriers, the mucociliary transport apparatus is the next line of defense and provides the primary mechanism for removing particulate matter from the ciliated airways. The respiratory mucosa is lined with columnar ciliated epithelium with lots of goblet cells at the beginning of the trachea, with gradually fewer cells towards the bronchioles; the terminal bronchioles are covered by the epithelium and a thin muscular layer. Goblet cells produce and secrete mucus into the airways which entraps the inhaled particles; they can vary depending upon the health status of the horse (Dixon, 1992). Ciliated cells transport the mucus out of the lung by beating in a coordinated fashion in the mucus layer, until it reaches the level of the esophagus where it is swallowed. Airway inflammation in horses with severe equine asthma is correlated with mucous cell metaplasia and the amount of stored mucosubstances within the epithelium (Lugo, Harkema et al., 2006). It appears that mucus accumulation in horses is partly caused by an increased number of mucous cells and is associated with airway inflammation.
In comparison to horses with severe equine asthma, after exposure to hay dust healthy controls exhibit an influx of neutrophils into the airways, but do not show increased mucus accumulation (Gerber, Lindberg et al., 2004).

**Respiratory immunoglobulins**

The mucus also contains immunoglobulins which can neutralize invading bacteria and viruses; primarily IgA which is produced in the lamina propria and transported across the epithelium. Infection with bacterial antigens and EHV-1 also promotes a mucosal IgA response; IgG isotypes can also be detected (Sheoran, Sponseller et al., 1997; Breathnach, Yeargan et al., 2001; Hooper-McGrevy, Giguere et al., 2001). An increase in BALF IgE levels in horses with severe asthma has been reported (Schmallenbach, Rahman et al., 1998), however there are presently no reports on the role of IgE in mild equine asthma. As discussed above under “Allergic asthma”, there is inconclusive evidence within the veterinary literature regarding the role of IgE in severe equine asthma.

**Alveolar macrophages**

In the alveolar wall, there are 3 major cell types: Type I pneumocytes which form the structure of the wall, Type II pneumocytes which secrete surfactant, and alveolar macrophages. Alveolar macrophages, together with the neutrophils, are the predominant effector cells of the innate immune system; the initial defense mechanism at the level of the lung. Furthermore, there is evidence that alveolar macrophage-epithelial cross-talk contributes to epithelial renewal and restoration of alveolar barrier function by inducing the release of epithelial granulocyte-macrophage colony-stimulating factor (GM-CSF), which can induce proliferation of type II alveolar epithelial cells (Churchill, Friedman et al., 1992; Fehrenbach, 2001; Cakarova, Marsh et al., 2009; Proud and Leigh, 2011). Epithelial GM-CSF expression also activates neutrophils, eosinophils, macrophages, and enhances eosinophil survival (Proud and Leigh, 2011). There is evidence that pro-inflammatory stimuli (Churchill, Friedman et al., 1992), and tumor necrosis factor (TNF)-α released from activated alveolar macrophages induce expression of GM-CSF (Cakarova, Marsh et al., 2009); GM-CSF expression is enhanced in patients with asthma and allergic
rhinitis (Nonaka, Nonaka et al., 1996). Particulate matter or pathogens penetrating to the alveolar surface are cleared through phagocytosis by alveolar macrophages, neutrophils and lymphocytes (Herold, von Wulffen et al., 2006; Martinez, Sica et al., 2008). After pathogen clearance, macrophages initiate the resolution of inflammation by phagocytosis of apoptotic neutrophils, releasing anti-inflammatory IL-10, amongst others (Serhan and Savill, 2005). Macrophages are also present in the mucociliary apparatus, as well as in the lymphoid tissues associated with the respiratory tract. Alveolar macrophages display altered expression of inflammatory cytokines in horses with severe equine asthma compared to non-susceptible (healthy) horses in response to aerosolized challenge; horses with severe equine asthma had greater expression of pro-inflammatory (TNF-α, IL-1β and IL-8) cytokines than non-susceptible (healthy) horses, whereas the latter had increased expression of IL-6, considered an anti-inflammatory cytokine (Laan, Bull et al., 2006a). Expression of both pro- and anti-inflammatory cytokines is likely initiated by a CD14 and Toll-like receptor dependent signaling pathway that activates the transcription factor NF-κB (Dobrovolskaia and Vogel, 2002; Strieter, Belperio et al., 2003). Whilst NF-κB activation in the surrounding tissues could contribute to an in vivo inflammatory response in the bronchoalveolar space (Bureau, Bonizzi et al., 2000), the differences found in cytokine expression following multiple different aerosolized challenges, as well as differences between horses with and without severe equine asthma, suggest that the alveolar macrophage plays a central role in the initiation of the inflammatory reaction (Laan, Bull et al., 2006a).

**Lymphocytes**

Lymphocytes are small white blood cells (7-15 µm in diameter), that are uniform in appearance, but vary in function. Lymphocytes include natural killer (NK) cells, which are involved in cell-mediated, cytotoxic innate immunity, T cells and B cells, which are the effectors of adaptive immunity. NK, B and T cells are derived from bone-marrow derived progenitors; T cells are derived from progenitors that migrate to the thymus (LaRosa and Orange, 2008). The specific recombination events that take place during T-cell development, and the molecular signals that mediate activation are beyond the scope
of this introduction and are discussed elsewhere (Nel, 2002). The initial antigen-specific activation and proliferation of naive T cells is referred to as “priming”.

Clearance of intracellular pathogens and tumors is dependent upon a cell-mediated immune response. CD8+ effector T cells (cytotoxic T lymphocytes) are important for cell-mediated immunity; when primed they produce perforin and granzyme, which is released at the point of contact resulting in antigen-specific cytotoxicity, causing apoptosis without damaging surrounding tissues. When primed, CD8+ cells also produce IFN-γ and TNF (LaRosa and Orange, 2008).

CD4+ T-cell priming results in the differentiation of various subsets - including Th1, Th2, Th17 and Treg cells - distinguished by the production of particular cytokines and effector functions (Weaver, Harrington et al., 2006). Th2 cells produce IL-4, IL-5, and IL-13, amongst others, and help facilitate B-cell antibody responses. They stimulate B-cell proliferation through IL-4 and contact-dependent CD40 ligand:CD40 binding, thus signaling B cells to augment the humoral immune response against extracellular pathogens (LaRosa and Orange, 2008). Furthermore, IL-4 and IL-5 stimulate IgE production and recruitment of eosinophils into the airways (Davis and Rush, 2002; Leguillette, 2003), which is highly relevant in the allergic asthma phenotype in humans. A Th-2 cytokine signature has been detected in BAL cells derived from mastocytic forms of mild equine asthma, characterized by increased expression of IL-4 and IL-5 mRNA (Lavoie, Cesarini et al., 2011; Beekman, Tohver et al., 2012).

Th1 cells specialize in macrophage activation through IFN-γ production and contact-dependent stimulation (via a variety of costimulatory ligands); they play a major role in intracellular pathogen clearance, as well as delayed-type hypersensitivity reactions (LaRosa and Orange, 2008). Evidence of a Th-1 response in the lower respiratory tract, characterized by upregulation of IFN-γ mRNA in BALF-derived cells, has repeatedly been reported in association with a generalized increase in BAL inflammatory cells, both in the presence and absence of clinical signs (Hughes, Nicolson et al., 2011; Lavoie, Cesarini et al., 2011; Richard, Depecker et al., 2014).
Th17 cells are now regarded as a distinct CD4+ T-cell subset, and produce IL-17, IL-17F, IL-6, and tumor necrosis factor (TNF). IL-17 is an inflammatory cytokine involved in the recruitment and proliferation of neutrophils. Th17 differentiation and survival requires IL-6 and IL-23, amongst others (LaRosa and Orange, 2008). A Th-17 response has been implicated in neutrophilic mild equine asthma, with an association between the BALF neutrophil ratio and increased IL-17 and IL-23 mRNA expression (Hughes, Nicolson et al., 2011; Beekman, Tohver et al., 2012).

Further clarification of lymphocytic cytokine responses in the lower respiratory tract of horses with chronic airway inflammation, and as well as investigations into the immunologic response to treatment would facilitate a greater understanding of the possible etiopathological pathways involved in mild equine asthma; this is explored in Chapters 3 and 4.

**Neutrophils**

The presence of neutrophils in the airway is often pathognomonic for mild and severe equine asthma (Couetil, Cardwell et al., 2016). Neutrophils are the most abundant type of granulocytes, are phagocytic, forming an essential part of the innate immune system, and are part of the polymorphonuclear family, together with basophils and eosinophils; they typically contain a nucleus divided into 2-5 lobes. They can be subdivided into banded and segmented neutrophils; they show increased segmentation as they mature. In BALF from healthy horses, neutrophils typically comprise less than 1% of cells (<5% is considered normal); in severe equine asthma their contribution can be > 50%. Mild equine asthma is characterized by a mild to moderate increase in neutrophils (>5%) (Couetil, Cardwell et al., 2016); however, it is not uncommon to find BALF neutrophil percentages >5% and <10% in asymptomatic horses (Couetil, 2014). Neutrophils accumulate in the lung within 6 to 8 hours after an inhalation challenge in horses with severe equine asthma, prior to the development of airway obstruction (Fairbairn, Page et al., 1993; Franchini, Gill et al., 2000; Brazil, Dagleish et al., 2005). In humans, neutrophil recruitment to the airway lumen is often seen with acute asthma exacerbations (Fahy, Kim et al., 1995; Norzila, Fakes et al., 2000; Lopuhaä, Out et al., 2002) and can be observed only 4 hours after antigenic challenge (Nocker, Out et al., 1999). This increased neutrophil recruitment in BALF
could be the result of neutrophil chemotactic cytokines (especially IL-8 and IL-4) being over-expressed (Franchini, Gill et al., 2000; Horohov, Beadle et al., 2005; Lavoie-Lamoureux, Moran et al., 2010).

Further clarification of neutrophilic cytokine responses in the lower respiratory tract of horses with chronic airway inflammation, and as well as investigations into the immunologic response to treatment would facilitate a greater understanding of the possible etiopathological pathways involved in mild equine asthma; this is explored in Chapters 3 and 4.

**Mast cells**

Mast cells are granulocytes which are well known for their role in allergic and anaphylactic reactions, where rapid degranulation is observed as part of a Th-2-biased response (Bradding, 2008). Mast cells are very similar in appearance and function to the basophil, although they have a single nucleus. Antigen-specific IgE becomes bound to mast cells via the Fc region; when the IgE-receptor complex on the plasma membrane binds to the specific circulating antigen, the mast cell degranulates, releasing granules of histamine, heparin and other inflammatory mediators. In chronic asthma sufferers, there is strong evidence of persistently activated mast cells in the bronchial mucosa, shown by persistently elevated cytokine expression and synthesis (Beasley, Roche et al., 1989; Broide, Gleich et al., 1991; Ying, Humbert et al., 1997). The pathophysiology of mild equine asthma is still unclear; some horses predominantly have airway mastocytosis - with degranulation sometimes observed - whereas others display neutrophilia, and some have a mixed inflammatory BAL profile. Clinical differences between different sub-types have been reported, with BAL mastocytosis and eosinophilia being associated with airway hyperresponsiveness (Hare and Viel, 1998; Hoffman, Mazan et al., 1998); only BAL neutrophilia has been associated with cough (Bedenice, Mazan et al., 2008a). Additionally, mast cells and neutrophils have different recruitment and activation pathways; there is evidence that the mastocytic sub-types of mild equine asthma has a different underlying pathophysiology compared to the neutrophilic sub-type (Beekman, Tohver et al., 2012). Additionally, an alternative role of mast cells in inflammation is emerging; they can exhibit “differential” or “selective” secretion of mediators without degranulation (Theoharides, Kempuraj et al., 2007). Whilst mast cell activation is often
assumed to be related to allergen exposure, there are multiple non-allergenic stimuli which can lead to activation, including proteases (Machado, Horton et al., 1996), cytokines (Okumura, Kashiwakura et al., 2003) and Toll-like receptor ligands (Okumura, Kashiwakura et al., 2003). These and other mechanistic pathways are described in detail in a review article (Bradding, 2008). Regardless of which pathway leads to mast cell degranulation, cytokines and chemokines secreted by activated mast cells affect smooth muscle contraction leading to bronchoconstriction, increase microvascular permeability and contribute to the inflammatory response observed in both human and equine asthma.

Further clarification of mastocytic cytokine responses in the lower respiratory tract of horses with chronic airway inflammation, and as well as investigations into the immunologic response to treatment would facilitate a greater understanding of the possible etiopathological pathways involved in mild equine asthma; this is explored in Chapters 3 and 4.

_Eosinophils_

Eosinophils are multilobulated granulocytes frequently associated with allergy, asthma and parasitic infections. They contain multiple inflammatory mediators (e.g. eosinophil peroxidase, ribonuclease, deoxyribonucleases, and lipase) which are released following activation of the eosinophil through a degranulation reaction, and are toxic to host tissue as well as the antigen/parasite. Cytological analysis of sputum derived from human asthmatics with allergic asthma often reveals eosinophilic airway inflammation and is usually associated with a past/family history of allergic disease (Moore, Meyers et al., 2010). In contrast, eosinophils are less frequently detected in equine BALF, with the exception of a sub-group of mild equine asthma predominantly observed in young horses – although it can be observed at any age, and be very severe - (McGorum and Dixon, 1994; Hughes, Malikides et al., 2003; Hughes, Nicolson et al., 2011; Wasko, Barkema et al., 2011) which appears to be associated with dust exposure (Riihimaki, Raine et al., 2008; Ivester, Couetil et al., 2014a). Increased BALF eosinophil ratios have been associated with pulmonary hyperresponsiveness (Hare and Viel, 1998). Further studies are required to further elucidate the role of eosinophils in the pathogenesis of mild equine asthma, as well as their effect on respiratory function (Couetil, Cardwell et al., 2016).
Treatment and prevention

The importance of prevention in mild and severe equine asthma cannot be overstated. Air quality within the horse’s living environment settings is determined by choice of feed and bedding, activity within the barn, ventilation, and ambient environmental conditions, such as bushfire smoke or traffic pollution (Ivester, Couetil et al., 2014b). Within an existing barn, management practices including feeding pelleted rations, steaming hay and ensuring horses are removed from the barn during cleaning of stalls or sweeping can help reduce exposure to poor air quality (Ivester, Couetil et al., 2014b). New barn constructions should be designed to maximize natural ventilation, and the location carefully considered to reduce potential external sources of pollution, including heavy road traffic (Ivester, Couetil et al., 2014b). However, veterinary recommendations regarding changes to environmental management practices in an effort to limit dust exposure often receive poor owner compliance, with medical treatment being the preferred option for many clients (Mair and Derksen, 2000). There is limited clinical research on the efficacy of treatments in horses with mild equine asthma, therefore treatment decisions are predominantly based on consideration of studies performed on horses with severe equine asthma (Mazan, 2015; Couetil, Cardwell et al., 2016), combined with clinical experience. Historically, research focused primarily on the use of bronchodilators in horses with severe equine asthma, to address increased respiratory effort at rest due to bronchoconstriction. However, bronchoconstriction in horses with mild asthma is so minor that there is no increase in respiratory effort at rest. Therefore, the little research that exists regarding effects of treatment in horses with mild equine asthma has focused on the control of airway inflammation.

Environmental control

Reducing exposure to dust and airborne particles is the cornerstone for both the management and prevention of mild and severe equine asthma; there is strong evidence that reduced exposure to particulate matter can improve clinical signs including coughing and poor performance (Nogradi, Couetil et al., 2015). There are two main ways in which this can be achieved: by improving ventilation
in the horses’ living environment, or by reducing exposure to dust from the feed and bedding. Other factors that also influence air quality in equine environments include the level of activity within the barn, and ambient environmental conditions (Ivester, Couetil et al., 2014b).

Effect of feed

The natural diet of free-ranging horses is grass, and horses are constantly feeding small amounts, typically high in fiber and calorically dilute; high performance domestic horses are usually fed large, infrequent meals which are often energy dense and low in fiber concentration (Elia, Erb et al., 2010). Whilst hay provides the bulk of energy requirements in conventional feeding programs, its use is associated with increased exposure to particulate matter, with even the best quality hay containing inorganic dust, insect, fungal, and plant fragments (Ivester, Couetil et al., 2014b). As the greatest exposure to respirable dust occurs during feeding, this can be reduced through changing a diet from dry hay to low-dust feed (hay cubes, pellets) (Woods, Robinson et al., 1993; Clements and Pirie, 2007a), or through soaking hay prior to feeding (Clements and Pirie, 2007b). Changing a horse’s diet from dry hay to haylage can reduce exposure to respirable particulate matter by 60–70% (Clements and Pirie, 2007a); similarly, soaking hay reduced exposure by half (Ferro, Ferrucci et al., 2000). Steaming hay significantly decreases its bacterial and mold content, however, in hay challenge trials in horses with severe equine asthma effects of steaming on clinical signs, BALF cytology and cytokine profiles are inconsistent (Orard, Hue et al., 2018; Blumerich, 2012). Feeding horses a complete pelleted feed reduced total and respirable particulate matter concentrations by 70% and 99% respectively (Hunt, 2000); however, being fed concentrates as opposed to a hay diet has a negative effect on the gastric health of horses (i.e. increased risk of ulcer formation) (Murray, 1994; Andrews, Buchanan et al., 2005). Hay which provides the lowest potential for respirable particulate and fungal release include second cutting hay baled at 85% dry matter (Séguin, Garon et al., 2012) and haylage (Seguin, Lemauviel-Lavenant et al., 2010; Séguin, Garon et al., 2012).
**Effect of bedding**

Choice of bedding material also impacts endotoxin and respirable particulate exposure. Respirable dust levels can be halved by changing bedding from straw to low-dust cardboard material (Kirschvink, Di Silvestro et al., 2002). Respirable particulate concentrations are highest in stalls bedded with straw, then wood shavings; straw pellets have been associated with the lowest inhalable (particulate matter <10μm; PM$_{10}$) concentrations (Fleming, Hessel et al., 2008). This finding was repeatable, with higher respirable dust concentrations in the breathing zone for horses on straw bedding, than those bedded on wood shavings; however, it must be noted that this difference was not statistically significant (Clements and Pirie, 2007a).

**Effect of activity within barn**

Barn activity has a significant effect on air quality, with total and respirable particulate matter doubling during the day from concentrations measured overnight (Woods, Robinson et al., 1993). Respirable particulate matter concentrations increase up to 19-fold within the stall during cleaning (Clements and Pirie, 2007b; Fleming, Hessel et al., 2008), and cleaning of adjacent stalls increases particulate matter 9-fold (Clements and Pirie, 2007b). Changing bedding in an adjacent stall from straw to wood shavings, and feed from hay to haylage reduces respirable particulate concentration by 71% (Clements and Pirie, 2007b). Furthermore, activities such as sweeping the isle are associated with peak times of exposure to barn workers, based on particulate concentrations measured in the breathing zone (Samadi, Wouters et al., 2009). Individual horse activity has a significant effect on inhalable and respirable particulate matter in the breathing zone in a barn with identical management practices (Ivester, Smith et al., 2012). However, the levels of inhalable dust in the breathing zone of horses are nearly 3 times greater than that of barn workers, across 4 barns with different management systems (Samadi, Wouters et al., 2009). Removing horses from the barn during cleaning can help reduce exposure during peak times of respirable particulate matter concentrations (Ivester, Couetil et al., 2014b).
**Effect of ventilation and external sources of particulate matter**

The installation of mechanical ventilation in a barn has been shown to decrease levels of CO₂, ammonia and ultrafine particles, resulting in a significant reduction of lower airway mucus (Wålinder, Riihimäki et al., 2011).

External sources of particulate matter, such as those generated by heavy road traffic, can also impact particulate concentrations within the barn (Robinson, Karmaus et al., 2006). Whilst not necessarily feasible or practical in existing barns, new barn constructions should be designed to maximize natural ventilation, including openings for airflow at the bottom of stall doors, and the location carefully considered to reduce potential external sources of pollution, including heavy road traffic (Ivester, Couetil et al., 2014b).

**Control of airway inflammation**

Horses with mild and severe asthma have empirically been treated with corticosteroids, with reduced inflammation alleviating clinical signs. It is well documented that activated T lymphocytes release inflammatory cytokines, resulting in an increased pulmonary concentration of IL-4 and IL-8 (Ainsworth, Grünig et al., 2003; Cordeau, Joubert et al., 2004; Lavoie, Cesarini et al., 2011; Beekman, Tohver et al., 2012). The potent chemokine IL-8 induces neutrophil chemotaxis (Hammond, Lapointe et al., 1995), contributing to enhanced mucous production, bronchospasm and coughing (Li, Tsang et al., 2006). Corticosteroids directly inhibit this inflammatory cycle, resulting in improved clinical signs, at the risk of potential detrimental systemic side effects such as laminitis and immunosuppression (Cornelisse and Robinson, 2013). Corticosteroids bind to glucocorticoid receptors present at the surface of bronchial epithelial and vascular endothelial cells, which activates a complex intracellular cascade of events; the end result is altered regulation of gene transcription of multiple genes via inhibition of transcription factors nuclear factor-κB (NF-κB) and activator protein (AP)-1 (Barnes, 1996). Of particular importance is the inhibition of inflammatory cytokine synthesis (including IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-11, IL-12, IL-13, TNF-α), and increased transcription of β₂-adrenoreceptors (Barnes, 1996), thereby working synergistically with β₂-agonists (described below). Historically, the name "glucocorticoid" is a
portmanteau (glucose + cortex + steroid); it is derived from early observations that these hormones were involved in regulation of glucose metabolism, was synthesized in the adrenal cortex, and its steroidal structure. With glucocorticoid receptors being present in almost every cell, effects may be broadly classified into 2 categories: immune and metabolic, which explains why the use of potent drugs acting on these receptors may lead to side effects such as immunosuppression and laminitis (Cornelisse and Robinson, 2013). However, although the mechanisms of corticosteroid-induced side effects in the horse have been studied, they are still not well understood (Eyre, Elmes et al., 1979; Slone, Purohit et al., 1983; French, Pollitt et al., 2000).

There have been a large number of studies investigating corticosteroid use in horses with severe equine asthma; succinctly, administration has been shown to improve clinical signs, reduce airway inflammation, reverse airway smooth muscle remodeling and improve pulmonary function (Lapointe, Lavoie et al., 1993; Ammann, Vrins et al., 1998; Lavoie, Leguillette et al., 2002; Robinson, Jackson et al., 2002; Leclere, Lefebvre-Lavoie et al., 2010; Leclere, Lavoie-Lamoureux et al., 2012; Bullone, Vargas et al., 2017; Mainguy-Seers, Bessonat et al., 2019). Recently, it has been shown that dexamethasone (0.05mg/kg IM q24h) and inhaled fluticasone (3,000µg q12h) are effective at reducing hypersensitivity and hyperreactivity in horses with mild equine asthma (Léguillette R., Tohver T. et al., 2017). There was a significant decrease in the number of lymphocytes after treatment with both systemic dexamethasone and inhaled fluticasone, however, there was no significant differences in any other BALF cell type (Léguillette R., Tohver T. et al., 2017). The absence of an effect on BAL neutrophil percentage following short-term administration of glucocorticoids was consistent with severe equine asthma studies where the air quality was not improved (Leguillette, Desevaux et al., 2002; Gerber, Schott Ii et al., 2011). Interestingly, without environmental modification, long-term dexamethasone and fluticasone administration have no effect on airway neutrophilia, even after 6-7 months, although clinical signs improved (Ivester and Couëtil, 2014). However, when corticosteroid treatment is combined with measures to improve air quality, it has been shown to improve clinical signs, airway neutrophilia and inflammatory cytokines in horses with severe equine asthma (Couëtil, Chilcoat et al., 2005; Leclere, Lavoie-Lamoureux et al., 2012). Recently, it has been shown that the nebulization of injectable dexamethasone does not
induce airway inflammation and has minimal systemic bioavailability (Haspel, Giguère et al., 2018). This represents an inexpensive method to deliver a corticosteroid directly to the lungs, with the aim of avoiding systemic side-effects. The efficacy of nebulized dexamethasone in horses with mild equine asthma is examined in Chapter 4.

**Bronchodilators**

Airway smooth muscle contraction is a significant contributing factor in the diffuse airway obstruction observed in severe equine asthma, with the administration of atropine or aerosolized pirbuterol both relieving bronchospasm rapidly (Murphy, McPherson et al., 1980; Broadstone, Scott et al., 1988; Lapointe, Lavoie et al., 1993; Derksen, Olszewski et al., 1996). However, the use of bronchodilators as monotherapy should be avoided, as bronchoconstriction is largely secondary to inflammation (Derksen, 1993). It is important to note that treatment with bronchodilators should always be in conjunction with environmental control strategies to reduce exposure to dust, thus ensuring that the antigenic load reaching the lower airways is not increased (Couetil, Cardwell et al., 2016). Bronchoconstriction in horses with mild equine asthma is so slight, and horses’ lung capacity so large, that there is no increase in respiratory effort at rest; however, lung function tests such as oscillometry can detect a functional respiratory impairment that can be measured, even given the absence of labored breathing at rest (Hoffman, 2002; Mazan and Hoffman, 2003; Richard, Fortier et al., 2009a). Regulation of airway smooth muscle contraction has been extensively studied in horses, however it is still not fully understood. The parasympathetic, sympathetic, and nonadrenergic noncholinergic nervous systems all contribute to airway smooth muscle contraction (Broadstone, LeBlanc et al., 1991; LeBlanc, Broadstone et al., 1991; Wang, Yu et al., 1992; Yu, Wang et al., 1994a; Yu, Wang et al., 1994b; Wang, Yu et al., 1995). The most commonly used classes of bronchodilators are anticholinergic agents (atropine and ipratropium), β2-agonists (clenbuterol and albuterol), and methylxanthines (theophylline and pentoxifylline) (Leguillette, 2003; Couetil, Cardwell et al., 2016).

The airways are primarily innervated by the parasympathetic nervous system, whereby the neurotransmitter acetylcholine acts on M3 muscarinic receptors to induce smooth muscle contraction.
Therefore, muscarinic antagonists are generally effective bronchodilators. Whilst systemic atropine is effective in alleviating clinical signs caused by bronchoconstriction in heaves (Muylle and Oyaert, 1973; Murphy, McPherson et al., 1980; Broadstone, Scott et al., 1988), due to its gastrointestinal side effects it is not commonly used in field practice (Ducharme and Fubini, 1983); it can be useful in a research context to quickly evaluate the reversibility of severe equine asthma (Pearson and Riebold, 1989). Anticholinergic agents can be administered via aerosolization or nebulization to avoid side effects, due to a lack of systemic absorption in this form. Administration of ipratropium bromide via a metered dose inhaler using a device such as Aeromask, AeroHippus or Equine Haler is recommended at a dosage of 0.2-0.4μg/kg every 8-12 hours; via a nebulizer the recommended dosage is 2-3μg/kg with a 0.02% solution every 8-12 h (Couetil, Cardwell et al., 2016). It has been found to relieve airway obstruction in horses with severe equine asthma in a dose-dependent manner for up to 6 hours (Robinson, Derksen et al., 1993; Duvivier, Votion et al., 1997).

The airway is also innervated with β2-adrenergic receptors, which binds with adrenaline (also known as epinephrine) to mediate smooth muscle relaxation and bronchodilation (Broadstone, LeBlanc et al., 1991; LeBlanc, Broadstone et al., 1991; Johnson, 2006). Therefore, it is logical that β2-agonist drugs are commonly used as bronchodilators. Clenbuterol is recommended at a dose rate 0.8–3.2 μg/kg PO (Couetil, Cardwell et al., 2016). Excessive tracheal mucus accumulation is a feature of mild equine asthma, and increased mucociliary clearance from clenbuterol administration might be beneficial (Norton, Jackson et al., 2013). However, 25% of horses with severe equine asthma are refractory to this treatment; horses can experience side effects (e.g. trembling, sweating, nervousness) at higher dose rates (Erichsen, Aviad et al., 1994). Albuterol is a short-acting β2-agonist agent which is administered via aerosolization, however, horses can still experience side effects; current dose rate recommendations are for 1-2μg/kg every 1-3 hours (Derksen, Olszewski et al., 1999; Couetil, Cardwell et al., 2016).

Methylxanthines inhibit phosphodiesterases, preventing the inactivation of (and thus increasing) intracellular cyclic adenosine monophosphate (cAMP), indirectly leading to smooth muscle relaxation. These drugs have a wide range of effects, including anti-inflammatory properties, however, they have a narrow therapeutic window and excitability appears at plasma levels close to those causing effective
bronchodilation (McKiernan, Koritz et al., 1990; Entzian, Bitter-Suermann et al., 1998). Both commercially available methylxanthines (theophylline and pentoxifylline) are recommended for oral administration (Couetil, Cardwell et al., 2016), making the avoidance of side effects challenging due to variability in absorption (Leguillette, 2003). Pentoxifylline is a non-specific phosphodiesterase inhibitor which is effective for the treatment of severe equine asthma at high doses, although it has not been determined whether this improvement is due to an anti-inflammatory or bronchodilator effect (Leguillette, Desevaux et al., 2002).

**Response to treatment**

Human asthma control is assessed in terms of both symptom control and risk of future adverse outcomes. The level of control is the extent to which symptoms are experienced by the patient, and is determined by interactions between the patient’s genetics, underlying disease processes, treatment, environment and psychosocial factors (Taylor, Bateman et al., 2008). In comparison, there are multiple challenges associated with assessing the control of symptoms in equine asthma; therefore, the majority of peer-reviewed studies are short-term therapeutic efficacy clinical trials. As maintaining appropriate air hygiene, through a reduction in antigen and airborne dust exposure, constitutes the most important therapeutic and prophylactic approach to both mild and severe equine asthma, one of the greatest challenges in the design of clinical trials is maintaining a degree of control over environmental exposures. Currently, there is a need for a long-term longitudinal study assessing the relative and combined beneficial effects of both drug therapy and environmental management on the control of clinical signs of mild equine asthma. Indeed, even clinical research on the efficacy of treatments on airway hypersensitivity and hyperreactivity in cases of mild equine asthma is limited to one study (Leguillette, Tohver et al., 2017), with treatment decisions typically based either on clinical experience and/or data derived from horses with severe equine asthma (Mazan, 2015; Couëtil, Cardwell et al., 2016). Initially, therapeutic trials investigating severe equine asthma focused primarily on the beneficial effects of bronchodilators, in light of the lower airway obstruction and increased respiratory effort at
rest exhibited by these cases. Recently, however, the therapeutic research focus in equine asthma has partly shifted towards the control of airway inflammation.

Airway inflammation is due in part to the increased activity of transcription factors that in turn lead to an increased production of inflammatory mediators and recruitment of inflammatory cells. Therefore, the efficacy of anti-inflammatory drugs, such as corticosteroids, in severe equine asthma has partly been evaluated via their influence on the expression of selected inflammatory genes in both BALF-derived cells (Giguere, Viel et al., 2002; DeLuca, Erb et al., 2008; Bullone, Vargas et al., 2017) and bronchial epithelium (DeLuca, Erb et al., 2008). Conversely, resistance to corticosteroids has been reported in human asthmatics, and there are anecdotal reports suggestive of decreased sensitivity in equine practice (Walsh, Sexton et al., 2003; Barnes, Ito et al., 2004; Laan, Bull et al., 2006b). Airway cytology has been used as a marker of therapeutic success with a reduction in airway neutrophilia being achieved by transferring horses to a low dust feed, with a greater level of improvement achieved by the additional administration of oral dexamethasone (DeLuca, Erb et al., 2008). However, in most studies, corticosteroids as sole therapy, whether administered systemically or by inhalation, failed to normalize the airway neutrophilia, even after up to 6 months of treatment, and this may also be true in MEA (Lavoie, Leguillette et al., 2002; Lavoie, Pasloske et al., 2006; Leclere, Lavoie-Lamoureux et al., 2012; Bullone, Vargas et al., 2017; Leguillette, Tohver et al., 2017). However, glucocorticoid therapy has been shown to down-regulate some of the neutrophil functions in the airways of horses with severe equine asthma (Vargas, Boivin et al., 2017). Compared to the use of low dust feed alone, dexamethasone administration resulted in a decreased expression of IL-8, chemokine (C-X-C motif) ligand 2 (CXCL2) and IL-1β in BALF derived cells; whereas, both treatments decreased expression of IL-8 and CXCL2 in airway epithelium, compared to baseline (DeLuca, Erb et al., 2008). Similarly, low dust feed resulted in a greater decrease of IL-8 expression than that of inhaled fluticasone (Leclere, Lavoie-Lamoureux et al., 2012). Furthermore, as the anti-inflammatory properties of glucocorticoids are thought to be mediated by suppression of inflammatory gene expression via inhibition of transcription factors NF-κB and AP-1, the effect of glucocorticoid administration on these factors in BALF-derived cells and bronchial epithelium in horses with severe equine asthma have also been investigated: no significant
treatment effect was observed on the expression of either transcription factor (Couëtil, Art et al., 2006). There are currently no published studies assessing the effects of glucocorticoid administration on the activity of transcription factors beyond a treatment period of 2 weeks.

New immunomodulatory agents have been investigated in both human and equine allergic (Th-2) asthma. Recently, non-specific CpG-GNP (Nanoparticle-bound cytosine-phosphate-guanosine oligodeoxynucleotides) based immunotherapy was shown to provide an effective, allergen-independent approach to treatment of horses with severe equine asthma (Klier, Geis et al., 2018). Briefly, CpG is recognized by Toll-like receptors (TLR9), that are expressed in equine pulmonary neutrophils, macrophages, and epithelial cells (Schneberger, Caldwell et al., 2009). Ligand binding results in the stimulation of a Th-1 response, leading to the down-regulation of any Th-2 bias associated with an allergenic trigger (as in seen during an exacerbation of severe equine asthma). Furthermore, Treg lymphocytes are stimulated, helping to re-establish T-helper cell homeostasis.

**Impact of airway inflammation on performance**

The ability of the athletic horse to compensate for any minor respiratory disease, such as mild equine asthma, is limited, particularly during high intensity exercise where obligatory locomotory-respiratory coupling imposes a mechanical constraint on respiration (Lafortuna, Reinach et al., 1996). Tracheal mucus scores ≥2/5 are associated with poor performance in racehorses (Holcombe, Robinson et al. 2006); scores ≥3/5 are associated with poor performance in sports horses and dressage horses (Widmer, Doherr et al. 2009). Furthermore, mild equine asthma has been shown to decrease racing performance in a population of Thoroughbred racehorses, based on Equibase speed figure (discussed further below under Measuring performance: exercise testing) and race place (Ivester K, 2017; Ivester, Couëtil et al., 2018). Mild equine asthma also impairs gas exchange after exercise, via a worsening of exercise-induced hypoxemia and hypercapnia (Couetil and Denicola, 1999; Courouce-Malblanc, Pronost et al., 2002; Sanchez, Couetil et al., 2005). Whilst lung function tests can be useful in determining the functional significance of respiratory signs and can detect disease before more obvious clinical signs
develop (Hoffman, Mazan et al., 1998), the measured outcomes don’t necessarily relate to an owner’s questions, particularly regarding performance: What is the impact of mild equine asthma on performance? How effective are treatments at improving performance? Given the economic impact of the horse industry worldwide, these are pertinent questions that many industry players are interested in.

It is estimated that the total contribution of the horse industry to the U.S. economy alone is $122 billion, with a total employment impact of 1.7 million jobs (Federation, 2017). To address these questions, we need to understand the role of the respiratory system, particularly during exercise, and determine appropriate measured outcomes to assess performance. The concept of “performance” is explored below under **Measuring performance: exercise testing**. Chapter 5 describes a study which was performed to determine a viable means to determine aerobic and anaerobic energy contributions in the field. Appendix A describes a study which, while not central to the topic of this thesis, was performed to determine the sensitivity of the ergospirometry mask (i.e. is it able to detect a change in aerobic capacity in response to training?), which would indicate whether the equipment was suitable for addressing the hypothesis central to this thesis: treatment targeting lung inflammation improves aerobic performance in horses with mild equine asthma. This study is discussed in Chapter 6.

**Equine respiratory physiology: response to exercise**

Complex interactions between multiple major body systems including the cardiovascular, respiratory, and musculoskeletal systems are required for athletic performance. Performance achieved in many athletic activities is highly dependent on the amount of oxygen consumed by the body ($\dot{V}O_2$). The aerobic capacity of a horse can be directly measured, as maximal oxygen consumption ($\dot{V}O_{2\text{max}}$); this is considered to be the reference technique for determining cardiorespiratory fitness. Typically, $\dot{V}O_{2\text{max}}$ is characterized by demonstrating no increase in $\dot{V}O_2$ despite an increase in workload; this is typically demonstrated using an incremental treadmill test to exhaustion. Under field conditions it is difficult to conclusively demonstrate $\dot{V}O_{2\text{max}}$ with the result that the variable, $\dot{V}O_2$peak is often preferred; therefore $\dot{V}O_2$peak was used in the study described in Chapter 6. Anaerobic contributions are also important to
total energy production during high-intensity exercise, and a horses’ performance discipline can dramatically alter the relative aerobic and anaerobic contributions to total energy production. For example, high-level endurance horses have a huge $\bar{V}O_{2\text{max}}$ of $>200 \text{ ml.(kg.min)}^{-1}$ and will have very low lactates in response to an incremental exercise test to exhaustion on a treadmill, whereas a good Thoroughbred racehorse will have a $\bar{V}O_{2\text{max}}$ of 170-180 ml.(kg.min)$^{-1}$ and will frequently have blood lactates over 20mmol/L in response to the same exercise test (Léguillette, Greco-Otto et al., 2018).

Oxygen uptake is a fundamental measurement (Hanak, Jahn et al., 2001) in any exercise test and the technique used is referred to as ergospirometry. Although this technique is used extensively in human sports medicine, until recently it has only been performed in the context of equine research activities using a high-speed treadmill (Evans and Rose, 1988a). Compared to other species, horses are considered elite athletes due to their large aerobic capacity (Poole, 2004) and physiological responses to exercise which increase oxygen transport within the body (McKeever, Hinchcliff et al., 1993). In contrast to humans, who have a typical $\bar{V}O_{2\text{max}}$ of 40-50 ml.(kg.min)$^{-1}$, with a record $\bar{V}O_{2\text{max}}$ of 97.5 ml.(kg.min)$^{-1}$; the average research racehorse usually has a $\bar{V}O_{2\text{max}}$ greater than 150 ml.(kg.min)$^{-1}$ (Rose, Hodgson et al., 1988). However, despite this massive aerobic capacity, relative hypoventilation and the resultant inappropriate pulmonary gas exchange remains the limiting factor of performance in sound horses (Art and Lekeux, 1993; Roberts, Marlin et al., 1999; Ainsworth, 2008).

**Gas exchange**

At very high exercise intensities, racehorses experience arterial hypoxemia and hypercapnia, as well as marked lactatemia. The arterial partial pressure of oxygen (PaO$_2$) can decrease from 92-99 mmHg at rest to <70 mmHg when running at maximal speeds and there is an associated desaturation of hemoglobin (Bayly, Hodgson et al., 1989). Accompanying the hypoxemia, CO$_2$ retention is observed during supramaximal exercise, with PaCO$_2$ increasing from resting values of 44 mmHg to 49-54 mmHg (Bayly, Hodgson et al., 1989). Theoretically, hypoxemia can be explained be one of 4 factors: right-to-left vascular shunts, ventilation/perfusion (V/Q) mismatching, diffusion impairment and alveolar hypoventilation (Art and Bayly, 2014). The exercise-induced hypoxemia can at least partly be explained
by the post-pulmonary shunts between the bronchial and pulmonary circulation; however, a reasonable approximation is a shunt of 1%, which would decrease the arterial PaO₂ by 5mmHg, which is less than the decrease observed during exercise (Wagner, Gillespie et al., 1989). The V/Q mismatch accounts for somewhere between 25-40% of the increase in the widening of the alveolar-arterial O₂ gradient (Wagner, Gillespie et al., 1989; Nyman, Björk et al., 1995), with the remaining 60-75% being related to diffusion limitations and/or alveolar hypoventilation. There are several factors which should contribute to improved diffusion during exercise. Horses experience a mixed venous O₂ pressure as low as 16mmHg during intense exercise (Bayly, Hodgson et al., 1989), thus widening the alveolar-arterial O₂ gradient and theoretically improving O₂ extraction and diffusion. Horses also increase the surface area available for gas exchange, via the dilatation and recruitment of poorly-perfused or non-perfused sections of the pulmonary vascular bed, increasing the volume by 50-60% (Wilkins, Gleed et al., 2001). Additionally, splenic contraction (discussed below) increases the hemoglobin concentration, and thus binding sites for O₂ (Persson, 1967; Persson, Ekman et al., 1973). However, horses exhibit an 8-fold increase in cardiac output during high-intensity exercise, which substantially decreases the capillary transit time, leading to a decrease in time available for O₂ equilibration, and thus diffusion impairment (Wilkins, Gleed et al., 2001; Art and Bayly, 2014). Alveolar hypoventilation is also a contributing factor to the development of exercise-induced hypoxemia; the alveolar-arterial O₂ gradient decreases when air is substituted for a helium-oxygen mixture, which is less dense and thus permits greater alveolar ventilation (Erickson, Seaman et al., 1994). It is therefore unsurprising that even mild airway inflammation adversely impacts the equine athlete; exercise-induced hypoxemia and hypercapnia during strenuous exercise on a treadmill is exacerbated in horses with mild equine asthma (Couetil and Denicola, 1999; Courouce-Malblanc, Pronost et al., 2002; Sanchez, Couetil et al., 2005), however the effect on VO₂max in the field is unknown. Whilst performance is impacted, it is important to note that we are describing high-performing equine athletes. Chronic cases of mild equine asthma do not experience respiratory compromise to the extent that quality of life is adversely impacted, as is observed in severe equine or human asthmatics.
Many factors may contribute toward this phenomenon, including mechanical ‘ventilation limitations’ due to a short inspiration-expiration cycle imposed by the strict phase coupling observed in horses between breathing and stride frequency during galloping (Lafortuna, Reinach et al., 1996; Ainsworth, Smith et al., 1997), and/or the very high ventilatory volumes required by the horse due to its extraordinary maximal CO$_2$ production, which can exceed 20 times the resting value (Bayly, Hodgson et al., 1989). Interestingly, the compulsory locomotor-respiratory coupling can be broken occasionally in healthy horses, resulting in a ‘big respiratory cycle’ (BRC). Each BRC is followed by a ‘readjustment cycle’ which recovers normal locomotor-respiratory coupling. It has been suggested that this mechanism facilitates a transient improvement in pulmonary gas exchange, or a readjustment of the end-expiratory lung volume in the galloping horse (Jolly, Art et al., 1995).

**Anaerobic contributions**

Blood lactate concentrations exceeding 20 mmol/L are commonly observed in Thoroughbred horses following supramaximal exercise (Evans, Rainger et al., 1995). The anaerobic contributions to total energy expenditure are therefore also very important to athletic performance. At sub-maximal exercise intensities, aerobic metabolism generates a large proportion of the ATP required for cross-bridge cycling, leading to muscle power and ultimately, performance; steady-state O$_2$ consumption is assumed to reflect the total rate of energy expenditure during exercise (Green and Dawson, 1993). As exercise intensity increases beyond the anaerobic threshold, anaerobic metabolic pathways (both lactic and alactic) contribute a greater proportion of the total energy required. Anaerobic capacity is the maximum amount of ATP resynthesized via anaerobic metabolism during maximal exercise of short duration. Energy released through hydrolysis of creatine phosphate and through anaerobic glycolysis contribute to the alactic and lactic components of the anaerobic capacity respectively. Anaerobic metabolism supplies between 21.3-30% of energy requirements in horses at exercise intensities of 110-115% $\dot{V}O_{2\text{max}}$ (Eaton, Evans et al., 1995; Hinchcliff, Lauderdale et al., 2002).

Additionally, it is also important to have an awareness of the impact a horse’s performance discipline has on the relative aerobic and anaerobic contributions to total energy production. For example, high-
level endurance horses have a huge $\dot{V}O_{2}\text{max}$ of $>200 \text{ ml.(kg.min)}^{-1}$ and will have relatively low lactates in response to incremental exercise tests to exhaustion on a treadmill (Léguillette, Greco-Otto et al., 2018). Horses are adapted to the demands of endurance exercise, which is predominantly aerobic (Rose, Ilkiw et al., 1979; Lucke and Hall, 1980), although races also contain phases of anaerobic work, with modern flat endurance racing requiring a strong anaerobic finish (Flaminio, Gaughan et al., 1996). In contrast, a good Thoroughbred racehorse will have a $\dot{V}O_{2}\text{max}$ of 170-180 ml.(kg.min)$^{-1}$ and will frequently have blood lactates over 20mmol/L in response to the same exercise test (Léguillette, Greco-Otto et al., 2018).

**Blood gas transport**

The oxygen concentration of blood is predominantly determined by the concentration of hemoglobin and its degree of $O_2$ saturation. At rest, hemoglobin is nearly fully saturated (97%) above an arterial $O_2$ partial pressure ($PaO_2$) of 70mmHg; At the partial pressure in the tissues (~40mmHg), roughly 25% of $O_2$ is released to the tissues. During exercise, the tissue partial pressure of $O_2$ is further decreased, increasing the diffusion gradient. There is also a right shift in the oxyhemoglobin dissociation curve related to the decrease in pH, and the increase in the temperature of the blood and $PaCO_2$; this results in a greater release of $O_2$ (Fenger, McKeever et al., 2000).

**Splenic contraction**

The horse has also adapted to contract its spleen at the onset of exercise, delivering up to 12L of splenic blood – with a hematocrit of ~80% - into the circulation (Persson, 1967; Persson, Ekman et al., 1973). This raises the resting hematocrit from ~35% to ~60% after short, intense exercise (Persson, 1967; McKeever, Schurg et al., 1987; Seeherman and Morris, 1990); evidence suggests that the initial increase in hematocrit is primarily due to splenic contraction and release of red blood cells at the start of exercise (McKeever, Hinchcliff et al., 1993). However, this increase in hematocrit can at least partly be attributed to a 5-10% decrease in plasma volume (McKeever, Hinchcliff et al., 1993). At the onset of exercise in humans there is a decrease in plasma volume prior to the onset of sweating; there is an isotonic shift of
fluid from the vascular compartment into the interstitial space, providing fluid for the production of sweat (Harrison, 1985; Convertino, 1987).

**Hypervolemia**

Another physiological adaptation that horses, humans and dogs experience in response to exercise is hypervolemia (Oscai, Williams et al., 1968; Convertino, Greenleaf et al., 1980; McKeever, Schurg et al., 1985; McKeever, Schurg et al., 1987). This expansion of vascular volume, with the corresponding decrease in hematocrit and hemoglobin concentration at rest, disappears when training ceases (Greenleaf, Bernauer et al., 1977), and likely helps ensure an adequate venous return once blood viscosity increases following splenic contraction, as well as help meet the large cardiac output required during intense exercise (Oscai, Williams et al., 1968; Nadel, Pandolf et al., 1974; Convertino, Greenleaf et al., 1980). It is important to note that viscosity is not a major contributor to vascular resistance in horses (Fedde and Wood, 1993). Pulmonary vascular resistance is low in horses, with a roughly 2-fold decrease with moderate exercise (Manohar and Goetz, 1999); major factors contributing to this decrease are likely dilatation and increased recruitment of the pulmonary vascular bed (Wilkins, Gleed et al., 2001). Exercise of greater intensity does not result in further changes to vascular resistance, despite the increased cardiac output (Manohar and Goetz, 1999).

**Intramuscular glycogen and mitochondria**

As with other athletic species such as dogs, horses have a very high intramuscular concentration of glycogen (~140mmol/kg of muscle; wet weight), compared to humans (~80-100 mmol/kg) (Essen-Gustavsson, McMiken et al., 1989; Weibel, Taylor et al., 1996). This is important, as intramuscular concentrations of substrate help fuel muscle contractions during exercise. Transport of glucose from blood into muscle, and subsequently into the mitochondria, only accounts for <10% of energy used during high-intensity exercise, possibly due to the rate-limiting step of transportation across the sarcolemma membrane (Weibel, Taylor et al., 1996; Geor, Hinchcliff et al., 2000). Therefore, having
large intramuscular stores of glycogen close to the mitochondria is essential in animals that frequently undertake high-intensity exercise.

Mitochondria provide aerobic energy for muscle contraction; the greater the concentration of mitochondria in the muscle, the greater the oxidative capacity. It is therefore unsurprising that horses contain roughly twice the concentration of mitochondria per unit of muscle weight, than cattle; a similarly sized animal with a far lower aerobic capacity (Kayar, Hoppeler et al., 1989). The greater aerobic capacity of equine muscle enables a higher maximal aerobic capacity, when adequately supported by substrate availability and oxygen delivery (Hinchcliff, 2013).

Measuring performance: exercise testing

There are two types of exercise tests which have been described: clinical exercise tests, which can be used for diagnostic purposes, and performance exercise tests, which are typically performed on healthy populations to provide insight into a horses’ physical condition, and can be a valuable, objective tool to validate or assess training programs. The latter can also be used to determine the effects of interventions on objective measures of performance (Franklin and Allen, 2013). Depending on the equine discipline there can be objective measures available; previous studies which have sought to find associations with Thoroughbred racing performance have used indices such as Timeform rating or “Equibase speed figure” which provide a numeric rating based on handicapping techniques or racing time, adjusted for factors such as distance, track conditions, and run-up distance (Evans, Harris et al., 1993; Ivester, Couëtil et al., 2018). Regardless of whether a discipline has such objective measures of performance or not, exercise tests can provide greater precision and accuracy in the measurement and calculation of a large number of physiologic response variables. Exercise testing can be performed either in a laboratory setting on a high-speed treadmill, or in the field (Franklin and Allen, 2013).
High-speed treadmill studies vs field studies

There are advantages and disadvantages for both treadmill studies and field tests. Laboratory exercise tests provide a controlled environment, with sophisticated equipment, enabling tests to be easily standardized. In particular, control of speed and workload are able to be very precise. However, a major limitation of high-speed treadmill tests performed in a laboratory is the fact that they do not reflect exercise performed under genuine field conditions. The locomotion biomechanics of running on a treadmill are different (Barrey, Galloux et al. 1993), and the workload is altered (Sloet and Barneveld 1995) as horses typically run at a 5-10% slope. The breathing strategy of horses can be impacted at steeper inclines as well, although there appears to be limits to peak airflows that can be generated, regardless of the frequency of breathing (Bayly, Redman et al., 1999). Another major disadvantage is that the artificial nature of the setting does not inspire competitive running behaviour observed on a track, and horses may learn behaviour required to stop the protocol. Therefore, it is desirable to measure performance in the field.

Exercise testing in the field

Measurements pertaining to performance which are relatively easily obtained in the field include heart rate, electrocardiography, blood or plasma lactate, red cell volume and hematocrit. Field exercise tests have been designed for many different performance disciplines, including trotters and pacing horses, Thoroughbreds, eventers, show jumpers, dressage horses and endurance horses (van Oldruitenborgh-Oosterbaan, Wensing et al., 1987; Munoz, Riber et al., 1998; Couroucé, 1999; Kobayashi, Kuribara et al., 1999; Fraipont, Van Erck et al., 2012).

Cardiac response to exercise

The heart rate (HR) is an indicator of cardiac output, which is the principal determinant of maximal oxygen consumption (\(\dot{V}O_2\text{max}\)) in horses. Telemetric electrocardiography has been widely used to study the HR and ECG of horses during exercise, historically on a racetrack (Marsland, 1968; Hall, Steel et
al., 1976). Horses have a low resting HR (~30bpm), which reaches an extremely high maximum HR (HRmax; >210bpm) during high-intensity exercise (Betros, McKeever et al., 2002). However, HRmax does not adapt in response to training (Betros, McKeever et al., 2002), and is not considered a good marker of fitness. However, the HR response to exercise is linear between 120bpm and 210bpm, and this linear regression is repeatable when measured during a standardized exercise test (Evans and Rose, 1988a). This linear relationship enables the calculation of speed-related HR indexes that are commonly used to assess or compare the fitness level of horses, such as the velocity at which the HR reaches 200 (V200). Typically, an increase in V200 represents an increase in fitness; conversely, loss of fitness, increased weight, stress, cardiovascular or respiratory disease, or lameness result in a decrease in V200 (Kobayashi, Kuribara et al., 1999; Couroucé-Malblanc, Pronost et al., 2002; Richard, Fortier et al., 2010b; Fraipont, Van Erck et al., 2011). It is important to note that multiple factors can impact on this HR/speed relationship, including level of excitement, training state, disease, environmental conditions and the length of the track (Foreman, Bayly et al., 1990; Kobayashi, Kuribara et al., 1999; Couroucé, Chrétien et al., 2002; Richard, Fortier et al., 2010b). Assessment of Warmbloods during a field exercise test can be difficult, as quite often the rider and trainer, as well as the horse, is unwilling to gallop at higher speeds. Whilst track tests suitable for Warmbloods and other horses competing in non-racing disciplines have been described (van Oldruitenborgh-Oosterbaan, Wensing et al., 1987; Munoz, Riber et al., 1998; Fraipont, Van Erck et al., 2012), the use of V160, rather than V200 has been proposed for use in endurance and other saddle horses, due to closer representation of these discipline’s physiologic demands (Léguillette, Greco-Otto et al., 2018).

Lactate response to exercise

Lactate is a product of muscular metabolism; glucose is broken down and oxidized to pyruvate, lactate is produced from the pyruvate faster than the capacity of clearance/consumption mechanisms. Lactate dissociates readily, diffusing from tissue to the red blood cells and plasma, causing lactate concentrations to rise. The concept of the anaerobic threshold is the level of work at which lactate accumulates in the blood, defined empirically in humans as 4mmol/L (Wasserman, Whipp et al., 1973).
The relationship between blood lactate levels and speed of exercise is exponential and can be calculated by resolving a speed-to-lactate exponential regression equation. Portable handheld analyzers have been validated for use on horses (Tennent-Brown, Wilkins et al., 2007; Hauss, Stablein et al., 2014). Factors including site of sample collected (venous vs arterial) and type of sample (whole blood vs plasma) can affect lactate concentrations, and therefore lactate derived parameters including VLa4 and VLa10 (Miller-Graber, Lawrence et al., 1988; Ferrante, Taylor et al., 1995; Poso, Lampinen et al., 1995; Taylor, Ferrante et al., 1995; Lehnhard, Bartlett et al., 2010). Blood samples are typically taken from the jugular vein for ease of sampling; given that that mixed venous blood lactate levels are not different from carotid arterial lactate concentrations following supramaximal exercise, it is reasonable to assume that the peak blood lactate value obtained is representative of the equilibrium lactate concentration throughout the body fluid compartments (Bayly, Kingston et al., 2006). The timing of the sample is important; lactate typically reaches peak concentration 2-5 mins after supramaximal exercise, whereas it decreases immediately after cessation of submaximal exercise (Evans, Harris et al., 1993). VLa4 can be useful to compare responses between horses, or to provide serial assessments of the same horse over a training season. Blood lactate 2 and 5 mins after submaximal treadmill exercise have previously been correlated with Timeform (Evans, Harris et al., 1993), and the VLa4 was significantly decreased in intermediate or poor performing French trotters, compared to better performing horses (Leleu, Cotrel et al., 2005). Underlying respiratory disease (including mild equine asthma) can impair oxygen exchange, and might contribute to a higher than expected peak lactate concentration during exercise, and therefore a low VLa4 (Courouce-Malblanc, Deniau et al., 2010).

Aerobic capacity

The aerobic capacity can be directly measured as the maximum rate of oxygen consumption ($\dot{V}O_{2\text{max}}$) and is considered to be the reference technique for determining cardiorespiratory fitness. A few attempts have been made to measure $\dot{V}O_{2\text{max}}$ in the field in horses (Karlsen and Nadaljak 1964, Hanak, Jahn et al. 2001, Van Erck, Jakesova et al. 2006). However, the minute ventilation of horses during maximal exercise is so great (>2000L/min in racehorses) that it represents a true technological challenge to
measure ventilatory and oxygen consumption parameters without having the equipment induce resistance to airflow. Unfortunately, because of increased resistance to airflow induced by the masks that have been trialed, the $\dot{V}O_{2}\text{max}$ measurements obtained were not reliable (Van Erck, Jakesova et al. 2006). In addition, two horses collapsed under intense exercise due to the resistance to airflow induced by the system (Van Erck, Jakesova et al. 2006) (data not reported in publication; personal communication from the author to Renaud Léguillette). Recently, a mask has been developed that can accurately measure $\dot{V}O_{2}\text{max}$, airflows and tidal volumes on a breath-by-breath basis under field conditions (Sides, Bayly et al. 2014). This technology, combined with the results of Chapter 5, enabled us to investigate the hypothesis central to this thesis: that treatment targeting lung inflammation improves aerobic performance in horses with mild equine asthma; this study is discussed in Chapter 6.

**Anaerobic capacity**

While the anaerobic contributions to performance are undeniably important, the anaerobic metabolism cannot be directly measured, and many techniques have been used in humans to estimate both anaerobic power and anaerobic capacity. Anaerobic power tests include cycle ergometer tests (Vandewalle, Peres et al., 1985), stair tests (Margaria, Aghemo et al., 1966), vertical jump tests (Vandewalle, Peres et al., 1987), and force-velocity tests (Perrine and Edgerton, 1978). However, as these require subject compliance they have not been adapted for use in the horse. Tests which aim to estimate the anaerobic capacity include blood lactate (Margaria, Edwards et al., 1933), ergometric tests (both maximal effort and constant load), excess post-exercise oxygen consumption (EPOC), previously termed oxygen debt (Margaria, Cerretelli et al., 1963), and the maximum accumulated oxygen deficit (MAOD) (Medbo, Mohn et al., 1988). The only methods which have been adapted for estimating the anaerobic capacity of horses are the MAOD (Rose, Hodgson et al., 1988; Eaton, Evans et al., 1995; Hinchcliff, Lauderdale et al., 2002) and EPOC (Rose, Hodgson et al., 1988). Calculation of the MAOD requires a controlled environment, where the exercise intensity is calculated by extrapolating theoretical oxygen demands from an incremental exercise test (Rose, Hodgson et al., 1988; Eaton, Evans et al., 1995).
Training demands of many equestrian disciplines are unable to be replicated on a high-speed treadmill. The ability to accurately assess aerobic and anaerobic contributions under field conditions, at both supramaximal and sub-maximal intensities would be highly advantageous. Since MAOD cannot be determined using the above approach in uncontrolled field conditions, an alternative method has been described in humans (MAOD\textsubscript{ALT}) which enables the characterization of differential energy contributions of the aerobic, alactic anaerobic and lactic anaerobic systems under conditions where the exercise intensity is unknown or fluctuates (de Moraes Bertuzzi, Franchini et al., 2007; Bertuzzi, Franchini et al., 2010; Artioli, Bertuzzi et al., 2012). As both the aerobic and anaerobic contributions to energy production in equine disciplines are undeniably important, and there was no published technique able to perform this analysis in the field, the aim of the study described in Chapter 5 was to assess the correlation between MAOD and MAOD\textsubscript{ALT} and determine the relative contributions of the aerobic and anaerobic energy systems. We hypothesized that there would be no significant difference in the anaerobic contributions between the two techniques, thus enabling MAOD\textsubscript{ALT} to be a useful, practical technique for in the calculation of the anaerobic capacity under field situations where the exercise intensity is unknown. This technique could have wide-spread future research and clinical applications, particularly once the equine ergospirometry mask becomes more widely available.

**Hypotheses and Specific Objectives**

A diagram of the research areas covered in this thesis are shown in Figure 1.1. Specific hypotheses and objectives regarding the different themes covered are outlined below.

**Theme 1: Gene expression**

**Hypotheses**

- Airway inflammation alters cells inflammatory cytokine gene expression in the lower respiratory tract
- Treatment targeting lung inflammation alters inflammatory cytokine gene expression in the lower respiratory tract of horses with mild equine asthma

**Specific Objectives**

- To determine whether airway inflammation is associated with changes in inflammatory mRNA cytokine expression in bronchoalveolar lavage fluid
- To determine whether dexamethasone administration via injected and nebulized routes of administration changes inflammatory mRNA cytokine expression in bronchoalveolar lavage fluid

**Theme 2: Microbiota/Mycobiota**

**Hypotheses**

- Airway inflammation alters the local bacterial, fungal and viral communities in the lower respiratory tract
- Treatment targeting lung inflammation alters local bacterial, fungal and viral communities in the lower respiratory tract of horses with mild equine asthma

**Specific Objectives**

- To characterize the respiratory microbiota and mycobiota of healthy horses, and those with mild equine asthma
- To determine whether dexamethasone administration via injected and nebulized routes of administration is associated with changes in the local bacterial, fungal and viral communities of the equine respiratory tract
Figure 1.1 Representation of research areas included in this thesis

Pathogenesis
- Gene expression (Chapter 3,4)
- Microbiota (Chapter 2,4)
- Mycobiota (Chapter 4)

Performance
How can we measure the aerobic/anaerobic contributions? (Chapter 5)

Functional effects of airway inflammation treatment (Chapter 6)
Theme 3: Aerobic Performance

Hypotheses

▪ Airway inflammation causes a measurable decrease in \( \dot{V}O_2\text{max} \) in a horse during a standardized field exercise test
▪ Treatment targeting lung inflammation improves \( \dot{V}O_2\text{max} \) in horses with mild equine asthma
▪ There is no significant difference in the anaerobic contributions between MAOD and MAOD\text{ALT} in horses
▪ It is possible to determine the relative contributions of the aerobic and anaerobic energy systems in a controlled environment, and in the field

Specific Objectives

▪ To determine whether airway inflammation can decrease \( \dot{V}O_2\text{max} \) in the field
▪ To determine whether dexamethasone, salbutamol and a reduction in inhaled particulate matter increase \( \dot{V}O_2\text{max} \) in the field in horses with mild equine asthma
▪ To determine if a correlation exists between MAOD and MAOD\text{ALT} in horses
▪ To determine the relative contributions of the aerobic and anaerobic energy systems in a controlled environment, and in the field
▪ To determine whether dexamethasone, salbutamol and a reduction in inhaled particulate matter changes the relative contributions of the aerobic and anaerobic energy systems in the field in horses with mild equine asthma

Statement of contribution

Equine asthma: Integrative biologic relevance of a recently proposed nomenclature

Stephanie Bond drafted the manuscript. All authors contributed to editing the manuscript and approved the final version of the manuscript for submission.
Upper and lower respiratory tract microbiota in horses: bacterial communities associated with health and mild asthma (inflammatory airway disease) and effects of dexamethasone

Stephanie Bond carried out the study design, sample collection, sample processing and extraction, statistical analysis and drafted the manuscript. Edouard Timsit conceived the study and participated in its design, coordination of materials for sample collection and helped to draft the manuscript. Matthew Workentine performed the OTU table construction, diversity analysis and helped to draft the manuscript. Trevor Alexander participated in the DNA extraction. Renaud Léguillette participated in study design, sample collection, BALF analysis and group allocation, and helped to draft the manuscript. All authors read and approved the final manuscript.

Effect of injected dexamethasone on relative cytokine mRNA expression in bronchoalveolar lavage fluid in horses with mild equine asthma

Stephanie Bond carried out the study design, sample collection, sample processing and extraction, statistical analysis and drafted the manuscript. Jana Hundt participated in sample processing and extraction. Renaud Léguillette participated in study design, sample collection, BALF analysis and group allocation. All authors read, edited and approved the final manuscript.

Investigation on the effects of nebulized dexamethasone on relative cytokine mRNA and EHV-1,2,4,5 expression and the respiratory microbiota and mycobiotas in an equine model of asthma

Stephanie Bond participated in the study design, sample collection, sample processing and extraction, statistical analysis and drafted the manuscript. Matthew Workentine performed the OTU table construction, diversity analysis and helped to draft the manuscript. Jana Hundt participated in sample processing and extraction. The UCVM Class of 2019 participated in the study design and sample collection and processing. Angela Galezowski assisted with BALF analysis. James Gilkerson collaborated with EHV primer design, data interpretation and edited the manuscript. Renaud Léguillette participated in study design, sample collection, BALF analysis, group allocation and edited the manuscript. All authors read and approved the final manuscript.
Assessment of 2 methods to determine the relative contributions of the aerobic and anaerobic energy systems in racehorses

Stephanie Bond designed the study, collected and analyzed data, and wrote and edited the manuscript. Warwick Bayly designed the study, collected and analyzed data, and edited the manuscript. Persephone Greco-Otto participated in data collection and edited the manuscript. Raymond Sides participated in data collection and analysis. Grace Kwong assisted with statistical analysis. Renaud Léguillette helped analyze the data and edited the manuscript. All authors read and approved the final version of the manuscript.

Efficacy of injected dexamethasone, aerosolized salbutamol and reduced respirable particulate concentration on respiratory performance in horses with smoke-induced mild equine asthma

Stephanie Bond designed the study, treated the horses, collected and analyzed data, and wrote and edited the manuscript. Renaud Léguillette designed the study, performed the BALF analysis, collected and analyzed data, and edited the manuscript. Jacqueline MacLeod participated in treatment and data collection. Persephone Greco-Otto participated in data collection. Angela Galezowski performed the BALF analysis. Warwick Bayly helped analyze the data and edited the manuscript. All authors read and approved the final version of the manuscript.

Assessment of high-intensity over-ground conditioning and simulated racing on aerobic and anaerobic capacities in racehorses

Stephanie Bond designed the study, collected and analyzed data, and wrote and edited the manuscript. Warwick Bayly designed the study, collected and analyzed data, and edited the manuscript. Persephone Greco-Otto participated in data collection and edited the manuscript. Raymond Sides participated in data collection and analysis. Renaud Léguillette analyzed the data and edited the manuscript. All authors read and approved the final version of the manuscript.
Chapter 2 - Upper and lower respiratory tract microbiota in horses: bacterial communities associated with health and mild asthma (inflammatory airway disease) and effects of dexamethasone

Summary

Background: The microbial composition of the equine respiratory tract, and differences due to mild equine asthma (also called Inflammatory Airway Disease [IAD]) have not been reported. The primary treatment for control of IAD in horses are corticosteroids. The objectives were to characterize the upper and lower respiratory tract microbiota associated with respiratory health and IAD, and to investigate the effects of dexamethasone on these bacterial communities using high throughput sequencing.

Results: The respiratory microbiota of horses was dominated by four major phyla, Proteobacteria (43.85%), Actinobacteria (21.63%), Firmicutes (16.82%), and Bacteroidetes (13.24%). Fifty genera had a relative abundance >0.1%, with Sphingomonas and Pantoea being the most abundant. The upper and lower respiratory tract microbiota differed in healthy horses, with a decrease in richness in the lower airways, and 2 OTUs that differed in abundance. There was a separation between bacterial communities in the lower respiratory tract of healthy and IAD horses; 6 OTUs in the tracheal community had different abundance with disease status, with *Streptococcus* being increased in IAD horses. Treatment with dexamethasone had an effect on the lower respiratory tract microbiota of both healthy and IAD horses, with 8 OTUs increasing in abundance (including *Streptococcus*) and 1 OTU decreasing.

Conclusions: The lower respiratory tract microbiota differed between healthy and IAD horses. Further research on the role of *Streptococcus* in IAD is warranted. Dexamethasone treatment affected the lower respiratory tract microbiota, which suggests that control of bacterial overgrowth in IAD horses treated with dexamethasone could be part of the treatment strategy.
Introduction

Horses can suffer from airway inflammation, resulting in severe or mild asthma (Robinson, 2001; Couetil, Hoffman et al., 2007). Severe equine asthma (Recurrent Airway Obstruction) is less common than mild asthma in North America, and manifest by increased respiratory effort at rest (Robinson, 2001; Leguillette, 2003). Mild equine asthma (also known as Inflammatory Airway Disease [IAD]) affects up to 66% of the equine population (Wasko, Barkema et al., 2011) and results in a mild increased resistance to airflow within the lower airways (Couetil, Rosenthal et al., 2001; Bedenice, Mazan et al., 2008b; Richard, Fortier et al., 2009b; Couetil, Cardwell et al., 2016). Clinical signs of IAD are typically subtle at rest, with horses exhibiting normal respiratory effort and occasional coughing; at work, increased nasal discharge, cough and poor performance are observed (Couetil, Cardwell et al., 2016).

Although IAD is primarily an inflammatory process, an infectious component is highly suspected (Burrell, Wood et al., 1996b; Christley, Hodgson et al., 2001b; Wood, Newton et al., 2005b). The presence of Streptococcus zooepidemicus, Streptococcus pneumoniae, Actinobacillus spp., and Mycoplasma equihinis in tracheal samples was recently associated with IAD, indicating that composition of the lower respiratory tract microbiota could contribute to the pathogenesis (Wood, Newton et al., 2005b). Furthermore, microbial composition and diversity of the bronchial airways in humans with sub-optimally controlled asthma has been associated with the degree of bronchial hyperresponsiveness, suggesting that lower airway bacterial communities play a role in asthma pathogenesis (Huang, Nelson et al., 2011). However, as there is limited evidence and controversy on associating IAD with bacterial populations in the trachea, further research is required to confirm this association in horses. Furthermore, there is a need to comprehensively describe the bacterial communities present in health and disease as the overall composition of the bacterial communities, rather than the presence of individual species, is important in defining health and disease (Hilty, Burke et al., 2010; Klepac-Ceraj, Lemon et al., 2010; Huang, Nelson et al., 2011).

Airway inflammation associated with IAD is primarily treated with parenteral corticosteroids (i.e. dexamethasone) (Couetil, Cardwell et al., 2016). Corticosteroids are effective at controlling airway
inflammation and inhibiting airway hypersensitivity and hyperreactivity, thus improving pulmonary function (Léguillette R., Tohver T. et al., 2017). However, as corticosteroids can cause immunosuppression within the respiratory tract (Cohn, 1991), treating IAD with dexamethasone could potentially influence the lower respiratory tract bacterial communities, promoting the overgrowth of specific bacteria, which may in turn contribute to recrudescence of disease upon cessation of treatment. Currently, the impact of dexamethasone on the equine respiratory microbiota is unknown.

The objectives of the current study were therefore (i) to characterize the upper and lower respiratory tract microbiota associated with health and mild IAD (mild asthma) and, (ii) to investigate the effects of dexamethasone on these bacterial communities, using high throughput sequencing.

Methods

Ethics statement

This study was conducted in strict accordance with the recommendations of the Canadian Council of Animal Care. The research protocol was reviewed and approved by the University of Calgary Veterinary Sciences Animal Care Committee (AC17-0036).

Animals and study design

Thirteen deconditioned Thoroughbred horses (geldings) used for chuckwagon racing were studied over a period of 12 days. All horses had a history of coughing, resided on the same property (Okotoks, AB, Canada) and were kept outside in dirt paddocks. They were fed a diet of second-cut alfalfa hay for the duration of the trial, beginning a minimum of 2 days before initial sampling. Nasopharyngeal swabs (NPS), percutaneous transtracheal washes (TTW) and bronchoalveolar lavages (BAL) were performed on all horses (n = 13) on day 0 (Fig. 2.1). Horses were then allocated on day 1 into one of three groups based on their BAL cytology (IAD versus healthy) and random selection (among healthy horses); IAD (horses with mild equine asthma; n = 7), DEX (healthy horses treated with dexamethasone; n = 3) and CONTROL (healthy horses not treated with dexamethasone; n = 3). Horses were considered to have
mild equine asthma based on the following inclusion criteria: 1. a BAL with increased percentage of mast cells (>3%) or/and eosinophils (>0.5%) or/and neutrophils (>10%), 2. absence of laboured breathing at rest (Couetil, Cardwell et al., 2016).

Horses in IAD and DEX groups were then administered dexamethasone (20mg, IM) every morning for 10 days. No other medications were given to horses for the duration of the trial. On day 11, NPS, TTW and BAL procedures were repeated (Fig. 2.1).

Figure 2.1- Representation of protocol and treatment group allocation. Horses (n = 13) were allocated into two groups on the basis of their bronchoalveolar lavage (BAL); healthy horses (n = 6) with a normal BAL, and horses with inflammatory airway disease (IAD, n = 7) with an inflammatory BAL.

Sampling procedures
Horses were pre-medicated with acepromazine maleate (0.07-0.08mg/kg, IM/IV) approx. 30 minutes prior to procedures. Horses were sedated to effect with xylazine hydrochloride (0.4 – 0.5mg/kg, IV) and butorphanol tartrate (0.05-0.1mg/kg). Following sedation, nasopharyngeal swabs (NPS) were performed first, followed by the percutaneous transtracheal washes (TTW) and then by the bronchoalveolar lavages.
Nasopharyngeal swabs (NPS) were collected as described previously (Pusterla, Mapes et al., 2008), using long guarded swabs (27cm) with a rayon bud (Dryswab Veterinary Laryngeal, Medical Wire and Equipment, Corsham, England). Two NPS were obtained per horse (one per nasal cavity). Control swabs (n = 2) were collected each sampling day, with the tip of the swab being exposed to the barn air. Immediately after collection, NPS were placed into an Amies transport media (1.0 mL) and refrigerated at 4°C. Samples were processed within 10 hours of collection. At processing, each rayon tip was removed from the Amies transport media, which was then transferred into a sterile 1.5mL microfuge tube. The Amies and tip were stored in the microfuge tube and original transport container respectively, at -80°C pending DNA extraction.

Percutaneous transtracheal washes were performed as described previously (Christley, Hodgson et al., 1999). Briefly, a 10cmx15cm area over the mid-cervical trachea was clipped and surgically prepared. After subcutaneous local anaesthesia (2% lidocaine, 5ml), a stab incision was made through the skin and subcutaneous tissues at the mid-tracheal level and an equine TTW kit was utilized (Mila International, Item number: TW1228) according to manufacturer’s instructions. Sterile saline (15ml) was injected through the catheter and aspirated immediately. The aspirate was immediately transferred into a sterile 10 mL plain tube (Stone B and G, 2010) and stored at 4°C. Controls (n=2) were also obtained using the same sterile saline, which was flushed into the catheter portion of a sterile TTW kit. Samples were processed within 10 hours of collection. At processing, particulate matter was re-suspended in aspirate through gentle agitation via pipette and 3.0 mL of aspirate was transferred into 2 sterile 1.5ml microfuge tubes, which were then centrifuged (14,000 rpm, 3 mins) to pellet the bacteria. The resulting pellet was stored in the microfuge tubes at -80°C pending DNA extraction.

Bronchoalveolar lavages were performed as described previously (Wasko, Barkema et al., 2011), with modifications. Briefly, a balloon-tipped BAL tube (Mila International, SKU: BAL300) was inserted into a nostril and progressively passed until its tip was wedged against the wall of a bronchus. During insertion of the BAL tube through the airways, several small boluses of a 0.5% solution (120 mL total/horse) of lidocaine hydrochloride were administered to desensitize the bronchial mucosa. Two
boluses (250 ml/bolus) of sterile isotonic saline (0.9% NaCl) solution were sequentially and rapidly instilled in the bronchus and then immediately aspirated by use of sterile 60ml syringes. Lavage fluid was recovered and transferred into two 10 mL sterile EDTA tubes and kept on ice until analysis, which was performed within 6 hours of sample collection. Preparation of slides was performed with 400 µl of BAL fluid, which was centrifuged using a Cytospin (90 X g for 5 minutes) to spread the pellet on the slide and stained with modified Wright-Giemsa stain. A differential count was performed on a minimum of 400 cells; epithelial cells were not included in the differential count. A paired nonparametric test (Wilcoxon Signed Rank Test) was used to compare BAL variables between day 0 and 12. A p-value <0.05 was considered significant.

DNA extraction

Total DNA was extracted from NPS and TTW samples using a Qiagen DNEasy Tissue kit (Qiagen Inc., Mississauga, ON, Canada) with the following modifications; briefly, after thawing, the microfuge tubes containing the Amies (NPS samples) and TTW fluid were centrifuged (13,000 × g for 5 min) again to ensure that bacteria were pelleted before the DNA extraction procedures. The supernatant was discarded. The rayon tip of the NPS was removed from the applicator and then placed in microfuge tube with bacterial pellet for each sample. The pellets were re-suspended in 180 µl of enzymatic buffer containing mutanolysin (300 U ml⁻¹) and lysozyme (20 mg ml⁻¹). The mixtures were vortexed and then incubated for one hour at 37 °C. Twenty-five µl of proteinase K and 200 µl Buffer AL (without ethanol) were then added, followed by vortexing and incubation at 56 °C for 30 min. Approximately 300 mg of 0.1 mm zircon/ silica beads were added and mixed using a Tissue Lyser II (Qiagen) at 30 Hz for 5 min. The mixtures were then centrifuged (13,000 × g for 5 min), and 200 µl of ethanol was added to the supernatants, followed by vortexing. The remainder of the protocol of the DNEasy Tissue Kit was followed as per manufacturer instructions. Extracted DNA was stored at -80°C until amplification and sequencing. Blank negative controls (kit only) were included in triplicate during DNA extraction.
Amplification and sequencing

The standard Illumina 16S metagenomics library preparation protocol was used (available at: https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf). All amplification and sequencing steps were carried out at University Core DNA Services, Sequencing and Genetic Analysis Lab (University of Calgary, AB, Canada). The 16S Amplicon PCR forward primer (5’TCGTCGGCACGCAGTGTGTATAAGAGACACGCTACGGGNGGCWGCAG) and reverse primer (5’GTCTCGTGGGCTCGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) were used to amplify the V3 and V4 regions of the 16S rRNA gene. Illumina sequencing adapters and dual-index barcodes were added to the amplicon target to allow for library pooling prior to sequencing. Briefly, 16S rRNA gene amplicons were generated using a KAPA HiFi HotStart ReadyMix Kit (Kapa Biosystems) with the following PCR conditions: a 3 min initial denaturation at 95 °C followed by 25 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension of 5 min at 72 °C. Amplicon were then purified with Agencourt AMPure XP beads (Beckman Coulter Inc., ON, Canada), and sequenced on an Illumina MiSeq system (Illumina Inc., Victoria, BC, Canada) using the 2 x 300 bp paired-end sequencing kit. Negative controls were included in triplicate during amplification and sequencing.

Operational Taxonomic Unit (OTU) table construction

Raw reads were processed with cutadapt 1.8.3 (Martin, 2011b) to remove the primer sequences and any preceding adaptors. Subsequent processing was done using the UPARSE pipeline (Edgar, 2013) as implemented in usearch 8.1.1861. The forward and reverse reads were merged using the fastq_mergepairs option of usearch and subsequently filtered with usearch –fastq_filter and an expected error (EE) cut-off of 1 (Edgar and Flyvbjerg, 2015) and truncated to maximum length of 420 bp. The filtered reads were de-replicated using usearch -derep_fulllength and then clustered at 97% identity with
usearch -cluster_otus and the option ‘-minsize 2’ to remove singleton reads prior to clustering. Taxonomy was assigned to the representative sequences using the RDP naïve Bayesian classifier (Wang, Garrity et al., 2007a) as implemented in ‘assign_taxonomy’ function in the R package dada2 (Callahan, McMurdie et al., 2016a) using the RDP training set 14. The final OTU table was constructed with usearch -usearch_global and the options ‘-strand plus -id 0.97’. OTU sequences were aligned using ssu-align 0.1.1 (Nawrocki, 2009b) and a phylogenetic tree built using FastTree 2.1.8. The entire procedure was run as a Snakemake pipeline (Koster and Rahmann, 2012) and code for the pipeline (version 1.0.1) is available on Github (https://github.com/ucvm/vmmp).

Diversity analysis

Downstream analysis was done in R 3.3.1 (RC, 2016) using phyloseq 1.16.2 (McMurdie and Holmes, 2013) and vegan 2.4-1 (Oksanen J BF, Kindt R et al., 2015). Mitochondrial and chloroplast sequences were removed as well as the top 20 most abundant OTUs that were present in the negative control samples (Fig S.1). Samples with less than 1000 sequences were also removed from downstream analysis (n = 1). Alpha-diversity was measured using Chao1 and Shannon index (CE, 1948) (using the whole OTU table) (Chao, 1987). Differences in alpha-diversity between groups was tested using a Mann-Whitney test, controlling the false discovery rate (Benjamini and Hochberg, 1995) and using a cut-off of p < 0.05 for rejecting the null hypothesis of no difference between groups. For β-diversity, only OTUs with a count of 2 or more in at least 10% of the samples were retained for further analysis. Between-sample diversity was evaluated using the Bray-Curtis distance metric on proportionally normalized OTU counts and visualized with non-metric multidimensional scaling (NMDS). In this manuscript, we refer to the OTU counts as abundance and the proportionally normalized counts as relative abundance. The generalized linear model framework as implemented in DESeq2 (Love, Huber et al., 2014b) was used to identify OTUs associated with sample type differences (upper versus lower airways), differences between untreated healthy and disease horses, and the effect of dexamethasone treatment. This approach appropriately controls for over-dispersed data and variable library sizes.
(McMurdie and Holmes, 2014). A p-value cut-off of 0.05 was specified for rejecting the null hypothesis of no difference between groups.

Results

Bronchoalveolar lavages results and enrolment

Bronchoalveolar lavages results are shown in Table 2.1. Seven horses were enrolled in the IAD group based on BAL cytology. Six horses were classified as healthy based on their BAL cytology; three were randomly enrolled in the DEX group and received dexamethasone for 10 days and three were enrolled in the CONTROL group. There was no significant difference in differential cell count for any cell type in the BAL fluid between day 0 and day 11 (Table 2.1).
Table 2.1: Median (IQR) values for cytologic evaluation of bronchoalveolar fluid obtained before (Day 0) and after 10 days of treatment (Day 11) with intramuscular dexamethasone (0.05 mg/kg SID) for horses with Inflammatory Airway Disease (IAD), and healthy horses (DEX). CONTROL horses were kept in the same environment as both IAD and DEX groups and were sampled at the same time-points, but were given no dexamethasone.

<table>
<thead>
<tr>
<th>Variable</th>
<th>IAD (7 horses)</th>
<th>DEX (3 horses)</th>
<th>CONTROL (3 horses)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 11</td>
<td>Day 0</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>10 (6.2 – 11.0)</td>
<td>11 (6.0 - 19.7)</td>
<td>3 (2.0 - 4.75)</td>
</tr>
<tr>
<td>Mast cells (%)</td>
<td>8 (5.2 – 11.0)</td>
<td>6.5 (4.2 - 7.2)</td>
<td>2 (2.0 – 2.0)</td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>0 (0.0 – 0.0)</td>
<td>0 (0.0 – 0.0)</td>
<td>0 (0.0 – 0.0)</td>
</tr>
<tr>
<td>Macrophages (%)</td>
<td>49 (45.5 – 53.0)</td>
<td>44 (37.0 - 55.5)</td>
<td>62 (53.5 – 64.0)</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>34.5 (31.7 – 36.0)</td>
<td>31 (23.2 - 42.5)</td>
<td>35 (32.0 - 40.7)</td>
</tr>
</tbody>
</table>
**Microbiota overview**

An average of 13,524 sequences per sample (min: 1,219; max: 81,289) were obtained after removal of (i) contaminating OTUs identified in the control samples (n = 20 OTUs; Supplementary Figure B1; Appendix B), and (ii) samples with less than 1000 sequences (n = 1). Using de-novo clustering, 2209 OTUs were identified and 963 OTUs remained after filtering low abundance and rare OTUs.

**Upper and lower respiratory tract microbiota in healthy horses**

Nineteen phyla were identified in the respiratory tract of healthy horses at day 0 (n = 6), with six phyla showing a relative abundance >0.1%: Actinobacteria, Bacteroidetes, Chloroflexi, Firmicutes, Proteobacteria and Verrucomicrobia (Table 2.2, Fig. 2.2). Four phyla represented 95.54% of the total abundance: Proteobacteria (43.85%), Firmicutes (16.82%), Bacteroidetes (13.24%) and Actinobacteria (21.63%) (Supplementary Figure B2). At the genus level, 50 genera had a relative abundance >0.1% with Sphingomonas and Pantoea being the most abundant (Table 2.2).

Nonmetric multidimensional scaling (NMDS) ordination with Bray-Curtis distance showed differences in bacterial communities between the upper and lower respiratory tract of healthy horses (Fig. 2.3A). Testing for a difference between the upper and lower respiratory tract microbiota revealed a change in the relative abundance of 2 OTUs, with Moraxella increased in the upper respiratory tract, and Cupriavidus increased in the lower respiratory tract (Supplementary Figure B3). There was also a significant decrease in richness at the lower respiratory tract level based on Chao1 (p=0.0043, Wilcoxon test) (Fig. 2.3B). However, Shannon index did not differ between the upper and lower respiratory tract (p=0.93, Wilcoxon test) (Fig. 2.3B), indicating that while there was an overall decrease in species richness, evenness remained unchanged.
Figure 2.2: Phyla relative abundance in the upper and lower respiratory tract in the healthy horses (n=6; H1 to H6). Note that the upper respiratory tract sample for horse 4 (H4) had low numbers of sequences after filtering contaminants and was discarded.
Table 2.2: Relative abundance of the 6 dominant phyla observed in the upper and lower respiratory tract of healthy horses (n = 6) at day 0, and relative abundance of genus within each phylum.

<table>
<thead>
<tr>
<th>Phylum (mean relative abundance, %)</th>
<th>Genus (mean relative abundance per phylum, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteobacteria (43.85%)</td>
<td>Sphingomonas (35.69%)</td>
</tr>
<tr>
<td></td>
<td>Pantoea (26.65%)</td>
</tr>
<tr>
<td></td>
<td>Pseudomonas (14.57%)</td>
</tr>
<tr>
<td></td>
<td>Massilia (5.59%)</td>
</tr>
<tr>
<td></td>
<td>Rhizobium (3.35%)</td>
</tr>
<tr>
<td></td>
<td>Mesorhizobium (2.20%)</td>
</tr>
<tr>
<td></td>
<td>Naxibacter (1.60%)</td>
</tr>
<tr>
<td></td>
<td>Serratia (1.39%)</td>
</tr>
<tr>
<td></td>
<td>Devosia (1.32%)</td>
</tr>
<tr>
<td>Actinobacteria (21.63%)</td>
<td>Agrococcus (15.67%)</td>
</tr>
<tr>
<td></td>
<td>Knoellia (12.98%)</td>
</tr>
<tr>
<td></td>
<td>Arthrobacter (12.1%)</td>
</tr>
<tr>
<td></td>
<td>Microbacterium (8.56%)</td>
</tr>
<tr>
<td></td>
<td>Corynebacterium (7.27%)</td>
</tr>
<tr>
<td></td>
<td>Brachybacterium (5.93%)</td>
</tr>
<tr>
<td></td>
<td>Ornithinimicrobium (5.06%)</td>
</tr>
<tr>
<td></td>
<td>Brevibacterium (4.06%)</td>
</tr>
<tr>
<td></td>
<td>Rhodococcus (4.03%)</td>
</tr>
<tr>
<td></td>
<td>Kocuria (3.88%)</td>
</tr>
<tr>
<td></td>
<td>Dietzia (3.71%)</td>
</tr>
<tr>
<td></td>
<td>Rothia (2.5%)</td>
</tr>
<tr>
<td></td>
<td>Clavibacter (1.87%)</td>
</tr>
<tr>
<td></td>
<td>Rathayibacter (1.65%)</td>
</tr>
<tr>
<td></td>
<td>Marmoricola (1.55%)</td>
</tr>
<tr>
<td></td>
<td>Streptomyces (1.29%)</td>
</tr>
<tr>
<td></td>
<td>Nocardoides (1.24%)</td>
</tr>
<tr>
<td>Firmicutes (16.82%)</td>
<td>Jeotgalicoccus (30.8%)</td>
</tr>
<tr>
<td></td>
<td>Planomicrobium (19.52%)</td>
</tr>
<tr>
<td></td>
<td>Gemella (16.63%)</td>
</tr>
<tr>
<td></td>
<td>Atopostipes (5.91%)</td>
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<tr>
<td></td>
<td>Bacillus (4.68%)</td>
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<tr>
<td></td>
<td>Staphylococcus (3.6%)</td>
</tr>
<tr>
<td></td>
<td>Sporosarcina (2.55%)</td>
</tr>
<tr>
<td></td>
<td>Facklamia (2.23%)</td>
</tr>
<tr>
<td></td>
<td>Trichococcus (1.85%)</td>
</tr>
<tr>
<td></td>
<td>Streptococcus (1.79%)</td>
</tr>
<tr>
<td></td>
<td>Carnobacterium (1.72%)</td>
</tr>
<tr>
<td>Phylum</td>
<td>Genus</td>
</tr>
<tr>
<td>------------------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>Bacteroidetes (13.24%)</td>
<td>Aerococcus (1.23%)</td>
</tr>
<tr>
<td></td>
<td>Hymenobacter (42.65%)</td>
</tr>
<tr>
<td></td>
<td>Pedobacter (17.93%)</td>
</tr>
<tr>
<td></td>
<td>Prevotella (17.42%)</td>
</tr>
<tr>
<td></td>
<td>Flavisolibacter (12.19%)</td>
</tr>
<tr>
<td></td>
<td>Gillisia (3.1%)</td>
</tr>
<tr>
<td></td>
<td>Chryseobacterium (2.69%)</td>
</tr>
<tr>
<td></td>
<td>Cloacibacterium (1.66%)</td>
</tr>
<tr>
<td></td>
<td>Ferruginibacter (1.51%)</td>
</tr>
<tr>
<td>Verrucomicrobia (0.79%)</td>
<td>Luteolibacter (20.93%)</td>
</tr>
<tr>
<td></td>
<td>Akkermansia (8.79%)</td>
</tr>
<tr>
<td>Chloroflexi (0.51%)</td>
<td>Sphaerobacter (97.84%)</td>
</tr>
<tr>
<td></td>
<td>Litorilinea (2.16%)</td>
</tr>
</tbody>
</table>
Figure 2.3A: Nonmetric multidimensional scaling (NMDS) ordination with Bray-Curtis distance of the upper respiratory tract (URT) and lower respiratory tract (LRT) in healthy horses (n = 6).

Figure 2.3B: Alpha diversity measures (Chao1 and Shannon) in both upper and lower respiratory tract samples. *** significant decrease in the richness of the local bacterial community in the lower respiratory tract (LRT) compared to the upper respiratory tract (URT) (p=0.0043, Wilcoxon test) in healthy horses (n = 6).
Differences between healthy and IAD horses

Differences in bacterial communities at the upper and lower respiratory tract level between disease status were visualized using NMDS (Fig. 2.4). Between healthy and IAD horses, 6 OTUs (Fig. 2.5) differed in the lower airways based on the generalized linear model. In horses with IAD, relative abundance of *Streptococcus* and an OTU assigned to the phylum Candidatus_Saccharibacteria were increased, whereas relative abundance of *Psychrobacter*, *Rhodococcus*, *Aerococcus* and *Hymenobacter spp.* were decreased (Fig. 2.5). No differentially abundant OTUs were identified between disease status in the upper respiratory tract.

Effects of dexamethasone on the upper and lower respiratory tract microbiota

Dexamethasone had an effect on the lower respiratory tract microbiota of both healthy and IAD horses (Fig. 2.6); the treatment effect was not different between disease status. After 10 days of dexamethasone administration, the lower respiratory tract of both healthy and IAD horses experienced a significant change in the abundance of 11 OTUs (Supplementary Figure B4), with 9 OTUs responding similarly between disease status (Fig. 2.7). *Peptostreptococcus*, *Porphyromonas*, *Filifactor*, *Streptococcus*, *Porphyromonas*, *Parvimonas*, *Fusobacterium* and *Bacteroides* spp. increased from day 0 to day 11 (pre vs post treatment), whereas Candidatus_Saccharibacteria OTU decreased. There was evidence that dexamethasone treatment also decreased evenness in the lower airways of both healthy and IAD horses, however, this decrease was not statistically significant based on our pre-set level of significance (Supplementary Figure B5). No treatment effect was observed on the upper respiratory tract microbiota (Fig. 2.6).

No differentially abundant OTUs were detected between time points (d 0 and d 11) for the CONTROL group (i.e. healthy horses not treated with dexamethasone; data not shown) indicating that time alone did not significantly influence the airways microbiota.
Figure 2.4: Nonmetric multidimensional scaling (NMDS) ordination with Bray-Curtis distance of the differences in bacterial communities at the upper respiratory tract and lower respiratory tract level between disease status (Inflammatory Airway Disease [IAD], n = 7; Normal, n = 6).
Figure 2.5: Abundance of 6 OTUs that are statistically different in horses with Inflammatory Airway Disease (IAD) (n = 7; red bars) compared to healthy controls (n=6; blue bars) in the Lower Respiratory Tract (LRT) from samples obtained on day 0 (prior to dexamethasone treatment). No differentially abundant OTUs were identified between disease status in the upper respiratory tract (URT). Each panel shows the abundance for an individual OTU and is labelled with the taxa and taxa rank (p: Phylum or g: Genus) that was assigned to it.
Figure 2.6: Nonmetric multidimensional scaling (NMDS) ordination with Bray-Curtis distance of the dexamethasone treatment effect on the lower respiratory tract microbiota of both healthy (Normal; n=6; Triangles) and Inflammatory Airway Disease (IAD; n=7; Circles) horses (“No”: indicates no treatment: n=3 Normal horses. “Yes”: indicates dexamethasone treatment for 10 days: n=3 Normal horses and n=7 IAD horses). The horizontal axis is the Upper Respiratory Tract (URT) and Lower Respiratory Tract (LRT), and the vertical axis is dexamethasone treatment (CONTROL group did not receive dexamethasone). Pre and Post indicate day 0 and day 11 sampling time-points, respectively.
Figure 2.7: Overlapping treatment effect of dexamethasone administration for 10 days (Pre: Day 0; green bars, and Post Day 11; purple bars) in healthy horses (Normal; n = 3) and horses with Inflammatory Airway Disease (IAD; n = 7). Each panel shows the abundance for an individual OTU and is labelled with the taxa and taxa rank (p: Phylum or g: Genus) that was assigned to it.
Discussion

This study investigated for the first time the differences in airway community profiles between healthy horses and horses with mild equine asthma (IAD), and the evolution of these communities after dexamethasone treatment. Using high throughput sequencing, we showed a difference in the lower respiratory tract between healthy and IAD horses, with 6 OTUs having different abundances. However, no differences were observed between disease status at the upper respiratory tract level. Dexamethasone induced a distinct shift in the community structure of the lower respiratory tract in both healthy horses and those with IAD, with a significant change in the abundance of 11 OTUs, with 9 OTUs responding similarly to treatment. A difference in community structure was also observed between the upper and lower respiratory tract of healthy horses.

Strengths of our study design include strict attention to controls, with the inclusion of negative controls at sample collection, during extraction, and throughout sequencing. This resulted in the removal of 20 potential contaminants from analysis. Previous reports warned that contamination is a critically important issue in sequence-based microbiome analysis, particularly when samples contain a low biomass (Salter, Cox et al., 2014; Weiss, Amir et al., 2014). Another strength of our study was the technique used to collect the lower respiratory tract samples, the percutaneous transtracheal method, which is superior at avoiding contamination than using a sheathed catheter through an endoscope (Sweeney, Sweeney et al., 1989; Christley, Hodgson et al., 1999). The OTUs filtering protocol used was also stringent, which limited the impact of contamination on results presented. Finally, all horses resided on the same property and paddock, which controlled for the potential confounding variables such as location, environmental management, and husbandry procedures. A limitation of this study is its small sample size (n = 13), which could have impaired our ability to detect differences in community profiles and/or OTUs between treatment groups (type 2 error). While we did not observe any apparent outliers in the studied population, the results presented in this manuscript must be interpreted with consideration of the small sample size; results might not generalise well to other equine communities.
The differences observed between healthy and IAD horses at the lower respiratory tract level concurred with the previous report indicating that bacteria could play a role in the pathogenesis of mild equine asthma (Burrell, Wood et al., 1996b; Christley, Hodgson et al., 2001b; Wood, Newton et al., 2005b). However, in the present study, only the abundance of Streptococcus spp. was increased in horses with IAD and we did not observe a significant increase in Actinobacillus spp., Acinetobacter spp. and Mycoplasma spp as reported previously (Wood, Newton et al., 2005b). Interestingly, the presence of Streptococcus (S. zooepidemicus and S. pneumonia) in tracheal washes were previously associated with lower airway inflammation in a study conducted on 278 Thoroughbred racehorses (Wood, Burrell et al., 1993b). Furthermore, a study on human asthma also reported that the genera with the highest relative abundance in bronchial biopsy samples was Streptococcus (Millares, Bermudo et al., 2017). Based on this finding, it seems that further research is warranted on the role of Streptococcus on asthma pathogenesis in horses.

As expected, dexamethasone had a significant treatment effect on the microbiota of the lower respiratory tract in horses. After dexamethasone treatment, numerous OTUs increased in abundance, including Streptococcus spp, while Candidatus_Saccaribacteria OTU decreased in abundance. This overgrowth of certain bacteria in the lower respiratory tract could be secondary to the immunomodulation induced by the dexamethasone treatment, with the reduction in Candidatus_Saccaribacteria OTU providing supportive evidence that dexamethasone treatment also decreased evenness in the lower airways of both healthy and IAD horses. In humans, macrolide antibiotic administration has been used in addition to bronchodilators and corticosteroids to improve lung function in asthmatic patients (Kostadima, Tsiodras et al., 2004). Perhaps bacterial overgrowth in IAD horses treated with dexamethasone should be controlled to improve treatment success, especially in cases with a poor response to corticosteroids therapy.

The treatment effect of dexamethasone was not different between healthy horses and those with IAD, indicating either (i) that inflammation present in the lower respiratory tract does not influence the immunosuppressive effects of dexamethasone, (ii) that dexamethasone has a stronger effect than the
disease on the microbiota, or (iii) that we did not have enough power to detect a difference. We did not include a control group of untreated horses with IAD for ethical reasons as they were privately owned horses; however, there were no OTUs that differed in abundance over the course of the trial in the CONTROL group, thus indicating that the difference observed in the groups treated with dexamethasone was due to the treatment. While dexamethasone administration has an effect on the lower respiratory tract microbiota, it is interesting to note its lack of effect on BAL cytology in the present study. This finding is consistent with other equine asthma studies, performed on horses with both mild and severe asthma, where an improvement in pulmonary function and clinical signs was not associated with a concurrent decrease in inflammatory cells in BAL (Lavoie, Leguillette et al., 2002; Robinson, Berney et al., 2009; Léguillette R., Tohver T. et al., 2017).

This study demonstrates that the equine lung is not sterile, with the lower respiratory tract possessing a unique microbiota. However, only 2 OTUs differed between upper and lower airways, indicating that the majority of OTUs were overlapping. Interestingly, in healthy humans, the nasal microbiota contributed very little to the composition of the local bacterial communities in the lung, however, there was an overlap with the communities observed in the mouth (Bassis, Erb-Downward et al., 2015). That numerous OTUs are shared by the upper and lower tract in the present study can be explained by the fact that horses are obligate nasal breathers and have complete separation of the nasopharynx and oral cavity, except when swallowing, due to an elongated soft palate.

**Conclusions**

The respiratory microbiome of horses is diverse, but dominated by four phyla, *Proteobacteria, Firmicutes, Bacteroidetes* and *Actinobacteria*. There was a clear separation between the bacterial community in the lower respiratory tract of healthy and IAD horses, with 6 OTUs in the tracheal community having different abundance with disease status, including an increase in *Streptococcus* in horses with IAD. Based on this finding, further research is warranted on the role of *Streptococcus* on IAD pathogenesis in horses. Treatment with dexamethasone has a significant treatment effect on the
lower respiratory tract microbiome in all horses, with numerous OTUs increasing in abundance, including *Streptococcus spp.* Perhaps control of bacterial overgrowth in IAD horses treated with dexamethasone could be part of the treatment strategy.
Chapter 3 - Effect of injected dexamethasone on relative cytokine mRNA expression in bronchoalveolar lavage fluid in horses with mild equine asthma

Summary

Background: Mild equine asthma is a common inflammatory airway disease of the horse. The primary treatment for control of mild equine asthma is corticosteroids. The purpose of this study was to investigate the effects of injected dexamethasone on relative IL-1β, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p35, IL-17, IL-23, IFN-γ, Eotaxin-2 and TNF-α mRNA expression in bronchoalveolar lavage fluid in healthy Thoroughbred horses, and those with mild equine asthma.

Results: Horses with mild equine asthma had a significantly greater bronchoalveolar lavage mast cell percentage than healthy horses both before and after treatment. Mild equine asthma was associated with a 4.95-fold up-regulation of IL-17 (p= 0.026) and a 2.54-fold down-regulation of IL-10 (p= 0.049) compared to healthy horses. TNF-α was down-regulated in response to dexamethasone treatment in both healthy horses (3.03-fold, p= 0.023) and those with mild equine asthma (1.75-fold, p= 0.023). IL-5 was also down-regulated in horses with mild asthma (2.17-fold, p=0.048).

Conclusions: Horses with mild equine asthma have a lower concentration of IL-10 in BAL fluid than healthy controls which concurs with human asthmatics. The marked up-regulation of IL-17 in horses with mild asthma suggests these horses had a true tendency of “allergic” airway inflammation in response to environmental antigens. Dexamethasone administration exerted anti-inflammatory effects associated with down-regulation of TNF-α in all horses, and decreased levels of IL-5 mRNA expression in horses with mild equine asthma. The inhibition of the Th-2 response, without any alterations to the airway cytology, indicates that maintained exposure to environmental antigens perpetuates airway inflammation.
Introduction

Mild equine asthma, previously known as inflammatory airway disease, is a non-infectious inflammatory airway disease in the horse (Couëtil, Cardwell et al., 2016), which affects up to 66% of the equine population (Wasko, Barkema et al., 2011). There is evidence that the Th-1, Th-2 and Th-17 immune responses are involved in the pathogenesis of various phenotypes of mild equine asthma, as indicated by multiple studies investigating the association between inflammatory cytokines and chemokines, and inflammatory bronchoalveolar lavage (BAL) cytology (Hughes, Nicolson et al., 2011; Lavoie, Cesarini et al., 2011; Beekman, Tohver et al., 2012; Richard, Depecker et al., 2014). Recently a review proposed a “non-allergic equine asthma” phenotype (Bond, Léguillette et al., 2018), citing evidence which correlated a Th-1 response characterized by upregulation of IFN-γ mRNA in BALF-derived cells (Hughes, Nicolson et al., 2011; Lavoie, Cesarini et al., 2011; Richard, Depecker et al., 2014) with a generalized increase in BAL inflammatory cells. Furthermore, this review also linked this “non-allergic” phenotype with a Th-17 response, evidenced by increased IL-17 and IL-23 mRNA expression (Hughes, Nicolson et al., 2011; Beekman, Tohver et al., 2012), which is associated with a neutrophilic BAL. This terminology was expanded to another commonly occurring human asthma phenotype, with justification provided for the introduction of an “allergic equine asthma” phenotype (Bond, Léguillette et al., 2018) associated with a Th-2 predominant response, characterized by increased expression of IL-4 and IL-5 in BAL-derived cells (Lavoie, Cesarini et al., 2011; Beekman, Tohver et al., 2012).

Inflammatory cells are recruited through increased production of inflammatory mediators, driven by increased activity of transcription factors. Logically, the efficacy of anti-inflammatory drugs, such as corticosteroids, has been evaluated in severe equine asthma through investigation of their impact on inflammatory gene expression in both BALF-derived cells (Giguere, Viel et al., 2002; DeLuca, Erb et al., 2008; Bullone, Vargas et al., 2017) and bronchial epithelium (DeLuca, Erb et al., 2008). Whilst there is a limited amount of clinical research on the efficacy of treatment on both airway hypersensitivity and hyperreactivity in horses with mild equine asthma (Leguillette, Tohver et al., 2017), the impact of
systemic corticosteroid administration on cytokine regulation in horses with mild equine asthma has not been investigated, and is the focus of the present study. Clarification of the cytokine responses in BALF-derived cells from horses undergoing treatment with dexamethasone would facilitate a greater understanding of the possible etiopathological pathways involved in mild equine asthma, and further elucidate how corticosteroids work to reduce inflammation in the lower respiratory tract of horses. Our hypothesis was that dexamethasone treatment alters cytokine gene expression in the lower respiratory tract of both healthy horses and those with mild asthma.

Methods

Animals and study design
This was a prospective, randomized, controlled clinical trial. The procedures performed on the horses enrolled in the present study have been previously published in an investigation of the upper and lower respiratory microbiota of the horse, associated with both health and mild equine asthma (Bond, Timsit et al., 2017). Briefly, BAL fluid was collected from a herd of 13 deconditioned Thoroughbred horses (geldings; 5-27 years old; weights not obtained) used for Chuckwagon racing. Horses had a history of coughing, resided on a single property (Okotoks, AB, Canada) and were kept outside in dirt paddocks. Coughing was observed over a period of at least 2 weeks by the owner, however, no cough data was recorded since no standardized validated method was available. With the exception of coughing, horses were judged to be clinically healthy based on thorough physical examination (performed by RL). They were fed a diet of second-cut alfalfa hay for the duration of the trial, beginning a minimum of 2 days before initial sampling. BAL were performed on all horses (n = 13) on day 0. On day 1, horses were allocated into one of three treatment groups based on their BAL cytology (mild equine asthma versus healthy) and random selection (among healthy horses); MEA (horses with mild equine asthma; n = 7), DEX (healthy horses treated with dexamethasone; n = 3) and CONTROL (healthy horses not treated with dexamethasone; n = 3). Horses were showing clinical signs of chronic coughing and were considered to have mild equine asthma based on the following inclusion criteria (defined in a consensus
publication (Couëtil, Cardwell et al., 2016)): 1. a BAL with increased percentage of mast cells (> 2%) or/and eosinophils (> 0.5%) or/and neutrophils (> 10%), 2. absence of labored breathing at rest. Horses in MEA and DEX groups were then administered dexamethasone (20 mg, IM) every morning for 10 days. No other medications were given to horses for the duration of the trial. On day 11, the BAL procedure was repeated.

**Sampling procedure**

Horses were pre-medicated with acepromazine maleate (0.07-0.08 mg/kg, IM/IV) approx. thirty minutes prior to procedures. Horses were sedated to effect with xylazine hydrochloride (0.4 – 0.5 mg/kg, IV) and butorphanol tartrate (0.05-0.1 mg/kg, IV). A BAL was then performed as previously described (Bond, Timsit et al., 2017). Briefly, a balloon-tipped BAL tube (Mila International, SKU: BAL300) was inserted until wedged against the wall of a bronchus, and 2 boluses (250 ml/bolus) of sterile isotonic saline (0.9% NaCl) solution were sequentially instilled. Lavage fluid was recovered and two 10 mL aliquots were immediately stored at 4°C. A differential cell count was performed within 6 h of sample collection and was performed on a minimum of 400 cells (Fernandez, Hecker et al., 2013); epithelial cells were not included in the differential count. Preparation of slides was performed with 400μL of BAL fluid, which was centrifuged using a Cytospin (90 x g for 5 min) and stained with modified Wright-Giemsa stain. Two 50 mL aliquots of BAL fluid were centrifuged at 700 × g for 10 minutes; the supernatant was then discarded, and the cell pellets resuspended in 1.5 mL of RNAlater (Qiagen, Mississauga, Ontario, Canada). Samples were stored at −80°C until RNA extraction.

**RNA extraction, cDNA synthesis and qPCR analysis**

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Mississauga, Ontario, Canada), using 40μL RNase-free water to elute samples; the initial eluate was re-applied directly to the spin column membrane and centrifuged at 8000g for 1 min. The quantity and quality of the extracted RNA were measured using the Nanodrop (ND 1000) spectrophotometer. Contaminating genomic DNA was removed prior to cDNA synthesis using dsDNase (Thermo Scientific, #EN0771, Wilmington, DE,
USA). Approximately 500 ng total RNA was retro-transcribed with the Omniscript® Reverse Transcription Kit (Qiagen, Mississauga, Ontario, Canada), as per manufacturer instructions, with RNaseOUT (Thermo Scientific, Wilmington, DE, USA) and Oligo(dT) primers (Invitrogen, Burlington, Ontario, Canada) included in the reaction mixture. Primer sequences used for IL-1β, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-17, IL-23, IFN-γ, Eotaxin-2 and TNF-α have been previously described (Giguère and Prescott, 1999; Hughes, Nicolson et al., 2011; Beekman, Tohver et al., 2012) (Table 1). Reference genes included GAPDH, SDHA, HPRT and RPL-32, which have been shown to provide accurate normalization for gene expression studies in BALF from horses with mild equine asthma, treated with dexamethasone (Beekman, 2011). Amplification of target RNA was in 25μL total reaction volume containing 13μL PerfeCta® SYBR® Green SuperMix, Low ROX™ (Quanta Biosciences), 50nM (Eotaxin-2 and IFN-γ) and 100nM (all other genes) forward and reverse gene-specific primers, 4μL nuclease-free H2O, and was completed by adding 4μL of cDNA template. Amplification was performed in 96-well skirted qPCR plates (VWR 82006-704) in a thermal cycler (BioRad CFX96 Touch™ Real-Time PCR Detection System). The reaction was initially denatured at 95°C for 3 min, which was followed by 45 cycles of 15s denaturation at 95°C, and 30s annealing at 62 or 64°C (gene specific; Table 3.1). Fluorescence data acquisitions occurred at the end of each annealing cycle. A final melt curve analysis was run from 60-90°C at .5°C increments for 5s, with a fluorescence data acquisition after each step. Reactions were executed in triplicate, with template from samples collected on day 0 and day 11 from the same horse included on the same plate. No RT and negative controls were included on each plate. Cycle threshold (Ct) values were generated from Bio-Rad CFX Manager 3.1 software, with a user defined baseline threshold of 1146.22 (genes with 62°C annealing temperature) and 984.66 (genes with 64°C annealing temperature).

Statistical analysis

The relative expression software tool (REST), which allows for correction for PCR efficiency normalization with multiple reference genes, was used for analysis, and has been previously validated (Pfaffl, Horgan et al., 2002; Léguillette, Laviolette et al., 2009) and shown to be a powerful tool in the
investigation of relative gene expression in BALF from horses with mild equine asthma (Beekman, Tohver et al., 2012). Briefly, the REST software uses a P(H1) test for statistical analysis which represents the probability of the alternate hypothesis; that the difference between the “sample” and the “control” group is due only to chance. The hypothesis test performs 2,000 random reallocations (“Iterations”) of “samples” and “controls” between the 2 groups and counts the number of times the relative expression on the randomly assigned group is greater than that of the sample data. Subsequently the expression ratio results of the investigated genes are tested for significance by a randomisation test, which accounts for multiple comparisons. In this study, “samples” referred to the post-treatment samples collected on day 11, and “control” referred to the pre-treatment samples collected on day 0; analysis was performed with horses separated by treatment group (MEA, DEX and CONTROL). Alternatively, when the effect of airway inflammation was examined, “sample” referred to the MEA group and “control” referred to horses with a normal BAL at day 0 (DEX and CONTROL groups). Normality of the distribution of the BALF differential cell counts were tested by a Shapiro-Wilk normality test. A two-way repeated measures ANOVA (controlling for treatment group and timepoint [Day 0 versus Day 11]) was used to assess differences in cell counts between groups. A p-value ≤ .05 was considered significant.

Results

Cytology results and enrolment

Of the seven horses enrolled in the MEA group based on BAL cytology; five horses had a mixed inflammatory profile (neutrophilic/mastocytic inflammation), and two horses exhibited mastocytic inflammation (Figure 3.1). Six horses were classified as healthy based on their BAL cytology and clinical examination; three were randomly enrolled in the DEX group and received dexamethasone for 10 days and three were enrolled in the CONTROL group. Bronchoalveolar lavage fluid differential cell counts (±SD) for each group on day 0 and day 11 are shown in Figure 3.1.
<table>
<thead>
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<th>Sequence (5’-3’)</th>
<th>PCR Product size (bp)</th>
<th>Annealing temperature (°C)</th>
<th>Sequence accession number(s)</th>
<th>Reference(s)</th>
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<td>Giguère, 1999</td>
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Figure 3.1: Bronchoalveolar lavage fluid differential cell counts (Mean ±SD) in 7 horses with mild equine asthma (MEA), and 3 healthy horses randomly allocated as a treatment control (DEX control), before (Day 0) and after (Day 11) treatment with 20mg Dexamethasone IM SID. BALF differential cell counts for no treatment controls (n=3, Control) are also provided.
After accounting for the treatment group, there was no significant difference in differential cell count for any cell type between day 0 and day 11. The MEA group had a significantly greater mast cell percentage than both the DEX control group (p= 0.003) and the CONTROL group (p= 0.004); there was no difference between the DEX control group and the CONTROL group (p= 0.80) (Figure 3.1). Although the MEA group had a greater neutrophil percentage than both the DEX group and the CONTROL group at both timepoints, after accounting for timepoint, there was no significant difference in the neutrophil cell count between treatment groups (p= 0.051). There was no significant difference in any other cell count (Lymphocyte, Eosinophil or Macrophage) between treatment groups.

Relative gene expression – healthy versus mild equine asthma

Mild equine asthma was associated with a 4.95-fold up-regulation of IL-17 (p= 0.026) and a 2.54-fold down-regulation of IL-10 (p= 0.049) compared to healthy horses (combined CONTROL group and DEX control group) on day 0 (Table 3.2).

Relative gene expression – effects of time/dexamethasone

There was no significant change in relative expression levels of any gene investigated (IL-1β, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-17, IL-23, IFN-γ, Eotaxin-2 or TNF-α) in the BALF from the CONTROL group between day 0 and day 11.

In the DEX control group, TNF-α was down-regulated in response to dexamethasone treatment 3.03-fold (p= 0.023) (Table 3.3). There was no dexamethasone treatment effect on relative expression of IL-1β, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-17, IL-23 or Eotaxin-2 (Table 3.3). IFN-γ was not present in detectable quantities at either timepoint in this treatment group.

In the MEA group, IL-5 was down-regulated 2.17-fold in response to treatment (p= 0.048) (Table 3.4). Furthermore, TNF-α was also down-regulated 1.75-fold in response to treatment (p= 0.023) (Table 3.4). There was no dexamethasone treatment effect on relative expression of IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17, IL-23, IFN-γ or Eotaxin-2 (Table 3.4).
Table 3.2: Relative gene expression in the BALF of 7 horses with mild equine asthma compared to healthy controls on day 0. The normalization factor (calculated from multiple reference genes) has a value of 1, and genes of interest are either up-regulated or down-regulated, in association with lower airway inflammation. * indicates a significant difference (p<0.05)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Expression</th>
<th>Regulation</th>
<th>Standard Error</th>
<th>95% C.I.</th>
<th>P value</th>
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<tbody>
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<td>IL-1B</td>
<td>1.15</td>
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<td>0.07 - 7.25</td>
<td>0.01 - 18.51</td>
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<td>1.96</td>
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<td>0.08</td>
</tr>
<tr>
<td>IL-10</td>
<td>2.56</td>
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<td>0.06 - 2.73</td>
<td>0.01 - 8.37</td>
<td>0.049*</td>
</tr>
<tr>
<td>IL-17</td>
<td>4.95</td>
<td>UP</td>
<td>0.24 - 74.33</td>
<td>0.01 - 1,407.10</td>
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<tr>
<td>IL-23</td>
<td>1.41</td>
<td>DOWN</td>
<td>0.14 - 3.39</td>
<td>0.01 - 7.63</td>
<td>0.42</td>
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<tr>
<td>Eotaxin-2</td>
<td>3.49</td>
<td>UP</td>
<td>0.31 - 35.10</td>
<td>0.12 - 2,104.64</td>
<td>0.053</td>
</tr>
<tr>
<td>TNF-a</td>
<td>1.01</td>
<td>DOWN</td>
<td>0.32 - 3.41</td>
<td>0.07 - 8.24</td>
<td>0.99</td>
</tr>
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</table>

Table 3.3: Relative gene expression in 3 healthy horses before and after treatment with 20mg dexamethasone IM SID for 10 days. The normalization factor (calculated from multiple reference genes) has a value of 1, and genes of interest are either up-regulated or down-regulated in response to treatment. * indicates a significant difference (p<0.05)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Expression</th>
<th>Regulation</th>
<th>Standard Error</th>
<th>95% C.I.</th>
<th>P value</th>
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<tr>
<td>IL-1B</td>
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<td>0.01 - 21.33</td>
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<tr>
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<td>0.16 - 4.04</td>
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<tr>
<td>IL-5</td>
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<td>0.01 - 17.84</td>
<td>0.01 - 29.49</td>
<td>0.5</td>
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<tr>
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<td>0.02 - 84.78</td>
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<td>IL-12</td>
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<td>0.02 - 2.85</td>
<td>0.01 - 4.56</td>
<td>0.11</td>
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<tr>
<td>Eotaxin-2</td>
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<td>DOWN</td>
<td>0.09 - 6.38</td>
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<tr>
<td>TNF-a</td>
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<td>DOWN</td>
<td>0.07 - 1.12</td>
<td>0.05 - 3.44</td>
<td>0.023*</td>
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</table>

Table 3.4: Relative gene expression in 7 horses with mild equine asthma before and after treatment with 20mg dexamethasone IM SID for 10 days. The normalization factor (calculated from multiple reference genes) has a value of 1, and genes of interest are either up-regulated or down-regulated in response to treatment. * indicate a significant difference (p<0.05)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Expression</th>
<th>Regulation</th>
<th>Standard Error</th>
<th>95% C.I.</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1B</td>
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<td>0.26 - 4.83</td>
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<td>1.82</td>
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<td>IL-12</td>
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<tr>
<td>Eotaxin-2</td>
<td>1.16</td>
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<td>0.04 - 42.07</td>
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<td>TNF-a</td>
<td>1.75</td>
<td>DOWN</td>
<td>0.19 - 1.69</td>
<td>0.07 - 3.55</td>
<td>0.023*</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>2.70</td>
<td>DOWN</td>
<td>0.01 - 10.68</td>
<td>0.01 - 22.52</td>
<td>0.21</td>
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</table>
Discussion

This study reports the effects of systemic administration of an anti-inflammatory corticosteroid medication, injected dexamethasone, on inflammatory gene expression in BAL-derived cells from healthy horses, and those with mild equine asthma. Horses with mild equine asthma had a significantly greater percentage of mast cell percentage than healthy horses before and also after dexamethasone treatment. Horses with mild equine asthma had up-regulation of IL-17 (4.95-fold) and down-regulation of IL-10 (2.56-fold) compared to healthy horses. In all horses, treatment with injected dexamethasone was associated with down-regulation of TNF-α. Dexamethasone administration was also associated with down-regulation of IL-5 in horses with mild equine asthma.

Airway cytology is used in clinics as an indicator of therapeutic success, however, a literature review shows that without environmental modifications, corticosteroid therapy alone fails to normalize airway neutrophilia, even after treatment periods of up to 6 months (Lavoie, Leguillette et al., 2002; Lavoie, Pasloske et al., 2006; Leclere, Lavoie-Lamoureux et al., 2012; Bullone, Vargas et al., 2017; Leguillette, Tohver et al., 2017). However, a reduction in airway neutrophilia has been achieved by transferring horses to a low dust feed, with the addition of oral dexamethasone administration being associated with greater improvement (DeLuca, Erb et al., 2008). In contrast to severe equine asthma, it could be hypothesized that a lesser degree of inflammation might be controlled with corticosteroid treatment only, even in the presence of suboptimal environmental conditions. However, the absence of an improvement in the mastocytic and mixed inflammatory BAL cytologic profiles of the MEA group in the present study after 10 days of dexamethasone treatment, without switching to a low-dust feed or other environmental modifications, dismisses such a hypothesis. Even for horses with mild asthma, poor environmental conditions have a greater impact than dexamethasone therapy, and if persistent, sustain lung inflammation. Furthermore, the benefit of using an intramuscular route of administration for dexamethasone treatment guarantees bioavailability (Picandet, Leguillette et al., 2003); the lack of cytological improvement was therefore not due to poor absorption.
The fact that environmental conditions were not changed avoided the potential for this to contribute as a confounding factor. The effect of environmental dust has been well described in horses with severe asthma, and the present study provides valuable information on the effects of treating horses with milder inflammation without changing environmental conditions. In reality, it is challenging for horse owners to decrease environmental dust and this study design therefore mimics current practice where horses with mild asthma are treated with corticosteroids without significant environmental changes.

A limitation that was considered during analysis was that the authors were unable to determine whether coughing increased or decreased in response to dexamethasone treatment, as no numerical cough data was recorded. However, standardized quantification of coughing in horses to assess the efficacy of treatments is challenging. For example, a previous study showed that contrary to horse owners, who spend more time with their horses on a daily basis, clinicians would not be able to notice an improvement in the clinical condition of horses with severe asthma after corticosteroid therapy (Gerber, Schott Ii et al., 2011). Furthermore, whilst the consensus statement inclusion criteria requires that coughing be chronic (>3 weeks) (Couëtil, Cardwell et al., 2016), due to limited access to the horses enrolled in the study, the authors believe the observed period of coughing of >2 weeks was sufficient to determine chronicity of disease in this instance. As these horses had evidence of lower airway inflammation, and the focus of this study was on the immunomodulatory effects of dexamethasone treatment and not on treatment effects on performance or clinical signs, it was felt that the lack of objective data regarding coughing was insufficient justification to preclude their enrollment in the study.

All horses with mild equine asthma were treated with dexamethasone for ethical reasons; furthermore, to include an additional category with horses with mild equine asthma without treatment would not assist in answering our question regarding the treatment effect of dexamethasone in horses with this disease. Therefore, the only treatment category not included was that of horses with mild asthma treated with a placebo. It is noteworthy that three healthy horses were not treated to control for the effect of
time and stress of sampling on BAL inflammatory cytokine expression. However, the authors do acknowledge that three horses in each of the control groups (CONTROL and DEX) is a small number, and results obtained and presented in this manuscript should thus be interpreted with caution.

In human asthma, there is accumulating evidence which suggests that IL-17 production plays a key role in severe forms of asthma (Wang and Wills-Karp, 2011). Since our results are based on mRNA, which can undergo post-transcriptional regulation and might therefore not reflect true protein concentrations, the presence of a 4.95-fold up-regulation of IL-17 is significant and might reflect horses with a true tendency of “allergic” airway inflammation in response to environmental antigens. Interestingly, in comparison with healthy horses, those with mild equine asthma also had a 2.56-fold down-regulation of IL-10. IL-10 is the intrinsic physiologic mechanism that inhibits pro-inflammatory cytokine synthesis (Borish, Aarons et al., 1996). In healthy human lungs, alveolar macrophages and circulating monocytes are the main sources of IL-10 (Rosenwasser and Borish, 1997). In human BAL fluid, alveolar macrophages constitute >80% of cells present; Th1/Th2 lymphocytes, cytotoxic B cells, B lymphocytes and mast cells comprise less than 10% of the cellular population (Borish, Aarons et al., 1996). In agreement with the findings of the present study, the concentration of IL-10 in BAL fluid of human asthmatic patients is lower than in healthy controls, and an inverse association between asthma severity and IL-10 concentration has been established (Borish, Aarons et al., 1996). The absence, or reduced concentration of IL-10, associated with asthma enables the continued secretion of pro-inflammatory cytokines that contribute to lower airway inflammation, including IL-6, IL-5, IL-4, TNF-α, and IL-1.

In horses with mild asthma, we observed a down-regulation of IL-5 in response to dexamethasone administration. In humans, IL-5 is highly specific for eosinophilic inflammation, and antibodies which block IL-5 actions are effective in reducing eosinophilic inflammation and airway hyperresponsiveness (Barnes, 2001). Whilst eosinophils are less commonly detected in equine BALF, excepting a sub-group of MEA reported predominantly in young horses associated with dust exposure (Riihimaki, Raine et
al., 2008; Ivester, Couetil et al., 2014a), it appears that environmental antigens are associated with both the clinical signs and lower airway inflammatory pathology observed in horses with MEA (Bond, Léguillette et al., 2018). Furthermore, while not a focus of the present study, corticosteroid administration has been shown to reduce both airway hypersensitivity and hyperreactivity in horses with MEA (Leguillette, Tohver et al., 2017). In mice, a single antigen challenge has been shown to increase IL-5 protein and mRNA in BALF and lung tissue, with dexamethasone treatment reducing both airway hyperresponsiveness, and IL-5 mRNA in BALF (Eum, Maghni et al., 2003). Consistent with this, the down-regulation of IL-5 observed in response to dexamethasone treatment in horses with MEA indicates a shift away from a dysregulated Th-2 response after allergen exposure.

Treatment with injected dexamethasone was also associated with down-regulation of TNF-α in all horses. In humans, it is acknowledged that TNF-α plays an important role in allergic inflammation of the bronchus, with increased levels of expression being reported in the serum of patients with allergic asthma in acute attack, compared to healthy individuals or asthmatics in clinical remission (Jiang, Yang et al., 2018). Furthermore, a week of oral glucocorticoid administration decreases serum TNF-α levels following an allergic asthma attack (Guanghui, Rongfei et al., 2005). As glucocorticoid administration exerts an anti-inflammatory effect and is capable of decreasing TNF-α levels, it is therefore logical that there was a larger anti-inflammatory effect exerted on horses experiencing airway inflammation.

Conclusions

Horses with mild equine asthma have a lower concentration of IL-10 in BAL fluid than healthy controls, which concurs with human asthmatics; the possible inverse association between equine asthma severity and IL-10 concentration warrants further investigation. The marked up-regulation of IL-17 in horses with mild asthma suggests these horses had a true tendency of “allergic” airway inflammation in response to environmental antigens. Dexamethasone administration exerted anti-inflammatory effects associated with down-regulation of TNF-α in all horses, and decreased levels of IL-5 mRNA expression
in horses with mild equine asthma. The inhibition of the Th-2 response, without any alterations to the airway cytology, indicates that while dexamethasone administration can help to reduce airway hypersensitivity and hyperreactivity, maintained exposure to environmental antigens perpetuates airway inflammation.
Chapter 4 - Effects of treatment on respiratory cytokine mRNA and micro/mycobiota in an equine model of asthma

Summary

Rationale: Prolonged exposure to environmental antigens elicits an immune response in both healthy horses and those with asthma. Corticosteroids are often used to treat lower airway inflammation in both horses and humans.

Objective: To investigate the changes in expression of inflammatory cytokine mRNA, equine herpesvirus (EHV)-1,2,4,5 glycoprotein B gene expression and changes in respiratory bacterial and fungal communities following nebulized dexamethasone treatment of horses in an equine model of asthma.

Methods: Prospective, randomized, controlled, blinded clinical trial (n=20). PCR amplification of inflammatory cytokine mRNA and EHV-1,2,4,5 in bronchoalveolar lavage fluid, and 16S (microbiome) and ITS2 (mycobiome) genes and subsequent sequencing was performed on DNA extracted from nasal swab and transendoscopic tracheal wash samples before and after 13 days treatment with nebulized dexamethasone (15mg SID) and saline.

Results: Nebulized dexamethasone treatment resulted in a significant drop in microbial diversity and a significant change in the relative abundance of eight genera in the upper respiratory tract. Treatment-specific effects were tested with two genera (Alysiella and Bordetella) showing a differential effect. Beta-diversity analysis showed that exposure to a dusty environment was more important than treatment effects. The mycobiota was dominated by timepoint effects. Treatment with saline altered the abundance of 12 genera; dexamethasone treatment altered nine genera. Alternaria, a known opportunistic pathogen and allergen in humans, was significantly increased in both treatment groups.

Conclusions: Nebulized dexamethasone treatment affected the upper respiratory tract microbiota, but not the mycobiota, which was overwhelmed by the effect of a sustained dusty environment.
Introduction

Mild equine asthma (MEA) is a commonly occurring inflammatory airway disease in the horse (Couëtil, Cardwell et al., 2016) which provides a biologically appropriate model for allergic and non-allergic phenotypes of human asthma (Bond, Léguillette et al., 2018). It is a naturally occurring disease that, similar to human asthma, is likely triggered by a combination of pathophysiological mechanisms. Interactions between environmental factors and immunological “defects” could possibly be central to the development of an exaggerated lower airway inflammatory immune response. Exposure to an allergenic environment for a prolonged period elicits an immune response in the respiratory tract of both healthy and asthmatic horses and humans, which appears to be dose-dependent (Tremblay, Ferland et al., 1993; Pirie, Dixon et al., 2001; Desjardins, Theoret et al., 2004; Gerber, Lindberg et al., 2004; Leclere, Lavoie-Lamoureux et al., 2011). However, the magnitude, rapidity of onset and immunological characteristics of this response, combined with adaptations to the local bacterial, fungal and viral communities of the respiratory tract, could provide key insights into asthma phenotype expression. It is as yet unknown whether these adaptations reflect the cause or effect of lower airway inflammation observed in asthma.

The large physical size and relative ease of respiratory sample collection in horses constitutes a clear benefit when compared to the invasive nature of performing these tests on humans (Bullone and Lavoie, 2015). Furthermore, inclusion of appropriate, untreated negative control groups that are a significant ethical difficulty in human studies, can be included in equine asthma studies. This represents a unique opportunity to perform a large-scale investigation into the interactions between the immune response and the local environment in the lungs, as well as looking at translocation of microbial flora and interactions between the upper and lower respiratory tract. Whilst the pathophysiologic mechanisms behind the disease are not fully elucidated, it is widely thought to be a multifactorial disease, with environmental factors central to the development of lower airway inflammation (Couëtil, Cardwell et al., 2016). High levels of airborne particulates including dust, endotoxin, fungi, molds, ultrafine particles and noxious gases are found in conventional horse stables (Ivester, Couëtil et al., 2014b), and
there is strong evidence that stabling of horses is a risk factor for mild equine asthma (Holcombe, Jackson et al., 2001; Gerber, Robinson et al., 2003; Millerick-May, Karmaus et al., 2013; Ivester, Couetil et al., 2014a). Furthermore, a study investigating biomarkers involved in the development of airway disease reported that some employees involved in the care and training of horses have signs of bronchial obstruction, which might be provoked by working in a stable environment (Elfman, Riihimäki et al., 2009). However, veterinary recommendations regarding changes to environmental management practices in an effort to limit dust exposure often receive poor owner compliance due to challenges with implementation, and medical treatment, primarily corticosteroids to reduce airway inflammation, is the preferred option for many clients (Mair and Derksen, 2000).

Although viral infection is a common cause of transient lower airway inflammation in horses, the role of viral infection in the pathogenesis of mild equine asthma is still controversial (Newton, Wood et al., 2003; Wood, Newton et al., 2005a; Fortier, Van Erck et al., 2009). Viral respiratory tract infections have a profound effect on the expression of asthma, as well as disease exacerbation, and are a major cause of morbidity (Busse, Lemanske Jr et al., 2010). While acute infection with viruses including equine herpesvirus (EHV)-1 and EHV-4 can be subclinical or present with mild clinical signs consistent with mild equine asthma; infections are usually self-limiting (Diaz-Mendez, Viel et al., 2010). Whilst EHV-2 and EHV-5 are ubiquitous in healthy horses (Fortier, Richard et al., 2013; Hartley, Dynon et al., 2013; Hue, Fortier et al., 2014) they are frequently identified in respiratory secretions of horses with respiratory disease (Fortier, Van Erck et al., 2009; Hue, Fortier et al., 2014), and there is some evidence that infection with EHV-2 is associated with poor performance and airway inflammation (Fortier, Van Erck et al., 2009). A frequent observation made after cessation of treatment for mild equine asthma is the re-emergence of clinical signs. Given the ubiquitous nature of EHV, as well as their ability to induce latent infection and recrudesce in times of immunosuppression or stress, it is logical to question their role in mild equine asthma.
Recently, it has been shown that the nebulization of injectable dexamethasone does not induce airway inflammation and has minimal systemic bioavailability (Haspel, Giguère et al., 2018). This represents an inexpensive method to deliver a corticosteroid directly to the lungs, with the intent to decrease possible systemic side-effects. As owner compliance with environmental improvements is often poor, the objective of this study was to investigate the effects of nebulized dexamethasone on relative inflammatory cytokine mRNA and EHV-1,2,4,5 expression in bronchoalveolar lavage (BAL) derived cells, and on the local bacterial and fungal communities of the respiratory tract whilst housing horses in indoor stables and feeding from hay nets.

**Materials and Methods**

For an extended description of Materials and Methods used for this study, please refer to Appendix C.

*Animals and study design*

This was a prospective, randomized, controlled, blinded clinical trial. Geldings (n=20, 435 - 612kg) were enrolled in the study based on an external veterinarian’s diagnosis of mild equine asthma (MEA); horses had a history of nasal mucus and coughing. Inclusion criteria were a clinical examination within normal limits, aside from nasal mucus and coughing, and no signs of systemic illness on complete blood count or chemistry analysis. Horses resided on 2 properties (Lake Louise and Cochrane, AB, Canada), and were transferred to an indoor stable on Day -7. Individual stalls with open tops, enabling free movement of air between stalls, with straw bedding were used. Horses were fed grass hay suspended in hay nets for the duration of the trial and were given free access to water. Horses were lightly exercised every second day, and cough scoring was performed during exercise and stall cleaning (data not shown). Respirable dust concentrations were measured for 4-minute sampling periods every 4 hours throughout the trial (SidePak AM520, TSI®, MN, USA) using a PM₄ impactor (4μm). Nasal swabs, transendoscopic tracheal washes and bronchoalveolar lavages (BAL) were performed on all horses (n = 20) on day 0 (Figure 4.1). On day 1, horses were allocated into one of three treatment groups (A, B, C) based on their BAL cytology (MEA *versus* horses with a non-inflammatory BAL profile
(healthy) and random selection (within horses with MEA) (Figure 4.1). Horses were considered to have MEA based on the following inclusion criteria: 1. inflammatory BAL with an increased percentage of mast cells (>2%) or/and eosinophils (> .5%) or/and neutrophils (> 5%); 2. History of nasal mucus, coughing or both; 3. absence of labored breathing at rest (Couëtil, Cardwell et al., 2016). Group A was treated with 15mg (5mg/mL) nebulized dexamethasone sodium phosphate SID (horses with MEA; n = 8), Group B was treated with 3mL of nebulized saline SID (horses with MEA; n = 8) and Group C was a no treatment environmental control (healthy horses; n = 4) (Figure 4.1). All horses were treated for 13 days, and the nasal swab, transendoscopic tracheal wash and BAL procedures were repeated on Day 14 (Figure 4.1). No other medications were given to horses for the duration of the trial. Those administering treatments (UCVM class) were blinded to the treatment provided to Groups A and B, as were the specialists (RL, AG) who reported the BAL results.

**Sampling procedures**

For details of the sampling procedures, which involved the collection of nasal swab samples first, then transendoscopic tracheal wash samples and finally bronchoalveolar lavage collection, whilst horses were sedated, please refer to Appendix C. A differential count of BALF was performed on a minimum of 400 cells for allocation of treatment groups; epithelial cells were not included in the differential count (Fernandez, Hecker et al., 2013). Details of slide preparation and statistical analysis are provided in the online data supplement. A two-way repeated measures ANOVA (controlling for treatment group and timepoint [Day 0 versus Day 14]) was used to assess differences in cell counts between groups. A p-value ≤ 0.05 was considered significant.

**RNA extraction, cDNA synthesis and qPCR analysis**

Details of RNA extraction, cDNA synthesis and qPCR analysis are provided in Appendix C. Total RNA was extracted from BAL samples using the RNeasy Mini Kit (Qiagen, Mississauga, Ontario, Canada), as per manufacturer instructions, using 40μL RNase-free water to elute samples. Amplification of target RNA (IL-1β, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-17, IL-23, IFN-γ, Eotaxin-2 and TNF-α) was
performed using previously optimized reaction conditions (Giguère and Prescott, 1999; Hughes, Nicolson et al., 2011; Beekman, Tohver et al., 2012). Reference genes included GAPDH, SDHA, HPRT and RPL-32 (Beekman, 2011).
Figure 4.1: Representation of protocol and treatment group allocation. Horses (n = 20) were allocated into two groups on the basis of their bronchoalveolar lavage (BAL): healthy horses (n = 4) with a normal BAL, and horses with mild equine asthma (MEA, n = 16). Dex = dexamethasone.
DNA extraction

Total DNA was extracted from nasal swab and transendoscopic tracheal wash samples using a Qiagen DNEasy Tissue kit (Qiagen Inc., Mississauga, ON, Canada) as previously described (Bond, Timsit et al., 2017).

EHV qPCR analysis

Primer sequences and reaction condition for EHV-1, EHV-2, EHV-4 and EHV-5 have been previously described (Dynon, 2010). To provide normalization, β2M was used as a reference gene (Dynon, 2010).

qPCR statistical analysis

The relative expression software tool (REST), which allows for correction for PCR efficiency and normalization with multiple reference genes, was used for analysis, which has been previously validated (Pfaffl, Horgan et al., 2002; Léguillette, Laviolette et al., 2009) and shown to be a powerful tool in the investigation of relative gene expression in BALF from horses with mild equine asthma (Beekman, Tohver et al., 2012).

16S and ITS amplification, sequencing, processing and analysis

The 16S amplicon PCR forward primer (5’GTGYCAGCMGCGCGGTAA) and reverse primer (5’GGACTACNVGGGTWTCTAAT) with forward-primer barcodes were used to amplify the V4 variable region. The ITS2 amplicon PCR ITS1F forward primer (5’CTTGGTCATTTAGAGGAAGTAA) and ITS2 reverse primer (5’GCTGCCGTTCCTCTCAGATGC) with forward-primer barcodes were used. Sequencing was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on an Illumina MiSeq system using the 2 × 300 bp paired-end sequencing kit following the manufacturer’s guidelines. All reaction conditions, sequence processing details and statistical analysis performed are provided in Appendix C.
Results

Dust concentrations
Dust concentrations of particulates <4μm (g/m$^3$) are shown in Figure 4.2. A malfunction in the monitor resulted in an absence of data collection between day 1 and 5. Data collected reflected a weather event on day 9. There was a steady circadian pattern due to husbandry (i.e. stall cleaning, feeding, lunging) between 0.05- 0.1 g/m$^3$.

Cytology results
Bronchoalveolar lavage fluid differential cell counts (Median [IQR]) for each treatment group on day 0 and day 14 are shown in Figure 4.3. There was no significant difference in the proportion of any cell type between groups (neutrophil, p=0.31; eosinophil, p=0.10; mast cell, p=0.55; alveolar macrophage, p=0.38; lymphocyte, p=0.84). Furthermore, there was no significant difference in the proportion of any cell type between day 0 and day 14 (neutrophil, p=0.91; eosinophil, p=0.60; mast cell, p=0.81; alveolar macrophage, p=0.49; lymphocyte, p=0.87). At day 14, 1 horse in Group C had a normal BAL cytology, whilst the other horses had developed a mixed inflammatory phenotype of mild equine asthma.

Effects of treatment on inflammatory cytokine expression
Horses were separated based on cytologic phenotype for REST analysis. IL-17 had very low levels of expression in most horses (Ct >45 cycles) at both Day 0 and Day 14 and was therefore excluded from analysis. In response to treatment with nebulized dexamethasone, the mixed phenotype of MEA (neutrophilic and mastocytic BAL inflammation) (n= 2) had a 1.80-fold up regulation of IL-12 (p= 0.045), and a 1.70-fold up regulation of IL-5 (p= 0.02) (Table 4.1). The neutrophilic phenotype of MEA (n= 3) had a 2.63-fold down regulation of IL-23 (p<0.00), and a 1.57-fold up regulation of IL-5 (p=0 .01) (Table 4.2), associated with nebulized dexamethasone administration.
There was no significant change in relative expression levels of any gene investigated (IL-1β, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-23, IFN-γ, Eotaxin-2 or TNF-α) in the BALF from horses treated with nebulized saline between day 0 and day 14.

In healthy horses, prolonged exposure to a dusty, stabled environment resulted in a 2.94-fold down regulation of IL-4 (p= 0.046), and a 2.53-fold up regulation of IL-12 (p< 0.00) (Table 4.3). There was no significant change in relative expression levels of IL-1β, IL-5, IL-6, IL-8, IL-10, IL-23, IFN-γ, Eotaxin-2 or TNF-α in the BALF from Group C between day 0 and day 14 (Table 4.3).

**Effect of treatment on EHV-1,2,4,5**

There was no detectable level of EHV-1 expression in any horse, at either timepoint (Table 4.4).

Out of 20 horses, EHV-4 was expressed in 8 horses on day 0, and 7 on day 14. Only 3 horses had expression at both timepoints; all 3 were in Group A. There was no difference in relative levels of expression of EHV-4 in response to treatment with nebulized dexamethasone (p= 0.933).

EHV-2 expression was up regulated in horses treated with nebulized dexamethasone by a factor of 28 (p = 0.001) (Table 4.4). The effect of nebulized dexamethasone on relative EHV-5 expression was unable to be quantified, as REST analysis requires a Ct value for both timepoints. Before treatment with nebulized dexamethasone, 4 horses had detectable levels of expression of EHV-5 (Ct<45); however, after treatment no horses from this group expressed EHV-5 (Table 4.4).

EHV-2 expression was up regulated in horses treated with nebulized saline by a factor of 16,327 (p = 0.000) (Table 4.4). Only 1 horse treated with nebulized saline had detectable levels of EHV-5 at both timepoints; in this horse EHV-5 was down regulated by factor of 1000 (p = 0.000). However, on day 0 there were 2 horses from group B with EHV-5 expression, and after treatment with nebulized saline there were 3 horses with very low levels of EHV-5 expression (Table 4.4).

There was no change in levels of EHV-2 expression in Group C (p = 0.866), however EHV-5 was up regulated by a factor of 263 (p = 0.007) (Table 4.4).
Figure 4.2: Dust concentrations of particulates <4μm (g/m³) from day 6 to 14. A malfunction in the monitor resulted in the absence of data between day 1 and 5. Each dot represents the average dust concentration obtained over a 4-minute sampling period; sampling timepoints are 4 hours apart.
Figure 4.3: Bronchoalveolar lavage fluid differential cell counts (Median [IQR]) in 16 horses with mild equine asthma before (Pre; Day 0) and after (Post; Day 14) treatment with nebulized dexamethasone (15mg SID, Group A, n=8), or nebulized saline (3mL SID, Group B, n=8). BALF differential cell counts for no treatment controls (n=4, Group C) are provided.
Table 4.1: Relative gene expression in 2 horses with the mixed phenotype of mild equine asthma over 13 days of treatment with nebulized dexamethasone (15mg, SID). The normalization factor (calculated from multiple reference genes) has a value of 1, and genes of interest are either up-regulated or down-regulated in response to treatment. * indicates a significant difference (p<0.05)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Expression</th>
<th>Regulation</th>
<th>Standard Error</th>
<th>95% C.I.</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>3.59</td>
<td>UP</td>
<td>.38 - 33.20</td>
<td>.36 - 44.20</td>
<td>.08</td>
</tr>
<tr>
<td>IL-5</td>
<td>1.7</td>
<td>UP</td>
<td>1.05 - 2.83</td>
<td>.90 - 3.81</td>
<td>.02*</td>
</tr>
<tr>
<td>IL-6</td>
<td>1.83</td>
<td>UP</td>
<td>.69 - 5.72</td>
<td>.24 - 7.05</td>
<td>.18</td>
</tr>
<tr>
<td>IL-8</td>
<td>1.64</td>
<td>UP</td>
<td>.20 - 13.78</td>
<td>.16 - 15.87</td>
<td>.49</td>
</tr>
<tr>
<td>IL-10</td>
<td>1.23</td>
<td>DOWN</td>
<td>.45 - 1.55</td>
<td>.32 - 1.80</td>
<td>.41</td>
</tr>
<tr>
<td>IL-12</td>
<td>1.8</td>
<td>UP</td>
<td>.98 - 3.28</td>
<td>.85 - 4.01</td>
<td>.047*</td>
</tr>
<tr>
<td>IL-23</td>
<td>2.27</td>
<td>DOWN</td>
<td>.05 - 4.36</td>
<td>.04 - 4.91</td>
<td>.24</td>
</tr>
<tr>
<td>Eotaxin-2</td>
<td>1.55</td>
<td>UP</td>
<td>.46 - 4.79</td>
<td>.39 - 8.80</td>
<td>.37</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1.79</td>
<td>UP</td>
<td>.87 - 3.71</td>
<td>.74 - 4.25</td>
<td>.051</td>
</tr>
</tbody>
</table>

Table 4.2: Relative gene expression in 3 horses with the neutrophilic phenotype of mild equine asthma over 13 days of treatment with nebulized dexamethasone (15mg, SID). The normalization factor (calculated from multiple reference genes) has a value of 1, and genes of interest are either up-regulated or down-regulated in response to treatment. * indicates a significant difference (p<0.05)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Expression</th>
<th>Regulation</th>
<th>Standard Error</th>
<th>95% C.I.</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>1.30</td>
<td>DOWN</td>
<td>.13 - 3.94</td>
<td>.11 - 5.05</td>
<td>.59</td>
</tr>
<tr>
<td>IL-4</td>
<td>1.37</td>
<td>DOWN</td>
<td>.15 - 3.15</td>
<td>.07 - 11.04</td>
<td>.53</td>
</tr>
<tr>
<td>IL-5</td>
<td>1.57</td>
<td>UP</td>
<td>1.02 - 2.63</td>
<td>.77 - 3.24</td>
<td>.01*</td>
</tr>
<tr>
<td>IL-6</td>
<td>1.25</td>
<td>UP</td>
<td>.30 - 3.97</td>
<td>.18 - 8.53</td>
<td>.58</td>
</tr>
<tr>
<td>IL-8</td>
<td>1.52</td>
<td>DOWN</td>
<td>.33 - 1.22</td>
<td>.27 - 1.88</td>
<td>.07</td>
</tr>
<tr>
<td>IL-10</td>
<td>1.16</td>
<td>DOWN</td>
<td>.43 - 1.61</td>
<td>.35 - 2.00</td>
<td>.46</td>
</tr>
<tr>
<td>IL-12</td>
<td>1.35</td>
<td>UP</td>
<td>.39 - 4.23</td>
<td>.27 - 5.12</td>
<td>.36</td>
</tr>
<tr>
<td>IL-23</td>
<td>2.63</td>
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<td>.23 - .81</td>
<td>.13 - .98</td>
<td>&lt;.000*</td>
</tr>
<tr>
<td>Eotaxin-2</td>
<td>1.34</td>
<td>UP</td>
<td>.26 - 5.21</td>
<td>.12 - 26.17</td>
<td>.58</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1.78</td>
<td>UP</td>
<td>.75 - 3.93</td>
<td>.42 - 8.69</td>
<td>.07</td>
</tr>
</tbody>
</table>

Table 4.3: Relative gene expression in 3 healthy horses used as environmental controls over 14 days in a dusty, stabled environment. The normalization factor (calculated from multiple reference genes) has a value of 1, and genes of interest are either up-regulated or down-regulated in response to treatment. * indicates a significant difference (p<0.05)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Expression</th>
<th>Regulation</th>
<th>Standard Error</th>
<th>95% C.I.</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-4</td>
<td>2.94</td>
<td>DOWN</td>
<td>.06 - 1.34</td>
<td>.02 - 3.17</td>
<td>.04*</td>
</tr>
<tr>
<td>IL-5</td>
<td>1.18</td>
<td>DOWN</td>
<td>.30 - 2.17</td>
<td>.15 - 4.15</td>
<td>.65</td>
</tr>
<tr>
<td>IL-6</td>
<td>1.52</td>
<td>UP</td>
<td>.38 - 8.26</td>
<td>.13 - 12.04</td>
<td>.38</td>
</tr>
<tr>
<td>IL-8</td>
<td>1.32</td>
<td>UP</td>
<td>.32 - 3.83</td>
<td>.28 - 7.57</td>
<td>.44</td>
</tr>
<tr>
<td>IL-12</td>
<td>2.53</td>
<td>UP</td>
<td>1.25 - 6.42</td>
<td>1.03 - 8.63</td>
<td>&lt;.000*</td>
</tr>
<tr>
<td>IL-23</td>
<td>1.05</td>
<td>DOWN</td>
<td>.29 - 2.64</td>
<td>.15 - 5.17</td>
<td>.89</td>
</tr>
<tr>
<td>Eotaxin-2</td>
<td>1.19</td>
<td>DOWN</td>
<td>.11 - 3.70</td>
<td>.06 - 18.10</td>
<td>.77</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1.56</td>
<td>UP</td>
<td>.72 - 2.79</td>
<td>.57 - 6.07</td>
<td>.1</td>
</tr>
</tbody>
</table>
Table 4.4: Number of horses in each treatment group with detectable levels of expression (Ct<40) of EHV-1,2,4,5 on day 0 and 14

<table>
<thead>
<tr>
<th></th>
<th># Horses with detectable levels of expression (Ct &lt; 40)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
</tr>
<tr>
<td></td>
<td>Dex</td>
</tr>
<tr>
<td>EHV-1</td>
<td>0</td>
</tr>
<tr>
<td>EHV-2</td>
<td>3</td>
</tr>
<tr>
<td>EHV-4</td>
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</tr>
<tr>
<td>EHV-5</td>
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</tbody>
</table>
**Upper and lower respiratory tract microbiota**

In total, twelve phyla were identified in nasal swab samples (Figure 4.4), and sixteen phyla were identified in tracheal wash samples (Figure 4.5), with five phyla showing a relative abundance > 0.1% at both the upper and lower respiratory tract level: Actinobacteria, Firmicutes, Proteobacteria, Bacteroidetes and Tenericutes (Table 4.5). Four phyla represented 99.25% of the total abundance across the respiratory tract: Proteobacteria (37.81%), Bacteroidetes (25.71%), Firmicutes (17.96%), and Actinobacteria (17.77%) (Table 4.5, Figure 4.4, Figure 4.5).

*Table 4.5: Relative abundance of the 5 dominant microbiota phyla observed in the upper and lower respiratory tract of horses (n = 20) over the duration of the trial, and relative abundance of genus within each phylum*

<table>
<thead>
<tr>
<th>Phylum (mean relative abundance, %)</th>
<th>Genus</th>
<th>Genus (mean relative abundance per phylum, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteobacteria (37.81%)</td>
<td>Moraxella</td>
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<td>Pseudomonas</td>
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</tr>
<tr>
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<td>Methylobacterium</td>
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<td>Bordetella</td>
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<td>Neorhizobium</td>
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</tr>
<tr>
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<td>Pasteurella</td>
<td>3.75%</td>
</tr>
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<td>Xylophilus</td>
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</tr>
<tr>
<td></td>
<td>Devosia</td>
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<tr>
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</tr>
<tr>
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<td>Rhizobium</td>
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<tr>
<td></td>
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<td>Stenotrophomonas</td>
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<td>Alkanindiges</td>
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<td>Bacteroidetes (25.71%)</td>
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<tr>
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<tr>
<td></td>
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<tr>
<td>Bacterium</td>
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<tr>
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<td>Anaerococcus</td>
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<td><em>Actinobacteria (17.77%)</em></td>
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<td>Bacterial Name</td>
<td>Percentage</td>
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</tr>
<tr>
<td>------------------------</td>
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<td>Aeromicrobiun</td>
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<tr>
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<td>Kocuria</td>
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<td>Nocardoides</td>
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<tr>
<td>Kineosporia</td>
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<tr>
<td>Nakamurella</td>
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<tr>
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<tr>
<td>Yonghahaparkia</td>
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<tr>
<td>Ornithinimicrobium</td>
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<td>Williamsia</td>
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<td>Iamia</td>
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<td>Saccharopolyspora</td>
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<td>Actinomycesporospora</td>
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<tr>
<td>Haloactinobacterium</td>
<td>0.04%</td>
<td></td>
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<tr>
<td>Frigoribacterium</td>
<td>0.03%</td>
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<tr>
<td>Streptomycetes</td>
<td>0.02%</td>
<td></td>
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<tr>
<td>Bifidobacterium</td>
<td>0.02%</td>
<td></td>
</tr>
<tr>
<td>Actinoplanes</td>
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<td></td>
</tr>
<tr>
<td>Glycomycetes</td>
<td>0.01%</td>
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</tr>
<tr>
<td>Mycobacterium</td>
<td>0.01%</td>
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</tr>
<tr>
<td>Tenericutes (0.52%)</td>
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<tr>
<td>Mycoplasma</td>
<td>100.00%</td>
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</table>
Figure 4.4: Relative abundance of microbiota phyla in the upper respiratory tract of 16 horses with mild equine asthma before (Dex_Pre; Day 0) and after (Dex_Post; Day 14) treatment with nebulized dexamethasone, or nebulized saline (Saline_Pre, Saline_Post). Relative abundance for no treatment controls (Control_Pre, Control_Post) are also provided.
Figure 4.5: Relative abundance of microbiota phyla in the lower respiratory tract of 16 horses with mild equine asthma before (Dex_Pre; Day 0) and after (Dex_Post; Day 14) treatment with nebulized dexamethasone, or nebulized saline (Saline_Pre, Saline_Post). Relative abundance for no treatment controls (Control_Pre, Control_Post) are also provided.
Upper and lower respiratory tract mycobiota

In total, 5 phyla were identified in nasal swab samples (Figure 4.6), and 4 phyla were identified in tracheal wash samples (Figure 4.7) with two phyla showing a relative abundance >0.1% and represented 99.78% of the total abundance: Ascomycota (42.92%) and Basidiomycota (56.86%) (Table 4.6).

Table 4.6: Relative abundance of the 2 dominant mycobiota phyla observed in the upper and lower respiratory tract of horses (n = 20) over the duration of the trial, and relative abundance of genus within each phylum

<table>
<thead>
<tr>
<th>Phylum (mean relative abundance, %)</th>
<th>Genus</th>
<th>Genus (mean relative abundance per phylum, %)</th>
</tr>
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<tbody>
<tr>
<td>Basidiomycota (56.86%)</td>
<td>Vishniacozyma</td>
<td>33.72%</td>
</tr>
<tr>
<td></td>
<td>Udeniomyces</td>
<td>22.81%</td>
</tr>
<tr>
<td></td>
<td>Dioszegia</td>
<td>15.57%</td>
</tr>
<tr>
<td></td>
<td>Filobasidium</td>
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</tr>
<tr>
<td></td>
<td>Kondoa</td>
<td>4.98%</td>
</tr>
<tr>
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<td>Wallemia</td>
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<td>Bullera</td>
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<tr>
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<td>Cristinia</td>
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<td>Cystobasidium</td>
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<td>Cryptococcus</td>
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<td>Papiliotrema</td>
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<td>Chaetospermum</td>
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<td>Tranzscheliella</td>
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<td>Rhodosporidiobolus</td>
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<td></td>
<td>Genolevuria</td>
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<tr>
<td></td>
<td>Phaeotremella</td>
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<td></td>
<td>Trechispora</td>
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<td>Itersonilia</td>
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<tr>
<td></td>
<td>Symmetrospora</td>
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<tr>
<td>菌名</td>
<td>百分比</td>
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<tr>
<td>Waitea</td>
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<td>Buckleyzyma</td>
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<tr>
<td>Serendipita</td>
<td>0.00%</td>
<td></td>
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<tr>
<td>Efibulobasidium</td>
<td>0.00%</td>
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<table>
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<th>Ascomycota (42.92%)</th>
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<td>17.68%</td>
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<tr>
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<td>15.26%</td>
</tr>
<tr>
<td>Alternaria</td>
<td>14.21%</td>
</tr>
<tr>
<td>Neoscychola</td>
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<td>Cladosporium</td>
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<tr>
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<td>5.14%</td>
</tr>
<tr>
<td>Septoria</td>
<td>3.20%</td>
</tr>
<tr>
<td>Nigrospora</td>
<td>2.42%</td>
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<tr>
<td>Mycosphaerella</td>
<td>2.41%</td>
</tr>
<tr>
<td>Aspergillus</td>
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<td>Sarocladium</td>
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<td>Ampelomyces</td>
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<tr>
<td>Ramularia</td>
<td>1.48%</td>
</tr>
<tr>
<td>Aureobasidium</td>
<td>1.14%</td>
</tr>
<tr>
<td>Articulosa</td>
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<tr>
<td>Zymoseptoria</td>
<td>0.98%</td>
</tr>
<tr>
<td>Helgardia</td>
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<td>Tetracladium</td>
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<td>Leptosphaeria</td>
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<tr>
<td>Gibberella</td>
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<tr>
<td>Radulidium</td>
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<td>Selenophoma</td>
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<td>Trichopeziza</td>
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<td>Trichometasphaeria</td>
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<td>Arthrinum</td>
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<tr>
<td>Podosphaera</td>
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<tr>
<td>Claviceps</td>
<td>0.41%</td>
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<tr>
<td>Chalaspospora</td>
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</tr>
<tr>
<td>Mycocentrospora</td>
<td>0.38%</td>
</tr>
<tr>
<td>Juncaceicola</td>
<td>0.38%</td>
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<tr>
<td>Pseudorobillarda</td>
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<td>Stagonospora</td>
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<td>Ascycha</td>
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<td>Pyrenophora</td>
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<tr>
<td>Torula</td>
<td>0.24%</td>
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<td>LECTERA</td>
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<td>Colletotrichum</td>
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<tr>
<td>Nectriopsis</td>
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<tr>
<td>Paradendryphiella</td>
<td>0.19%</td>
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<tr>
<td>Species</td>
<td>Percentage</td>
</tr>
<tr>
<td>--------------------------</td>
<td>------------</td>
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<tr>
<td>Taphrina</td>
<td>0.18%</td>
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<tr>
<td>Monographella</td>
<td>0.18%</td>
</tr>
<tr>
<td>Dinemasporium</td>
<td>0.17%</td>
</tr>
<tr>
<td>Hymenula</td>
<td>0.16%</td>
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<tr>
<td>Protomyces</td>
<td>0.15%</td>
</tr>
<tr>
<td>Rhynchosporium</td>
<td>0.10%</td>
</tr>
<tr>
<td>Stemphylium</td>
<td>0.10%</td>
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<tr>
<td>Oculimacula</td>
<td>0.10%</td>
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<tr>
<td>Plenodomus</td>
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<tr>
<td>Plectosphaerella</td>
<td>0.08%</td>
</tr>
<tr>
<td>Fusarium</td>
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<tr>
<td>Thelebolus</td>
<td>0.07%</td>
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<tr>
<td>Didymella</td>
<td>0.07%</td>
</tr>
<tr>
<td>Debaryomyces</td>
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<tr>
<td>Myrothecium</td>
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</tr>
<tr>
<td>Periconia</td>
<td>0.06%</td>
</tr>
<tr>
<td>Calycina</td>
<td>0.06%</td>
</tr>
<tr>
<td>Candida</td>
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</tr>
<tr>
<td>Dissoconium</td>
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<td>Comoclathris</td>
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<tr>
<td>Blumeria</td>
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</tr>
<tr>
<td>Saccharomycetes</td>
<td>0.04%</td>
</tr>
<tr>
<td>Ramichloridium</td>
<td>0.04%</td>
</tr>
<tr>
<td>Botrytis</td>
<td>0.04%</td>
</tr>
<tr>
<td>Setomelanomma</td>
<td>0.04%</td>
</tr>
<tr>
<td>Knufia</td>
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</tr>
<tr>
<td>Stachybotrys</td>
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<tr>
<td>Rachicladosporium</td>
<td>0.03%</td>
</tr>
<tr>
<td>Apenidiella</td>
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</tr>
<tr>
<td>Dipodascus</td>
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<tr>
<td>Tetrachaetum</td>
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<tr>
<td>Boeremia</td>
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<td>Keissleriella</td>
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</tr>
<tr>
<td>Periconiella</td>
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</tr>
<tr>
<td>Paraphaeosphaeria</td>
<td>0.01%</td>
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<tr>
<td>Penicillium</td>
<td>0.01%</td>
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<tr>
<td>Dendryphion</td>
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<td>Pithoascus</td>
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<tr>
<td>Pseudogymnoascus</td>
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<tr>
<td>Venturia</td>
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<tr>
<td>Crocicreas</td>
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<td>Phaeococcomyces</td>
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<tr>
<td>Pyrenochaetopsis</td>
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<tr>
<td>Cyberlindnera</td>
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</tr>
<tr>
<td>Preussia</td>
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</table>
Figure 4.6: Relative abundance of mycobiota phyla in the upper respiratory tract of 16 horses with mild equine asthma before (Dex_Pre; Day 0) and after (Dex_Post; Day 14) treatment with nebulized dexamethasone, or nebulized saline (Saline_Pre, Saline_Post). Relative abundance for no treatment controls (Control_Pre, Control_Post) are also provided.
Figure 4.7: Relative abundance of mycobiota phyla in the lower respiratory tract of 16 horses with mild equine asthma before (Dex_Pre; Day 0) and after (Dex_Post; Day 14) treatment with nebulized dexamethasone, or nebulized saline (Saline_Pre, Saline_Post). Relative abundance for no treatment controls (Control_Pre, Control_Post) are also provided.
Effects on nebulized dexamethasone and saline on the upper and lower respiratory tract environments

Community differences with treatment at the upper respiratory tract level were visualized using principal coordinates analysis (PCoA) and were dominated by timepoint effects (i.e. differences between day 0 and day 14, after controlling for treatment group; the effect of sustained exposure to a dusty environment), which were more prominent than treatment effects (Figure 4.8A and 4.8C). In the upper respiratory tract dexamethasone treatment resulted in a significant decrease in microbiota diversity based on Chao1 (p=0.004) and Shannon (p=0.004) indexes (Figure 4.8B); saline treatment resulted in a significant increase in mycobiota diversity based on Chao1 (p=0.04) and Shannon (p=0.01) indices (Figure 4.8D). There were no significant differences in either beta or alpha diversity at the lower respiratory tract level with treatment (Figure 4.9).

At the OTU level in the microbiota, there was a significant change in the abundance of eight genera with dexamethasone treatment (Table 4.7), with *Alysiella*, *Bordetella*, *Acinetobacter*, *Staphylococcus* and *Pedobacter* being increased with treatment, whereas *Brevundimonas*, *Pigmentiphaga*, and an OTU assigned to the genus *Corynebacterium_1* decreased with treatment. Treatment with nebulized saline had no significant effect on diversity but did alter the abundance of three genera, including a significant decrease in *Streptococcus* and *Brevundimonas*; *Pedobacter* was increased with treatment (Table 4.7). Treatment-specific effects were tested (interaction) with two genera (*Alysiella* and *Bordetella*) showing a differential effect between treatments, although manual inspection of the counts for each group showed a few samples dominated by high counts suggesting a false-positive leading us to interpret the interaction effects with caution (Figure 4.10).

The mycobiota was dominated by timepoint effects; when the interaction between treatment group and timepoint was tested no significant genera were detected. Treatment with nebulized saline altered the abundance of ten genera (Table 4.8), with *Mycocentrospora*, *Acremonium*, *Kondoa*, *Alternaria*, *Aspergillus*, *Wallemia* and *Pseudorobillarda* increasing with treatment; *Leucosporidium*, *Septoriella* and *Vishniacozyma* decreased with treatment. Nebulized dexamethasone treatment altered eight genera
(Table 4.8) with *Paradendryphiella, Radulidium, Pseudorobillarda, Wallemia* and *Kondoia* increasing with treatment; *Vishniaczyma, Mycosphaerella* and *Leucosporidium* decreased with treatment.
Table 4.7: Differential abundance associated with 14 days of nebulized dexamethasone (Dex) or saline treatment (shown as Log₂ fold-change) of individual microbiota OTUs in 16 horses with mild equine asthma

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Genus</th>
<th>Treatment</th>
<th>Log₂ fold-change</th>
<th>S.E.</th>
<th>P-value</th>
<th>P_adj</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteroidetes</td>
<td>Pedobacter</td>
<td>Saline</td>
<td>1.41</td>
<td>0.31</td>
<td>4.78E-06</td>
<td>0.0005</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>Brevundimonas</td>
<td>Saline</td>
<td>-1.45</td>
<td>0.37</td>
<td>0.0001</td>
<td>0.0054</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>Streptococcus</td>
<td>Saline</td>
<td>-6.99</td>
<td>1.89</td>
<td>0.00022</td>
<td>0.0076</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>Alysiella</td>
<td>Dex</td>
<td>25.83</td>
<td>3.03</td>
<td>1.41E-17</td>
<td>8.16E-16</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>Bordetella</td>
<td>Dex</td>
<td>22.73</td>
<td>3.82</td>
<td>2.62E-09</td>
<td>7.59E-08</td>
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<tr>
<td>Bacteroidetes</td>
<td>Pedobacter</td>
<td>Dex</td>
<td>1.72</td>
<td>0.31</td>
<td>2.27E-08</td>
<td>4.39E-07</td>
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<tr>
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<td>Brevundimonas</td>
<td>Dex</td>
<td>-1.50</td>
<td>0.38</td>
<td>0.000083</td>
<td>0.0012</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>Corynebacterium_1</td>
<td>Dex</td>
<td>-6.69</td>
<td>1.87</td>
<td>0.00035</td>
<td>0.0041</td>
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<tr>
<td>Proteobacteria</td>
<td>Acinetobacter</td>
<td>Dex</td>
<td>5.28</td>
<td>1.63</td>
<td>0.0012</td>
<td>0.011</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>Pigmentiphaga</td>
<td>Dex</td>
<td>-3.85</td>
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<td>0.0016</td>
<td>0.013</td>
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<td>Firmicutes</td>
<td>Staphylococcus</td>
<td>Dex</td>
<td>3.90</td>
<td>1.30</td>
<td>0.0027</td>
<td>0.019</td>
</tr>
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</table>

Table 4.8: Differential abundance associated with 14 days of nebulized dexamethasone (Dex) or saline treatment (shown as Log₂ fold-change) of individual mycobiota OTUs in 16 horses with mild equine asthma

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Genus</th>
<th>Treatment</th>
<th>Log₂ fold-change</th>
<th>S.E.</th>
<th>P-value</th>
<th>P_adj</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basidiomycota</td>
<td>Vishniacozyma</td>
<td>Saline</td>
<td>-1.20</td>
<td>0.24</td>
<td>4.96E-07</td>
<td>6.1E-05</td>
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<tr>
<td>Basidiomycota</td>
<td>Wallemia</td>
<td>Saline</td>
<td>2.94</td>
<td>0.62</td>
<td>2.08E-06</td>
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<td>Ascomycota</td>
<td>Septoriella</td>
<td>Saline</td>
<td>-2.07</td>
<td>0.45</td>
<td>5.06E-06</td>
<td>0.00021</td>
</tr>
<tr>
<td>Ascomycota</td>
<td>Mycocentrospora</td>
<td>Saline</td>
<td>5.95</td>
<td>1.42</td>
<td>2.64E-05</td>
<td>0.00065</td>
</tr>
<tr>
<td>Basidiomycota</td>
<td>Leucosporidium</td>
<td>Saline</td>
<td>-4.07</td>
<td>0.96</td>
<td>2.51E-05</td>
<td>0.00065</td>
</tr>
<tr>
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<td>Pseudobilliardia</td>
<td>Saline</td>
<td>3.76</td>
<td>0.99</td>
<td>0.00014</td>
<td>0.0029</td>
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<td>Ascomycota</td>
<td>Acremonium</td>
<td>Saline</td>
<td>5.07</td>
<td>1.38</td>
<td>0.00023</td>
<td>0.0041</td>
</tr>
<tr>
<td>Ascomycota</td>
<td>Alternaria</td>
<td>Saline</td>
<td>0.73</td>
<td>0.22</td>
<td>0.0012</td>
<td>0.019</td>
</tr>
<tr>
<td>Ascomycota</td>
<td>Aspergillus</td>
<td>Saline</td>
<td>2.86</td>
<td>0.96</td>
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<td>Kondoa</td>
<td>Saline</td>
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<td>Kondoa</td>
<td>Dex</td>
<td>1.19</td>
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<td>1.33E-06</td>
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<td>Radulidium</td>
<td>Dex</td>
<td>3.95</td>
<td>0.93</td>
<td>2.22E-05</td>
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<td>0.61</td>
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<td>Dex</td>
<td>-1.01</td>
<td>0.24</td>
<td>2.4E-05</td>
<td>0.00061</td>
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<tr>
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<td>Pseudobilliardia</td>
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<td>3.65</td>
<td>0.96</td>
<td>0.00015</td>
<td>0.0022</td>
</tr>
<tr>
<td>Basidiomycota</td>
<td>Leucosporidium</td>
<td>Dex</td>
<td>-3.65</td>
<td>0.96</td>
<td>0.00015</td>
<td>0.0022</td>
</tr>
<tr>
<td>Ascomycota</td>
<td>Paradendryphiella</td>
<td>Dex</td>
<td>7.12</td>
<td>2.40</td>
<td>0.0031</td>
<td>0.037</td>
</tr>
<tr>
<td>Ascomycota</td>
<td>Mycosphaerella</td>
<td>Dex</td>
<td>-1.65</td>
<td>0.57</td>
<td>0.004</td>
<td>0.042</td>
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</table>
Figure 4.8: Principal coordinate analysis (PCoA) with Bray-Curtis distance of the equine upper respiratory tract microbiota (8A) and mycobiota (8C). Alpha diversity measures (Chao1 and Shannon) in upper respiratory tract samples of the microbiota (8B) and mycobiota (8D).
Figure 4.9: Principal coordinate analysis (PCoA) with Bray-Curtis distance of the equine lower respiratory tract microbiota (8A) and mycobiota (8C). Alpha diversity measures (Chao1 and Shannon) in lower respiratory tract samples of the microbiota (8B) and mycobiota (8D).

A

Axis 2 [3.4%]

Axis 1 [12.5%]

B

Alpha diversity value

Chao1

Shannon

Treatment

Control Dex Saline

Control Dex Saline

Group

Saline Pre

Dex Pre

Saline Post

Dex Post

C

Axis 2 [9.2%]

Axis 1 [16%]

D

Alpha diversity value

Chao1

Shannon

Treatment

Control Dex Saline

Control Dex Saline
Figure 4.10: Treatment-specific effects were tested (interaction) in the microbiota (Pre = Day 0; Post = Day 14) of 16 horses with mild equine asthma, treated with nebulized dexamethasone (Dex; n=8) and nebulized saline (Saline; n=8). Each panel shows the abundance for an individual OTU and is labelled with the Genus that was assigned to it.
Discussion

This study describes a comprehensive investigation into the effects of 14 days of nebulized dexamethasone and saline administration in a sustained dusty environment on the respiratory tract in horses with mild equine asthma. Nebulized dexamethasone treatment affected the upper respiratory tract microbiota, but not the mycobiota, which was overwhelmed by the effect of a sustained dusty environment. Notably, the genus *Alternaria*, a known opportunistic pathogen and allergen in humans, was significantly increased in both treatment groups. Relative expression of the equine gammaherpesviruses (EHV-2 and -5) were affected by treatment, with EHV-2 being upregulated in response to both nebulized dexamethasone and saline, and EHV-5 being downregulated. Evidence of a Th1 response in control horses could suggest a “non-allergic asthma” phenotype in response to exposure to a sub-optimal environment. This highlights the importance of environmental modification as part of the treatment strategy for MEA.

Importantly, a strength of this study is the investigation and report of the respiratory mycobiota. The contribution of fungi to the overall community structure of the equine airways is unknown. A recent, culture-based study investigated the prevalence of fungi in respiratory samples of horses diagnosed with mild equine asthma, and assessed risk factors associated with the presence of fungi in the airways (Dauvillier, ter Woort et al., 2018). They obtained a positive fungal culture in 55% (402/731) horses referred for signs of poor performance or respiratory disease (Dauvillier, ter Woort et al., 2018). Horses with fungi present in tracheal wash cytology are 2 times more likely to have mild equine asthma than those without (Dauvillier, ter Woort et al., 2018). Risk factors associated with mild equine asthma and with the presence of fungi in tracheal wash cytology included straw bedding and being fed dry hay (Dauvillier, ter Woort et al., 2018). The most commonly isolated fungi were Penicillium (53%), Aspergillus (34%), Rhizomucor (5%), and Candida (5%) (Dauvillier, ter Woort et al., 2018). Our study was not designed to test differences between healthy horses and those with asthma however, we found that time (pre vs. post) had the largest effect on the mycobiota. Interestingly, only one of the commonly isolated fungi, *Aspergillus*, was found to increase following treatment with nebulized saline. Perhaps
more importantly, the genus *Alternaria*, a known opportunistic pathogen and allergen in humans which has been increasingly recognized as a risk factor for asthma, asthma severity and exacerbations (Bush and Prochnau, 2004), was significantly increased in both treatment groups. As a major aeroallergen, prolonged exposure to *Alternaria* has been recognized as a risk factor for the development of asthma (Bush and Prochnau, 2004).

A frequent observation made after cessation of corticosteroid treatment in horses with MEA is the re-emergence of clinical signs. Equine herpesviruses are ubiquitous in the equine population, establish lifelong latent infections and reactivate in times of immunosuppression or stress. It is logical to question whether the re-emergence of clinical signs is due to an insufficient improvement in exposure to inhalable particulate matter, or whether corticosteroid-derived immunosuppression leads to recrudescence of latent infection. Acute EHV-1 and -4 infection present with mild clinical signs consistent with MEA (Diaz-Mendez, Viel et al., 2010). EHV-2 and EHV-5 are identified frequently in samples from horses with respiratory disease (Fortier, Van Erck et al., 2009; Hue, Fortier et al., 2014), and there is some evidence that EHV-2 is associated with poor performance and airway inflammation (Fortier, Van Erck et al., 2009). In the present study, we found no difference in relative expression in EHV-1 or -4 in response to nebulized dexamethasone. Interestingly, relative expression of the equine gammaherpesviruses (EHV-2 and -5) were affected by treatment, with EHV-2 being upregulated in response to both nebulized dexamethasone and saline, and EHV-5 being downregulated. To account for the potential for location to confound results – there was definitely the potential for one horse to infect others in neighboring stalls – we investigated the stall locations of horses with up-regulation of EHV-2. Nebulizers were also disinfected thoroughly between horses. None of the horses that exhibited relative up-regulation of EHV-2 were next to, or across from others; this is likely therefore a true response to nebulization. Furthermore, none of the control horses exhibited any changes in relative expression of any equine herpesvirus. Nebulization of isotonic saline can relieve breathlessness (a subjective parameter only assessable in humans), possibly through an increased rate of mucociliary clearance, however, it does not affect lung function or increase bronchial hyperresponsiveness to
bronchoprovocative challenge, allowing it to be used as a placebo (Bacci, Cianchetti et al., 1996; Khan and O'Driscoll, 2004). The role of EHV-2 in perpetuating the clinical signs of mild equine asthma after cessation of treatment warrants further investigation.

Many aeroantigens, including dust, endotoxin, fungi, molds, ultrafine particles and noxious gases are found in conventional stables (Ivester, Couetil et al., 2014b), and there is strong evidence that without environmental modifications, corticosteroid therapy alone fails to normalize airway neutrophilia, even after treatment periods of up to 6 months (Lavoie, Leguillette et al., 2002; Lavoie, Pasloske et al., 2006; Leclere, Lavoie-Lamoureux et al., 2012; Bullone, Vargas et al., 2017; Leguillette, Tohver et al., 2017). In a single study investigating the effects of nebulized dexamethasone in horses with asthma, a cytological improvement was reported (Haspel, Giguère et al., 2018), however, there were changes to the horse’s environment not reported in the paper, as this was not the primary focus of the study (RL personal communication). It is therefore not surprising that in the present study there was no change in the inflammatory cytology in response to nebulized dexamethasone or saline; there was no significant difference in the proportion of any cell type between day 0 and day 14. Furthermore, prolonged exposure to the dusty stable environment induced airway inflammation in 75% of our control horses (3/4). This is consistent with previous reports, where healthy mature horses develop airway neutrophilia in response to an organic dust challenge by exposure to straw and moldy hay (Pirie, Dixon et al., 2001; Gerber, Lindberg et al., 2004; Leclere, Lavoie-Lamoureux et al., 2012).

In healthy horses, a prolonged exposure to a dusty, stabled environment resulted in a 2.94-fold down regulation of IL-4 and a 2.53-fold up regulation of IL-12. IL-4 is a potent activator of inflammatory responses and is considered to be a central Th2 cytokine of the immune system. On the other hand, IL-12 is secreted by T lymphocytes, and plays as a key role in the differentiation of Th0 into Th1 cells. As horses were exposed to these environmental conditions for 7 days prior to the initial sample collection; that they still retained a healthy BAL cytology at the initial testing suggests that these horses are not
predisposed to equine asthma, and the immune response exhibited by them in response to prolonged exposure might indicate how a “healthy” horse with no predisposing factors to lower airway inflammation responds to sustained exposure to airborne irritants. Together, this information indicates that this subset of horses responds with a shift away from a Th2 cytokine response, towards a Th1 cytokine response, suggesting a “non-allergic asthma” phenotype in response to sustained exposure to a sub-optimal environment.

In horses with the mixed phenotype of MEA, IL-12 and IL-5 were up-regulated in response to treatment with nebulized dexamethasone. In contrast, the neutrophilic phenotype of MEA responded to treatment with down-regulation of IL-23 and up regulation of IL-5. Typically, the production of eosinopoietic cytokines such as IL-5 is inhibited by glucocorticoid administration. IL-5 plays a role in asthma and other acute hypersensitivity manifestations. Yet we observed an increase in IL-5 in not only the mixed phenotype, but also the neutrophilic phenotype of MEA. Furthermore, the up-regulation of IL-12 in the mixed phenotype typically results in the differentiation of T progenitor cells to Th1 cells. IL-23 is an IL-12-related cytokine essential for the maintenance of Th17 cells (Langrish, Chen et al., 2005), and plays a key role in the development of inflammatory disease. That IL-23 was down-regulated in the neutrophilic phenotype is consistent with a non-significant decrease in neutrophil % observed in this group. It is plausible that these contradictory results indicate the overwhelming influence of the environment on the disease process, as is observed in analysis of the respiratory mycobiota. In the microbiota, whilst timepoint effects predominated, there was a treatment-specific effect, with two genera (Alysiella and Bordetella) showing a differential effect between treatment with nebulized dexamethasone, and nebulized saline. Interestingly, the relative abundance of Streptococcus was not influenced by nebulized dexamethasone administration, as was observed in a trial investigating the effects of injected dexamethasone, whilst the horses were kept outside (Bond, Timsit et al., 2017). However, whilst treatment with nebulized saline had no significant effect on diversity, it was associated with a significant decrease in Streptococcus in horses with mild equine asthma.
The findings of this study highlight the importance of environmental modification as part of the treatment strategy for MEA. Prolonged exposure to a dusty stable environment induced lower airway inflammation in healthy adult horses, with a Th1 immune response. The role of EHV-2 in perpetuating clinical signs of MEA after completion of treatment warrants further investigation. Nebulized saline provided an ideal control; there was no up- or down-regulation of any inflammatory cytokine mRNA in BAL fluid in this group. However, nebulized saline was associated with a significant decrease in *Streptococcus* in the upper respiratory tract. Nebulized dexamethasone treatment resulted in a non-specific immune response, and affected the upper respiratory tract microbiota, but not the mycobiota, which was overwhelmed by the effect of a sustained dusty environment.
Chapter 5 - Assessment of 2 methods to determine the relative contributions of the aerobic and anaerobic energy systems in racehorses

Summary

A prospective, randomised, controlled study was designed to determine relative aerobic and anaerobic (lactic and alactic) contributions at supramaximal exercise intensities using 2 different methods. Thoroughbred racehorses (n=5) performed a $\dot{V}O_{2\text{max}}$ test and 3 supramaximal treadmill runs (105%, 115% and 125%$\dot{V}O_{2\text{max}}$). Blood lactate concentration (BL) was measured at rest, every 15s during runs, and 2, 5, 10, 20, 30, 40, 50 and 60mins post-exercise. Method 1: Oxygen demand was calculated for each supramaximal intensity based on the $\dot{V}O_{2\text{max}}$ test, and relative aerobic and anaerobic contributions were calculated from measured $\dot{V}O_{2}$ and the accumulated oxygen deficit. Method 2: Aerobic contribution was calculated using the trapezoidal method to determine $\dot{V}O_{2}$ during exercise. A mono-exponential model was fitted to the post-exercise $\dot{V}O_{2}$ curve. Alactic contribution was calculated using the coefficients of this model. Lactate anaerobic contribution was calculated by multiplying the $\Delta$BL$_{\text{Peak-Resting}}$ by 3. Linear mixed effects models were used to examine the effects of exercise intensity and method (as fixed effects) on measured outcomes (p≤0.05). Relative aerobic and anaerobic contributions were not different between methods (p=0.20). Horses’ mean contributions were 81.4%, 77.6% and 72.5% (aerobic), and 18.5%, 22.3% and 27.4% (anaerobic) at 105%, 115%, 125%$\dot{V}O_{2\text{max}}$. Individual alactic anaerobic energy was not different between supramaximal exercise intensities (p=0.43), and was negligible, contributing a mean of 0.11% of the total energy. Relative energy contributions can be calculated using measured $\dot{V}O_{2}$ and BL in situations where the exercise intensity is unknown. Understanding relative metabolic demands could help develop tailored training programs.
Introduction

Understanding energy contributions from aerobic and anaerobic energy systems is essential to training athletes safely and effectively. Horses have an extraordinarily large aerobic capacity which is directly measured as the maximal rate of oxygen consumption (\(\dot{V}O_{2\text{max}}\)); the average racehorse typically has a \(\dot{V}O_{2\text{max}}\) greater than 150 ml.(kg.min\(^{-1}\)) \(^1\) (Rose, Hodgson et al., 1988). At sub-maximal exercise intensities aerobic metabolism generates a large proportion of the ATP required for cross-bridge cycling, leading to muscle power and ultimately, performance; steady-state \(O_2\) consumption is assumed to reflect the total rate of energy expenditure during exercise (Green and Dawson, 1993). As exercise intensity increases beyond the anaerobic threshold, anaerobic metabolic pathways (both lactic and alactic) contribute a greater proportion of the total energy required. Anaerobic capacity is the maximum amount of ATP resynthesized via anaerobic metabolism during maximal exercise of short duration. Energy released through hydrolysis of creatine phosphate and through anaerobic glycolysis contribute to the alactic and lactic components of the anaerobic capacity respectively. Anaerobic metabolism supplies between 21.3-30% of energy requirements in horses at exercise intensities of 110-115% \(\dot{V}O_{2\text{max}}\) (Eaton, Evans et al., 1995; Hinchcliff, Lauderdale et al., 2002). Anaerobic metabolism cannot be directly measured, and many techniques have been used in humans to estimate both anaerobic power and anaerobic capacity. Anaerobic power tests include cycle ergometer tests (Vandewalle, Peres et al., 1985), stair tests (Margaria, Aghemo et al., 1966), vertical jump tests (Vandewalle, Peres et al., 1987), and force-velocity tests (Perrine and Edgerton, 1978). However, as these require subject compliance they have not been adapted for use in the horse. Tests which aim to estimate the anaerobic capacity include blood lactate (Margaria, Edwards et al., 1933), ergometric tests (both maximal effort and constant load), excess post-exercise oxygen consumption (EPOC), previously termed oxygen debt (Margaria, Cerretelli et al., 1963), and the maximum accumulated oxygen deficit (MAOD)(Medbo, Mohn et al., 1988). The only methods which have been adapted for estimating the anaerobic capacity of horses are the MAOD (Rose, Hodgson et al., 1988; Eaton, Evans et al., 1995; Hinchcliff, Lauderdale et al., 2002) and EPOC (Rose, Hodgson et al., 1988). Calculation of the MAOD requires a controlled
environment, where the exercise intensity is calculated by extrapolating theoretical oxygen demands from an incremental exercise test (Rose, Hodgson et al., 1988; Eaton, Evans et al., 1995).

Training demands of many equestrian disciplines are unable to be replicated on a high-speed treadmill. The ability to accurately assess aerobic and anaerobic contributions under field conditions, at both supramaximal and sub-maximal intensities would be highly advantageous. Since MAOD could not be determined using the above approach in uncontrolled field conditions, an alternative method has been described in humans (MAODALT) which enables the characterization of differential energy contributions of the aerobic, alactic anaerobic and lactic anaerobic systems under conditions where the exercise intensity is unknown or fluctuates (de Moraes Bertuzzi, Franchini et al., 2007; Bertuzzi, Franchini et al., 2010; Artioli, Bertuzzi et al., 2012). The aerobic contribution is measured as the $\dot{V}O_2$ during exercise using the trapezoidal method, the anaerobic alactic contribution is calculated from the fast component of the post-exercise $\dot{V}O_2$ curve (Roberts and Morton, 1978; Beneke, Pollmann et al., 2002; Beneke, Beyer et al., 2004) and the anaerobic lactate contribution is calculated by determining the energetic equivalent of lactate accumulation in the blood (Margaria, Cerretelli et al., 1963; di Prampero and Ferretti, 1999; Artioli, Bertuzzi et al., 2012). The aim of this study was therefore to assess the correlation between MAOD and MAODALT and determine the relative contributions of the aerobic and anaerobic energy systems at multiple supramaximal intensities in racehorses. We hypothesized that there would be no significant difference in the anaerobic contributions between the two techniques, thus enabling MAODALT to be used in the calculation of the anaerobic capacity under field situations where the exercise intensity is unknown.

Materials and Methods

Experimental design

This was a prospective, randomized, controlled study designed to determine relative aerobic and anaerobic contributions at supramaximal exercise intensities using 2 different methods. Five retired
Thoroughbred racehorses (1 mare, 3 geldings, 1 stallion) aged 6-8 years were enrolled in the study. Each horse completed 4 runs on a high-speed treadmill inclined at 10%: an initial incremental exercise test to determine $\dot{V}O_{2\text{max}}$, followed by 3 supramaximal runs at speeds calculated to induce an oxygen demand of 105% ($V_{105\%}$), 115% ($V_{115\%}$) and 125% ($V_{125\%}$) of $\dot{V}O_{2\text{max}}$ for each horse. All horses were judged to be clinically healthy based on physical, lameness and endoscopic examinations, and had a history of exercise-induced pulmonary hemorrhage (EIPH). Sub-clinical degenerative joint disease or other mild joint changes are common in retired racehorses aged 6-8 years old. Whilst these changes did not preclude enrollment in the study, due to the demands of the high-intensity exercise conditioning, a lameness examination was performed on every horse each day. For humane reasons, treatments were prescribed to all horses as required, including either phenylbutazone (1-2g PO SID) or flunixin meglumine (1.1mg/kg IV SID). Horses were housed in small groups of 2-3 horses/paddock, and had ad libitum access to pasture, alfalfa and grass hay, and were fed senior ration and beet pulp. This study was approved by the Institutional Animal Care and Use Committee at Washington State University.

**Instrumentation**

Horses were weighed using a digital weigh scale (Brecknell, model PS-3000HD, Fairmont, MN, USA) immediately before or after instrumentation. Catheterization of the jugular vein (14-gauge, 5½” Abbocath-T, Hospira Inc., Lake Forest, IL, USA) was performed on alternating sides to facilitate blood sampling during exercise. A digital-display polar cardiotachometer (Polar Equine RS800CX G3, Bethpage, NY, USA) was attached to a surcingle to record the heart rate (HR). Horses were hand walked for a minimum of 10 minutes prior to application of a mask fitted with an ergospirometer (Sides, Bayly et al., 2014); the horse was then walked immediately onto the high-speed treadmill (SÄTO, Knivsta, Sweden).

**Incremental exercise test: measurement of maximum $O_2$ consumption ($\dot{V}O_{2\text{max}}$)**

$\dot{V}O_{2\text{max}}$ measurements were performed on the high-speed treadmill using a full mask ergospirometry system (Sides, Bayly et al., 2014). Horses were acclimatized to exercise on a high-speed treadmill whilst
wearing the mask prior to commencement of the study, through participation in previous studies. The mask was internally padded to minimize dead space. The system was calibrated (flowmeter and gas analyzer) as previously reported (Sides, Bayly et al., 2014) prior to application on the horse. The incremental exercise test was performed with the treadmill inclined at 10% and consisted of a 4-minute warm-up period at 4m.s\(^{-1}\), after which the treadmill speed was increased to 6m.s\(^{-1}\) for 60s, followed by 1m.s\(^{-1}\) increments in speed every 60s until the horse could not maintain speed on the treadmill despite strong verbal encouragement. The treadmill was then rapidly slowed and stopped (<10s). The same person (RS) judged when to stop all exercise tests (both the incremental and supramaximal intensity tests). Oxygen consumption (\(\dot{V}O_{2}\)) was calculated based on data acquired in the last 15s of each speed, or the last 15s before the incremental test terminated for the final speed using the software provided (Sides, Bayly et al., 2014). Venous blood and a HR measurement were obtained concurrently at the following timepoints: before exercise (at rest), at the end of the warm-up period, in the last 10s of each speed, within 10s of the end of the test, 2, 5 and 10 minutes post exercise, and then every 10 minutes until 60 minutes post exercise. Venous blood was collected for lactate analysis into sterile 4.0mL EDTA tubes and kept on ice until analysis (Lactate Pro 2, Arkray, Edina, MN, USA) which occurred within 10 mins of sample collection.

**Supramaximal exercise tests**

After completion of the incremental exercise test and identification of the \(\dot{V}O_{2}\)\(_{\text{max}}\) plateau, a \(\dot{V}O_{2}\) versus speed regression equation for the linear portion of the curve was determined for each horse, as previously described (Rose, Hodgson et al., 1988). From this equation, speeds were calculated that would induce an oxygen demand of 105% (\(V_{105}\)), 115% (\(V_{115}\)) and 125% (\(V_{125}\)) of \(\dot{V}O_{2}\)\(_{\text{max}}\) for each horse. High intensity exercise tests consisted of the horse trotting on the inclined treadmill at 4m/s for 4 minutes, after which the speed was rapidly increased (<12s) to \(V_{105}\), \(V_{115}\) or \(V_{125}\). Horses ran at this speed until they were unable to maintain speed on the treadmill despite strong verbal encouragement; the treadmill was then rapidly slowed and stopped (<10s). Horses had a minimum of 48 hours to recover between high intensity exercise tests, and the order of the intensity of the tests was
randomized. \( \dot{V}O_2 \) was measured using the same mask system as for the incremental exercise test, recorded and was later calculated every 15s during the run. Venous blood was collected for lactate analysis, and a HR obtained concurrently at the following timepoints: before exercise, at the end of the warm-up period, every 15s during the test, 2, 5 and 10 minutes post exercise, and then every 10 minutes until 60 minutes post exercise.

**Assessment of anaerobic capacity: Method one (MAOD)**

Anaerobic capacity was indirectly calculated from the MAOD during the supramaximal exercise tests (Figure 5.1). Oxygen demand during each high intensity test was extrapolated from the \( \dot{V}O_2 \) versus speed regression equation calculated for each horse after the incremental exercise test as previously reported (Rose, Hodgson et al., 1988). Maximum accumulated oxygen deficit (MAOD) was therefore calculated as the difference between the measured oxygen consumption and the estimated oxygen demand for the duration of each high intensity exercise test (Figure 5.1), starting from the moment the speed was rapidly increased from 4m/s to \( V \)105\%, \( V \)115\% or \( V \)125\% (Hinchcliff, McKeever et al., 1996; Geor, McCutcheon et al., 2000).

**Assessment of anaerobic capacity: Method two (MAOD\textsubscript{ALT})**

Aerobic contribution was calculated using the trapezoidal method (subtracting resting \( O_2 \) consumption) to determine \( \dot{V}O_2 \) during exercise (Figure 5.1) (de Moraes Bertuzzi, Franchini et al., 2007; Artioli, Bertuzzi et al., 2012). A mono-exponential model was fitted to the post-exercise \( \dot{V}O_2 \) curve (Figure 1). Alactic contribution was calculated using the coefficients of this model as previously described (Beneke, Pollmann et al., 2002; Beneke, Beyer et al., 2004; Bertuzzi, Franchini et al., 2010; Artioli, Bertuzzi et al., 2012). Venous blood was collected for lactate analysis during the supramaximal runs at the following timepoints: before exercise, at the end of the warm-up period, every 15s during the test, 2, 5 and 10 minutes post exercise, and then every 10 minutes until 60 minutes post exercise. Resting and peak blood lactate were recorded. Lactic anaerobic contribution was calculated by multiplying the \( \Delta BL \text{Peak-Resting} \) by 3 as previously described using this estimation method in human subjects (Margaria,
Cerretelli et al., 1963; Di Prampero, Capelli et al., 1993; Zagatto, Redkva et al., 2011; Artioli, Bertuzzi et al., 2012; Bertuzzi, Kiss et al., 2015). Calculated contributions (mL) were then converted into kJ (1L O₂= 20.92kJ) to determine relative contributions. MAOD\textsubscript{ALT} was calculated as the sum of the alactic and lactic metabolism contributions.

Statistical analysis

A linear mixed effects model was used to examine the effects of method (MAOD, MAOD\textsubscript{ALT}) and exercise intensity (V105%, V115%, V125%) (as fixed effects) on the anaerobic and anaerobic contributions to total energy production (%) (as the outcomes), after accounting for the nested data structure from horses (as a random effect). A linear mixed effects model was also used to examine the effects of exercise intensity (V105%, V115%, V125%) (fixed effect) on numerous outcomes (shown in table 5.1), after accounting for the nested data structure from horses (as a random effect). The assumptions of normality and equal variance were assessed. Analysis was performed using R version 3.4.1, and ‘nlme’ package version 3.1-137 was used for linear mixed effects model analysis. Statistical significance was set at p ≤ 0.05 for all tests. Values are reported as median and interquartile range (IQR) to accommodate non-normal data, except where stated as mean (SD).
Figure 5.1: Schematic illustration of a typical equine oxygen consumption curve during and after exercise.
Results

The aerobic and anaerobic (lactic and alactic) variables measured and calculated using the trapezoid method and MAOD<sub>ALT</sub> (method 2) at each exercise intensity are shown in Table 1. Heart rate data was measured to ensure a plateau was reached (Figure 5.2). There was no significant difference in VO<sub>2max</sub> between supramaximal exercise intensities (Table 5.1); horses had a mean VO<sub>2max</sub> of 155.42 ml.kg<sup>-1</sup>.min<sup>-1</sup> (SD 10.80) at V105%, 153.47 ml.kg<sup>-1</sup>.min<sup>-1</sup> (SD 7.90) at V115% and 155.62 ml.kg<sup>-1</sup>.min<sup>-1</sup> (SD 5.90) at V125%. After accounting for inter-horse variation, horses ran significantly longer at V105% than at V115% (P=0.007) and V125% (P=0.0001). There was a significant increase in mass specific (ml.kg<sup>-1</sup>) net O<sub>2</sub> consumption from V105% to V115% (p=0.005) to V125% (p=0.0001) (Table 1). Furthermore, there was a significant increase in the absolute (L) net O<sub>2</sub> consumption from V105% to V115% (p=0.0047) to V125% (p=0.0001) (Table 5.1). Therefore, there was a corresponding significant increase in the total aerobic energy (kJ) from V105% to V115% (p=0.0047) to V125% (p=0.0001). There was no difference in resting or peak lactate between exercise intensities. Lactate clearance after the supramaximal runs is shown in Figure 5.3. There was no difference in the net anaerobic (lactate) energy between V105% and either V115% (p=0.45) or V125% (p=0.52). Individual alactic anaerobic energy was unaltered with supramaximal exercise intensity with no difference between V105% and either V115% (p=0.43) or V125% (p=0.43), and was negligible, contributing a mean of 0.11% of the total energy. The rate of energy production (kJ/s) increased with exercise intensity; there was a significant increase from V105% to V125% (p=0.006), however there was no difference between V105% and V115% (p=0.86). The relative aerobic and anaerobic contributions of both methods at each supramaximal intensity are shown in Figure 5.4. Horses’ mean aerobic and anaerobic energy contributions significantly decreased and increased respectively with exercise intensity; with 81.4%, 77.6% and 72.5% of energy demands being met by aerobic contributions, and 18.5%, 22.3% and 27.4% of requirements being met by anaerobic contributions at 105%, 115%, 125% VO<sub>2max</sub> (Figure 5.4, Table 5.1). There was no significant difference in the relative aerobic or anaerobic (p=0.20) contributions between methods (Figure 5.4); the linear mixed-effects model reported a non-significant (p=0.20) difference of 1.88% (SE 1.43) between methods. Anaerobic contributions calculated using both
methods at each exercise intensity are shown in Figure 5.5; the standardized residuals by method (Figure 5.6), and the normal QQ plot (Figure 5.7) are also reported.
Table 5.1: Median (IQR) values for aerobic and anaerobic (lactic and alactic) variables measured and calculated from 5 horses during supramaximal exercise to exhaustion on a high-speed treadmill at speeds corresponding to 105%, 115% and 125% \( \dot{V}O_2\text{max} \)

<table>
<thead>
<tr>
<th>Variable</th>
<th>105%</th>
<th>115%</th>
<th>125%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (kg)</td>
<td>496 (485-527)</td>
<td>497 (482-530)</td>
<td>492 (483-529.5)</td>
</tr>
<tr>
<td>Run duration (s)</td>
<td>166 (113-168)*</td>
<td>131 (84.5-144.5)*</td>
<td>83 (68.5-102)*</td>
</tr>
<tr>
<td>Resting VO(_2) (ml.(min.kg(^{-1}))</td>
<td>4.26 (1.97-5.72)</td>
<td>5.31 (3.64-8.09)</td>
<td>4.16 (2.45-7.57)</td>
</tr>
<tr>
<td>(\dot{V}O_2\text{max} ) (ml.kg(^{-1}).min(^{-1}))</td>
<td>151.79 (147.74-164.93)</td>
<td>149.90 (148.03-160.70)</td>
<td>155.23 (150.11-161.33)</td>
</tr>
<tr>
<td>Net O(_2) consumption (ml.kg(^{-1}))</td>
<td>348.27 (231.0-360.1)*</td>
<td>280.06 (161.41-294.52)*</td>
<td>173.62 (144.9-208.0)*</td>
</tr>
<tr>
<td>Net O(_2) consumption (L)</td>
<td>172.74 (113.29-187.95)*</td>
<td>139.19 (79.1-153.37)*</td>
<td>85.42 (70.80-110.01)*</td>
</tr>
<tr>
<td>Net aerobic energy (kJ)</td>
<td>3613.73 (2370.04-3931.89)*</td>
<td>2911.84 (1654.68-3208.58)*</td>
<td>1787.05 (1481.16-2301.46)*</td>
</tr>
<tr>
<td>Resting lactate (mmol/L)</td>
<td>0.6 (0.5-0.75)</td>
<td>0.5 (0.45-0.55)</td>
<td>0.5 (0.35-0.65)</td>
</tr>
<tr>
<td>Peak lactate (mmol/L)</td>
<td>24.4 (20.55-24.85)</td>
<td>22.8 (20.05-24)</td>
<td>23.3 (20.1-24.05)</td>
</tr>
<tr>
<td>Net anaerobic (lactate) energy (kJ)</td>
<td>750.36 (609.17-792.05)</td>
<td>715.07 (601.18-760.46)</td>
<td>714.91 (607.0-763.07)</td>
</tr>
<tr>
<td>Alactic energy (kJ)</td>
<td>3.39 (2.56-4.11)</td>
<td>3.13 (1.3-3.93)</td>
<td>3.75 (3.26-4.79)</td>
</tr>
<tr>
<td>Total energy (kJ)</td>
<td>4217.01 (3059.15-4725.91)*</td>
<td>3610.54 (2285.8-3954.08)*</td>
<td>2500.67 (2110.29-3052.99)*</td>
</tr>
<tr>
<td>Rate of energy production (kJ/s)</td>
<td>26.12 (25.53-29.36)*</td>
<td>26.39 (25.80-29.34)</td>
<td>31.00 (27.45-32.65)*</td>
</tr>
<tr>
<td>Aerobic contribution (%)</td>
<td>82.82 (77.52-84.61)*</td>
<td>80.65 (72.63-81.15)*</td>
<td>72.09 (69.71-75.37)*</td>
</tr>
<tr>
<td>Anaerobic (lactate) contribution (%)</td>
<td>17.1 (15.31-22.38)*</td>
<td>19.27 (18.77-27.27)*</td>
<td>27.79 (24.47-30.1)*</td>
</tr>
<tr>
<td>Anaerobic (alactic contribution) (%)</td>
<td>0.089 (0.07-0.1)</td>
<td>0.09 (0.03-0.15)</td>
<td>0.14 (0.12-0.22)</td>
</tr>
</tbody>
</table>

*Indicates significant difference (p<0.05) between variables in row with symbol
Figure 5.2: Heart rates (bpm) of five horses during supramaximal exercise to exhaustion performed on a high-speed treadmill inclined at 10%. Data are reported as mean (SD).
Figure 5.3: Blood lactate concentrations of five horses after supramaximal runs performed on a high-speed treadmill inclined at 10%. Data are reported as mean (SD)
Figure 5.4: Relative aerobic and anaerobic contributions of five horses during supramaximal exercise to exhaustion on a high-speed treadmill inclined at 10%, calculated using MAOD (method 1), and MAOD_{ALT} (method 2). Data are reported as mean (SD). * indicate a significant difference between contributions (p<0.05).

<table>
<thead>
<tr>
<th>Intensity</th>
<th>Aerobic contribution (Method 1)</th>
<th>Anaerobic contribution (Method 1)</th>
<th>Aerobic contribution (Method 2)</th>
<th>Anaerobic contribution (Method 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>105%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>115%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>125%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* indicate a significant difference between contributions (p<0.05).
Figure 5.5: Anaerobic contributions (%) of five Thoroughbred horses calculated using MAOD and MAOD_{ALT} during supramaximal exercise to exhaustion on a high-speed treadmill inclined at 10%
Figure 5.6: Standardized residuals by method (MAOD and MAOD_{ALT})

Figure 5.7: Normal QQ plot
Discussion

This study reports a method which can be used to determine the relative energy contributions in horses, in situations where the exercise intensity is unknown or cannot be controlled. The maximum accumulated oxygen deficit, used as the reference method, was not significantly different to a method which determines the energetic equivalence of lactate accumulation in the blood following exercise; there was no difference in the relative aerobic or anaerobic contributions between methods. Individual alactic anaerobic capacity provided a negligible contribution to the total energy requirements.

Whilst we used MAOD as our “reference” method, it is important to note that it has never been validated for use in the horse, as there was no prior standard to which to compare it (Eaton, Evans et al., 1995). Therefore, this manuscript provides an ideal opportunity for the discussion of the physiological strengths and weaknesses of both methods examined in this study. Theoretically, an ideal model of anaerobic capacity would give repeatable results regardless of whether one method or another was used; the difference between the two methods would be zero. However, the measurement of any variable always implies some degree of error (Giavarina, 2015). As we do not have the ability to measure the true value of the anaerobic capacity in the intact horse, the mean of the two measurements is the best estimate available (Giavarina, 2015). We determined that there was a non-significant 1.88% difference between the two methods. In humans, absolute MAOD values are reduced by 10% to account for intrinsic O₂ contributions (Medbo, Mohn et al., 1988). However, the body oxygen stores in the equine athlete are not well understood and this reduction was not performed, which may have contributed to this small, non-significant difference. As the anaerobic capacity is unable to be directly measured, tests which attempt to estimate it are dependent upon assumptions; the transferability of these assumptions to the equine athlete must be discussed.

The calculation of MAOD in both humans and horses is dependent on two major assumptions; firstly, that the O₂ demand of a supramaximal exercise test can be extrapolated from submaximal VO₂
measurements, i.e. that the relationship between substrates (ATP), oxygen consumption, and work performed is a relatively stable relationship, and secondly, that \( \text{O}_2 \) demand is constant during the supramaximal exercise test (Green and Dawson, 1993). In humans, it is probable that the first assumption is violated, as the efficiency of muscle contraction at submaximal intensities is higher than at supramaximal power outputs, representing an impairment of ATP utilization (Hansen, Casaburi et al., 1988; Luhtanen, Rahkila et al., 1990). Furthermore, it is increasingly recognized that ATP production can also be impaired; factors which affect the ratio between ATP re-synthesis and \( \text{O}_2 \) consumption by the mitochondria in muscle, such as mitochondrial hyperthermia (as experienced during a supramaximal exercise test) can uncouple this relationship (Grassi, Rossiter et al., 2015). On a macroscopic scale, both impairment of ATP production and utilization would result in progressively greater anaerobic contribution in order to maintain a fixed rate of supramaximal exercise, which would lead to an underestimation of the \( \text{O}_2 \) demand and therefore the \( \text{O}_2 \) deficit (MAOD) (Green and Dawson, 1993).

In contrast, horses are able to maintain a high mechanical energy through the storage and release of elastic energy in both the lower limb apparatus and the nuchal ligament, which enables an exchange of potential and kinetic energy at the gallop; the ‘apparent efficiency’ of galloping horses is greater than 100% (Minetti, Ardigo et al., 1999). In humans the individual work economy, which is dependent on both internal and external muscle efficiency factors, can vary at submaximal exercise intensities by up to 16% (Medbo, Mohn et al., 1988). At high exercise intensities, lactate accumulates and decreases the muscle efficiency, possibly decreasing the accuracy with which \( \text{O}_2 \) consumption reflects the \( \text{O}_2 \) demand (Vandewalle, Kapitaniak et al., 1989; Green and Dawson, 1993). In both human and equine athletes, muscle lactate concentration increases up to 100mmol/kg dry weight after maximal exercise (Juel, Bangsbo et al., 1990), and concentrations of 200mmol/kg dry weight have been reported in horses (Snow, Harris et al., 1985). Whilst the buffering capacity of trained muscle can be 50% higher in horses than in human athletes (McCutcheon LJ, 1987), it is not enough to stop acidification, with the pH reported to drop as low as 6.57 after repeated bouts of intense exercise (Gollnick, Bertocci et al., 1990).
Furthermore, variation in MAOD between studies can be attributed to multiple factors: the exercise mode and exercising muscle mass, exercise duration, technological and biological variability, and differences in methods between studies (Green and Dawson, 1993).

The validity of using the MAOD to quantify the anaerobic capacity has previously been directly and indirectly supported. Indirectly, sprint athletes consistently display greater MAOD values than endurance athletes during exhaustive treadmill tests (Hermansen and Medbø, 1984), which is consistent with the increased metabolic indicators of anaerobic potential (i.e. buffer values, glycolytic enzyme activities) observed in sprint athletes. Directly, the oxygen deficit accumulated during maximal exercise was almost identical to the ATP yield calculated from metabolite changes and estimated skeletal muscle lactate clearance during exercise (Bangsbo, Gollnick et al., 1990). At present, MAOD is considered the most accurate method to quantify the anaerobic capacity in both horses and humans and can therefore provide insight into the validity of using MAOD$_{ALT}$ to estimate the anaerobic capacity in horses.

An alternative method used in people, MAOD$_{ALT}$ (Bertuzzi, Franchini et al., 2010), estimates the alactic and lactic components of the anaerobic capacity separately. To apply this technique to horses, the underlying basis for each component requires examination. The fast component of the post-exercise $\dot{V}O_2$ curve has been associated with the rate of resynthesis of high energy phosphates ($\sim P_i$ i.e. phosphocreatine (PCr), adenine nucleotides) in both canine gastrocnemius muscle (Piiper, Di Prampero et al., 1968) and the rate of PCr resynthesis in human quadriceps muscle (Hultman, Bergström et al., 1967). Therefore, it is considered that the energy yield from phosphagen hydrolysis during exercise reflects the alactic O$_2$ debt (di Prampero and Ferretti, 1999); the alactic anaerobic system is estimated by the fast component of the post-exercise $\dot{V}O_2$ curve (Haseler, Hogan et al., 1999). This is well-described by a monoexponential time equation, the constant of which is independent of exercise intensity and PCr concentration at the end of exercise (Thompson, Kemp et al., 1995). Furthermore, PCr recovery after exercise in trained humans is altered by the fraction of inspired O$_2$ (FiO$_2$); PCr
recovery is longer with hypoxia and shorter with hyperoxia, and is therefore considered to be limited by O₂ availability (Haseler, Hogan et al., 1999). High energy phosphate recovery in our horses was correlated with the fast component of the post-exercise VO₂ curve (Rose, Hodgson et al., 1988), suggesting that the alactic anaerobic system can be estimated by the fast component of the post-exercise VO₂ curve in horses. In the present study, the individual alactic anaerobic energy was unaltered with supramaximal exercise intensity, and was negligible, contributing a mean 0.11% of the total energy required. Equine muscle ATP is depleted by 30% and 47% after 800m and 2000m gallops respectively, and muscle PCr decreased below resting concentrations for ≤20 mins (Harris, Marlin et al., 1987). The relatively small contribution of the alactic anaerobic system is not surprising, given i) that its contributions to metabolism are predominantly in the first 10 seconds of exercise, and ii) the large overall ATP expenditure associated with the entire exercise.

A method has been suggested which calculates the glycolytic energetic equivalent of lactate accumulation in the blood and is equated with the anaerobic lactate contribution (Margaria, Cerretelli et al., 1963; di Prampero and Ferretti, 1999; Artioli, Bertuzzi et al., 2012). Lactate concentration at any given time is a balance between production and its removal and metabolism. It is likely that the tight coupling between ATP production and VO₂ observed at moderate intensity in humans (Cannon, Bimson et al., 2014) is lost during high-intensity exercise in horses. It is important to consider that due to the probable impairment of ATP production, this is likely a limitation of both MAOD,ALT and MAOD; especially given that MAOD,ALT relies on an assumption of a fixed constant representing the oxygen equivalents for the lactic anaerobic contribution. At supramaximal exercise intensities, glycogen resynthesis is depressed to the extent that lactate is removed via its oxidation. Pyruvate removal into the Krebs cycle, as part of this process, requires oxygen consumption with the same stoichiometric coefficient (di Prampero and Ferretti, 1999). During intense exercise the total amount of energy required is larger than the oxygen consumption by an amount proportional to the net lactate accumulation (di Prampero and Ferretti, 1999). Lactate “production” per kilo of body mass was therefore estimated based on two assumptions; firstly, that the peak blood lactate value obtained is representative of the
equilibrium lactate concentration throughout the body fluid compartments, and secondly, that the water fractions of the blood and of the whole body are 0.8 and 0.6 respectively (Margaria, Cerretelli et al., 1963). These assumptions are readily transferrable to horses. The initial calculation of energy released in vivo by the “production” of 1g of lactic acid was 222 cal g\(^{-1}\) (Margaria, Cerretelli et al., 1963); this result was confirmed in multiple studies in humans (Margaria, Aghemo et al., 1971), running dogs (Cerretelli, Piiper et al., 1964) and in an isolated-perfused system of the canine gastrocnemii muscle (Cerretelli, Di Prampero et al., 1969). Given that there is no difference between lactate in horses, dogs and people, when both \(\text{O}_2\) consumption and lactate production are expressed in energy units, 1ml \(\text{O}_2\) = 5 cal = 22.5mg lactic acid (Margaria, Cerretelli et al., 1963). Assuming an equine body water content of 0.6 and blood water fraction of 0.8, 22.5mg/kg lactic acid = 3.3mg/100ml lactic acid in blood (Margaria, Cerretelli et al., 1963). The energy equivalent of blood lactate accumulation is derived from accumulated oxygen deficit and \(\Delta\text{BL}_{\text{Peak-Resting}}\) (Di Prampero and Ferretti, 1999). Reported values range from 2.7 (Pendergast, Di Prampero et al., 1977) to 3.58 mL \(\text{O}_2\).kg\(^{-1}\) (Tanji, Tsuji et al., 2017) and vary with the type and intensity of exercise. A smaller range of values have been derived under a variety of supramaximal running conditions. We noted that other studies aiming to determine MAOD, rather than the energy equivalent of lactate, used the value of 3.0 mL \(\text{O}_2\).kg\(^{-1}\) for their calculations (Di Prampero, Capelli et al., 1993; Zagatto, Redkva et al., 2011; Bertuzzi, Kiss et al., 2015) and elected to follow suit in the absence of any other more definitive information. Thus, lactate anaerobic contribution was calculated by multiplying the \(\Delta\text{BL}_{\text{Peak-Resting}}\) by 3: 1mmol/L BL increase = 3mL \(\text{O}_2\).kg\(^{-1}\). The argument can therefore be made, bearing in mind the underlying assumptions, that this is a biologically valid and applicable technique to apply to the estimation of the lactic anaerobic system of horses.

Horses are incredible athletes, with the supramaximal exhaustive exercise described in this study yielding up to 4217.01kJ of total energy at \(\text{V}_{105}\)%. Increasing exercise intensity was associated with an increased rate of energy production, from 26 to 31 kJ/s, markedly higher than reported values of 1.15kJ/s in humans, although this was in recreational rock-climbers (de Moraes Bertuzzi, Franchini et al., 2007; Artioli, Bertuzzi et al., 2012). However, increasing exercise intensity also resulted in a lower
total energy cost (kJ). This reflects that it is harder to maintain a higher speed due to the various factors responsible for the onset of fatigue, in particular, intramuscular acidification (Lovell DK, 1987). One of the limitations of treadmill studies when compared to field exercise, is that upon reaching fatigue, in the field horses can keep going, albeit at a slowing speed, whereas on a treadmill, the test is ended. Therefore, the method applied in this paper will be highly useful for calculating total energy expenditure and the contribution of the energy systems in the field where the exercise intensity is not only unknown but is able to fluctuate. In field situations, it will be important to ensure the protocol is designed to ensure the peak lactate value is obtained, as the accuracy of MAOD_{ALT} is dependent on detecting the maximum change in lactate from rest. In the present study, peak lactate concentration was obtained two minutes after the cessation of supramaximal exercise, which is similar to previous studies where the peak lactate was obtained two to five minutes post-exercise (Evans, Harris et al., 1993; Evans, Rainger et al., 1995).

The methods reported in this paper allow for the measurement of the aerobic capacity and give a reasonable estimate of the anaerobic capacity of horses. That there was no difference between MAOD and MAOD_{ALT} will enable the utilization of MAOD_{ALT} in the field where the exercise intensity is unknown or varies, or on a high-speed treadmill in instances where a single supramaximal run is preferable to an incremental exercise test. The ability to understand the relative metabolic contributions of equine athletes using a single, supramaximal test could have many possible applications. Tailored training programs might be developed, where owners or trainers are able to track progress by performing serial measurements over time to ensure that horses are being trained in the correct “zone” for the performance discipline.
Chapter 6 - Efficacy of injected dexamethasone, aerosolized salbutamol and reduced respirable particulate concentration on respiratory performance in horses with smoke-induced mild equine asthma

Summary

Background – Mild equine asthma (MEA) decreases racing performance and impairs gas exchange after exercise. The efficacy of treatment on performance is unknown.

Hypothesis – Our hypothesis was that treatment targeting lung inflammation improves $\dot{V}O_2$peak in horses with MEA.

Animals – Argentinean Thoroughbred polo horses (n=12) with smoke-induced MEA.

Methods – Prospective, randomized, placebo-controlled, double-blinded clinical trial. All horses completed three $\dot{V}O_2$peak tests, measuring aerobic and anaerobic variables: T1 (day 0) – baseline; T2 (day 16) – after dexamethasone (20mg IM SID; DEX, n=6) or saline treatment (SALINE, n=6), with improved environmental respirable particulate mass; T3 (day 17) – 15-30mins after a single dose of inhaled salbutamol (1500μg). Bronchoalveolar lavage and mucus scoring were performed on day -8 and day 20. Horses were treated for 20 days (commencing day 1). Linear mixed effects models were used to examine the effects of timepoint and treatment group (as fixed effects) on BAL differential cell counts, mucus scores, and aerobic and anaerobic variables.

Results – Horses’ mucus scores improved significantly from day -8 to day 20 by 1.27 ± .38 (mean ± S.E.; p=.008). There was a significant increase in $\dot{V}O_2$peak of 15.5 ± 4.0 ml(min.kg)$^{-1}$ from T1 to T3 (p=0.002). Individual increases in $\dot{V}O_2$peak ranged from 11.7% (SALINE) to 14.6% (DEX). There was no difference in $\dot{V}O_2$peak between treatment groups (SALINE versus DEX) at any timepoint (T1, T2 or T3) (p=.91).

Conclusions and clinical importance – Results of this study highlighted the importance of improved air quality on functionally significant airway inflammation. The evidence regarding the corrective efficacy
of treatment provided in this study is central to increasing owner compliance with veterinary recommendations regarding improved air quality for the treatment and prevention of MEA.

**Introduction**

It has recently been shown that mild equine asthma decreases racing performance in a population of Thoroughbred racehorses (Ivester K, 2017). Furthermore, in studies performed in a controlled environment on a high-speed treadmill, it has been shown that gas exchange is impaired after exercise in horses with mild equine asthma (Couetil and Denicola, 1999; Courouce-Malblanc, Pronost et al., 2002; Sanchez, Couetil et al., 2005). Therefore, given that one of the presenting complaints of mild equine asthma is poor performance (Couetil, Cardwell et al., 2016), evidence regarding the corrective efficacy of treatment on performance would be invaluable.

Horses with mild equine asthma have empirically been treated with glucocorticoids. Recently, it has been shown that dexamethasone (0.05mg/kg IM q24h) and inhaled fluticasone (3,000µg q12h) are effective at reducing hypersensitivity and hyperreactivity in horses with mild equine asthma (Leguillette, Tohver et al., 2017). However, no improvement in bronchoalveolar lavage (BAL) neutrophil percentage was observed following short-term administration of glucocorticoids, which is consistent with findings in severe equine asthma studies where the air quality was not improved (Leguillette, Desevaux et al., 2002; Gerber, Schott Ii et al., 2011). Interestingly, without environmental modification, long-term dexamethasone and fluticasone administration have no effect on airway neutrophilia, even after 6-7 months (Ivester and Couëtil, 2014). However, when corticosteroid treatment is combined with measures to improve air quality, it has been shown to improve clinical signs, airway neutrophilia and inflammatory cytokines in horses with severe equine asthma (Couëtil, Chilcoat et al., 2005; Leclere, Lavoie-Lamoureux et al., 2012). Excessive tracheal mucus accumulation is a feature of mild equine asthma, and increased mucociliary clearance might be also be beneficial in the amelioration of the condition (Norton, Jackson et al., 2013). Airway hyperresponsiveness is also observed in horses.
with mild equine asthma, and has been associated with respiratory clinical signs and exercise intolerance (Klein and Deegen, 1986; Hare, Viel et al., 1994). Airway hyperresponsiveness is often treated with an inhaled bronchodilator, even though bronchoconstriction is not severe enough to increase respiratory effort at rest and its effect is not well documented during exercise. Treatment with bronchodilators should always be in conjunction with environmental control strategies to reduce exposure to dust to ensure that the amount of particulates reaching the lower airways is not increased (Couetil, Cardwell et al., 2016).

The aerobic capacity of a horse can be directly measured, as maximal oxygen consumption ($\dot{V}O_2$max). Typically, $\dot{V}O_2$max is characterized by demonstrating no increase in $\dot{V}O_2$ despite an increase in workload. Under field conditions this can be difficult to demonstrate conclusively with the result that the variable, $\dot{V}O_2$peak is often preferred. Traditionally, $\dot{V}O_2$ has been measured in equine sports medicine using stationary equipment under laboratory conditions, while a horse performs a standardized treadmill incremental speed test (Eaton, Evans et al., 1995). A major limitation of these laboratory tests is the fact that they do not reflect exercise performed under genuine field conditions. Attempts have been made to measure $\dot{V}O_2$peak in the field in horses (Karlsen and Nadaljak, 1964; Hanak, Jahn et al., 2001; Van Erck, Jakesova et al., 2006) but, unfortunately, because of increased resistance to airflow induced by the masks that have been worn, the $\dot{V}O_2$peak measurements obtained were not reliable and presented risks to the horses (Van Erck, Jakesova et al., 2006). Recently, a mask has been developed and validated that can accurately measure $\dot{V}O_2$peak, airflows and tidal volumes on a breath-by-breath basis under field conditions (Sides, Bayly et al., 2014). The overall aim of this study was to evaluate the hypothesis that treatment targeting lung inflammation improves $\dot{V}O_2$peak in horses with mild equine asthma. Our specific objective was to determine whether dexamethasone, salbutamol and a reduction in inhaled particulate mass increase $\dot{V}O_2$peak in the field in horses with mild equine asthma.
Materials and Methods

Animal care

This study was conducted in accordance with the recommendations of the Canadian Council of Animal Care. The research protocol was reviewed and approved by the University of Calgary Veterinary Sciences Animal Care Committee (AC18-0133). Informed consent was obtained from the owners of the horses enrolled in the study.

Horse enrollment and study design

This was a prospective, randomized, controlled, double-blinded clinical trial. Argentinean Thoroughbred horses (n=12; 10 mares, 2 geldings; 6-17 years old; mean weight 493±26kg) used for polo were recruited at the end of the competition season when horses were at a maintenance level of fitness. All horses continued their maintenance exercise regime throughout the trial to ensure no deconditioning occurred (5-10 mins walk, 15-20 mins canter/extended trot, 15 mins walk, turned out; 5-6 days/week). Air quality was poor due to bushfire smoke for one month prior to the initial performance analysis (T1; Figure 1), with an average daily respirable particulate mass (<2.5μm; PM$_{2.5}$) of 35.51µg/m$^3$ from day -33 to day 0. Air quality improved on day 0, with an average daily value of 7.04µg/m$^3$ (PM$_{2.5}$) from day 0 to day 20. This average approved air quality data was obtained from the City of Calgary under the Open Government License. Horses had a history of coughing and decreased performance during the period of exposure to smoke, and resided on two properties in close proximity to each other. Of the 12 horses, 10 were turned out together in a 30-acre grass paddock; the other two horses were kept outside in a smaller grass/dirt paddock at the polo club. Except for clinical signs consistent with mild equine asthma, horses were judged to be healthy, based on thorough physical, lameness and respiratory examinations. The horses also had a history of no health issues or respiratory infections during the polo season. For the duration of the trial, the horses’ diet consisted of pasture supplemented with senior feed, with alfalfa hay also being provided (spread out on ground) for the two horses housed at the polo club. BAL fluid was obtained from all horses (n = 12), and respiratory endoscopy (Karl Storz Endoscope, Mississauga, ON, Canada) was performed for mucus scoring twice,
on day -8 and day 20 (Figure 6.1). On day -7, horses were randomly allocated into one of two treatment groups: DEX (horses treated with 20mg dexamethasone IM SID; n = 6) and SALINE (horses treated with 4mL saline IM SID; n = 6). The person administering the treatments and performing the respiratory and statistical analysis (SB) was blinded to the treatment groups. All horses had chronic coughs and were considered to have smoke-induced mild equine asthma based on the following inclusion criteria (defined in a consensus publication (Couëtil, Cardwell et al., 2016)): 1. a BAL with increased percentage of mast cells (> 2%) or/and eosinophils (> 0.5%) or/and neutrophils (> 5%), and 2. absence of labored breathing at rest. On day 0 (T1; Figure 6.1), horses completed their first \( \dot{V}O_2 \) peak test. Treatment commenced on day 1. \( \dot{V}O_2 \) peak was measured again on day 16. On day 17, horses were administered salbutamol (1500μg) 13-30 minutes prior to completing their third performance analysis (T3; Figure 6.1). Ambient temperature ranged from 19-20°C (T1), 5-9°C (T2) and 3-6°C (T3). As exercise in cold conditions has been shown to induce a transient airway neutrophilia (Davis, Williams et al., 2007) and is associated with higher respiratory impedance and resistance 48 hours after exercise (Davis, Royer et al., 2006), horses were given 3 days to recover from the runs prior to the BAL and scoping procedure being repeated on day 20 (Figure 6.1). Horses were treated for 20 days, from day 1 until day 20.

**Procedures**

Horses were sedated to effect with xylazine hydrochloride (0.4 – 0.5 mg/kg, IV) and butorphanol tartrate (0.05-0.1 mg/kg, IV). Horses were then endoscopically scored for tracheal mucus (Gerber, Straub et al., 2004) (Karl Storz, Mississauga, ON, Canada). A blind BAL was then performed as previously described (Bond, Timsit et al., 2017). Lavage fluid was stored immediately after collection at 4°C. A differential cell count was performed within 6 h of sample collection and was performed on a minimum of 400 cells (Fernandez, Hecker et al., 2013). Epithelial cells were not included in the differential count. Preparation of slides was performed with 400μL of BAL fluid (Fernandez, Hecker et al., 2013), which was centrifuged using a Cytospin (90 x g for 5 min) and stained with modified Wright-Giemsa stain. A differential cell count was later performed on a minimum of 2,000 cells by a board-certified pathologist.
Normality of the distribution of the BALF differential cell counts were tested by a Shapiro-Wilk normality test. A two-way repeated measures ANOVA (controlling for treatment group and timepoint [Day -8 versus Day 20]) was used to assess differences in cell counts between groups. A p-value < .05 was considered significant.

Performance Analysis

Horses performed a maximal intensity exercise test, consisting of a standardized warm-up, followed by a 600m gallop at maximal intensity, while wearing a mask capable of accurately measuring VO₂peak, airflow and tidal volume on a breath-by-breath basis under field conditions (Sides, Bayly et al., 2014). Calibration of the system (flowmeter and gas analyzer) was conducted as previously reported (Sides, Bayly et al., 2014) before and after each horse was exercised. The mask was internally padded and adjusted for each horse to minimize dead space. Results were calculated using customized software provided with the system. Environmental conditions (ambient temperature, barometric pressure and humidity) were recorded and included in ventilation calculations. Results are reported as STPD.

Jugular venous blood samples (2ml) were collected in lithium-heparin containing vacutainer tubes at rest, and 5, 10 and 15 minutes post-exercise to ensure peak blood lactate concentration was obtained (Evans, Harris et al., 1993). A handheld analyzer (Lactate Scout+, EKF Diagnostics, Penarth, Wales) was used to immediately measure the blood lactate concentration.

Heart rate (HR) was monitored continuously during exercise using a telemetric ECG device and software (Televet 100, Engel Engineering Service, Heusenstamm, Germany). A base/apex configuration was used. Tracings were analyzed to ensure HR plateaued, indicating a maximal effort was obtained.

Split times were obtained for the second and third 200m exercise sectionals using timing lights (Farmtek, Wylie, TX, USA).
Figure 6.1: Representation of protocol and treatment group allocation. Horses were randomly allocated into two treatment groups, DEX (n=6) and SALINE (n=6).
The aerobic contribution to the metabolic energy consumed during the exercise test was calculated using the trapezoidal method (subtracting resting O\(_2\) consumption) (de Moraes Bertuzzi, Franchini et al., 2007; Artioli, Bertuzzi et al., 2012). Resting and peak blood lactate were recorded. Lactic anaerobic contribution was calculated by multiplying the ΔBL\(_{\text{Peak-Resting}}\) by 3 as previously described using this estimation method in human subjects (Margaria, Cerretelli et al., 1963; Di Prampero, Capelli et al., 1993; Zagatto, Redkva et al., 2011; Artioli, Bertuzzi et al., 2012; Bertuzzi, Kiss et al., 2015), and horses (Bond, Greco-Otto et al., 2019). Calculated contributions (mL) were then converted into kJ (1L O\(_2\) = 20.92kJ) to determine the relative contributions.

**Statistical analysis**

Linear mixed effects models were used to examine the effects of timepoint (T1, T2 and T3, Figure 6.1) and treatment group (DEX and SALINE) (as fixed effects) on BAL differential cell counts, mucus scores, and anaerobic and anaerobic variables outlined in Table 6.1 (as the outcomes), after accounting for the nested data structure from horses (as a random effect). The assumptions of normality and equal variance were assessed. Analysis was performed using R version 3.4.1, and ‘nlme’ package version 3.1-137 was used for linear mixed effects model analysis. Statistical significance was set at \(p \leq 0.05\) for all tests. Values are reported as mean ± S.E, except where stated as median and interquartile range (IQR) to accommodate non-normal data.

**Results**

**Cytology**

Bronchoalveolar lavage fluid differential cell counts for each treatment group on day -8 and day 20 are shown in Figure 6.2. The proportion of alveolar macrophages in the BAL fluid significantly increased by 10.8% ± 3.5% from day -8 to day 20 (\(p=0.005\)). The proportion of lymphocytes in the BAL fluid significantly decreased by 10.1% ± 3.4% from day -8 to day 20 (\(p=0.007\)), as did the proportion of eosinophils (0.8% ± 0.3%; \(p=0.01\)). There was no change in the proportion of neutrophils (0.8%, \(p=0.39\), nor
mast cells (p=.39) in the BAL fluid from day -8 to day 20. There was no significant difference in the proportion of any cell type between treatment groups (neutrophils: p=.13; eosinophils: p=.34; mast cells: p=.09; alveolar macrophages: p=.92; lymphocytes: p=.66). Abundant extracellular debris and pollen were present in every BAL on both day -8 and day 20, with some horses also displaying evidence of erythrophagocytosis (Figure 6.3). Curshmann’s spirals were observed on both day -8 (two horses) and day 20 (different two horses) (Figure 6.4).

Mucus scoring

On day -8, the median (IQR) mucus score of horses was 1.5 (.5-3) (SALINE) and 1 (.63-2.5) (DEX).

On day 20, the median mucus score was 0.25 (0-.88) (SALINE) and 0 (0-0) (DEX).

Horses’ mucus score improved significantly from day -8 to day 20 by 1.27 ± .38 (p=.008). There was no difference in mucus score between treatment groups (p=.44).
Figure 6.2: Bronchoalveolar lavage fluid differential cell count percentages (n=12 horses) for each treatment group (SALINE=A and DEX=B) on day -8 (Pre) and day 20 (Post)
Figure 6.3: Intracellular debris and pollen present in BAL slides on both day -8 and day 20. These were also typical findings in our clinical BAL activities during the bushfire season, where horses were exposed to smoke.
Figure 6.4: Curshmann’s spirals in slides made from bronchoalveolar lavage fluid from 4 horses, obtained on day -8 (top 2 pictures) and day 20 (bottom 2 pictures)
Performance analysis

For descriptive values for aerobic and anaerobic variables measured and calculated for both treatment groups before treatment (T1), after treatment with dexamethasone or a saline control (T2) and with the addition of inhaled salbutamol <30 mins prior to the performance test (T3), see Table 6.1. Heart rate data for each run was analysed to ensure a plateau was reached (data not shown).

Horses were 6.7kg ± 1.9kg heavier at T1 than at T2 and T3 (p=.002); there was no difference in weight between T2 and T3 (p=.1). There was no difference in weight between treatment groups (p=.72) at any timepoint.

Horses were significantly faster at T2 and T3 than at T1, with the run duration decreasing from T1 by 6.6s ± 1.4s at T2 (p=.0006), and by 3.9s ± 1.3s at T3 (p=.01), respectively. There was no significant difference in run duration between T2 and T3 (p=.1). There was no significant difference in run duration between treatment groups (p=.3).

After controlling for treatment group, there was no difference in the 200m sectional time (s), obtained between 200m and 400m of the exercise test between T1 and T3 (p=.95), T1 and T2 (p=.78) or T2 and T3 (p=.83). After controlling for timepoint, there was no difference in the same sectional time between SALINE and DEX (p=.83). The average time it took horses to gallop this section (across all timepoints and groups) was 13.6s (See Table 6.1).

After controlling for treatment group, there was no difference in the sectional time (s) obtained between 400m and 600m of the exercise test between T1 and T3 (p=.07), T1 and T2 (p=.07) or T2 and T3 (p=.98). After controlling for timepoint, there was no difference in the sectional times between SALINE and DEX (p=.39). The average time it took horses to gallop this section (across all timepoints and groups) was 15.6s (See Table 6.1).

There was a significant increase in \( \dot{V}O_2 \text{peak} \) of 15.5 ± 4.0ml(min.kg\(^{-1}\)) from T1 to T3 (p=.002). There was a non-significant increase in \( \dot{V}O_2 \text{peak} \) of 6.3 ± 4.5ml(min.kg\(^{-1}\)) from T1 to T2 (p=.19). There was no significant difference between T2 and T3, although there was a strong trend for horses to have a \( \dot{V}O_2 \text{peak} \) greater (9.2 ± 4.7ml(min.kg\(^{-1}\)) at T3 than at T2 (p=.07). At the pre-determined level of
Table 6.1: Mean ± S.E. values for aerobic and anaerobic variables measured and calculated from 12 horses with smoke-induced mild equine asthma during performance tests on a racetrack before treatment (T1), after treatment with dexamethasone or a saline control (T2) and with the addition of inhaled salbutamol <30 mins prior to the performance test (T3). Sectional time 1 refers to the average time to gallop from 200-400m of the 600m exercise test. Sectional speed 2 refers to the time taken to gallop from 400-600m.

<table>
<thead>
<tr>
<th>Variable</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (kg)</td>
<td>492.2 ± 5.8</td>
<td>490.5 ± 15.3</td>
<td>488.3 ± 4.5</td>
</tr>
<tr>
<td>Run duration (s)</td>
<td>53.0 ± .8</td>
<td>54.8 ± 1.3</td>
<td>45.0 ± 1.7</td>
</tr>
<tr>
<td>Sectional time 1 (s)</td>
<td>13.7 ± .5</td>
<td>13.5 ± .5</td>
<td>13.6 ± .3</td>
</tr>
<tr>
<td>Sectional time 2 (s)</td>
<td>14.9 ± .3</td>
<td>15.7 ± 1.0</td>
<td>15.4 ± .4</td>
</tr>
<tr>
<td>Resting VO2 (ml(min.kg)^{-1})</td>
<td>2.2 ± .5</td>
<td>4.4 ± .7</td>
<td>2.9 ± .6</td>
</tr>
<tr>
<td>VO2peak (ml(kg.min)^{-1})</td>
<td>111.2 ± 2.0</td>
<td>108.9 ± 2.7</td>
<td>115.2 ± 4.6</td>
</tr>
<tr>
<td>Net O2 consumption (ml.kg^{-1})</td>
<td>77.8 ± 1.9</td>
<td>79.8 ± 2.2</td>
<td>70 ± 6.8</td>
</tr>
<tr>
<td>Net O2 consumption (L)</td>
<td>38.2 ± 8.8</td>
<td>39.0 ± 8.8</td>
<td>34.5 ± 3.6</td>
</tr>
<tr>
<td>Net aerobic energy (kJ)</td>
<td>800.0 ± 16.8</td>
<td>815.9 ± 17.5</td>
<td>722.0 ± 75.3</td>
</tr>
<tr>
<td>Resting lactate (mmol/L)</td>
<td>.7 ± .02</td>
<td>.7 ± .08</td>
<td>.7 ± 1.3</td>
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<tr>
<td>Peak lactate (mmol/L)</td>
<td>16.5 ± 1.3</td>
<td>17.0 ± .9</td>
<td>17.8 ± 1.1</td>
</tr>
<tr>
<td>Net anaerobic energy (kJ)</td>
<td>488.4 ± 40.1</td>
<td>499.7 ± 19.7</td>
<td>490.3 ± 18.7</td>
</tr>
<tr>
<td>Aerobic contribution (%)</td>
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<td>62.1 ± 1.0</td>
<td>59.3 ± 1.5</td>
</tr>
<tr>
<td>Anaerobic contribution (%)</td>
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<td>37.9 ± 1.0</td>
<td>40.7 ± 1.5</td>
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<table>
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<th>Saline Dexamethasone</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Run duration (s)</td>
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<td>54.8 ± 1.3</td>
<td>45.0 ± 1.7</td>
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<td>15.4 ± .4</td>
</tr>
<tr>
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<td>4.4 ± .7</td>
<td>2.9 ± .6</td>
</tr>
<tr>
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<td>115.2 ± 4.6</td>
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</tr>
</tbody>
</table>
significance (p≤0.05), it is possible that this lack of statistical difference between T2 and T3 is due to a type II error. After controlling for timepoint, there was no significant difference between treatment groups at any timepoint (p=.91).

After controlling for treatment group, there was no difference in peak lactate between T1 and T2 (p=.77), T1 and T3 (p=.13), or T2 and T3 (p=.22) (Table 6.1). After controlling for timepoint, there was no difference in peak lactate between treatment groups (p=.78).

After controlling for timepoint, there was no difference in total exercise aerobic (p=.88) or anaerobic (p=.49) energy (kJ) between treatment groups. There was no significant difference in total exercise aerobic or anaerobic energy between any timepoints (p values all >>.05) (Table 6.1). Consequently, there was no difference in aerobic or anaerobic contributions to total energy production (%) between treatment groups (p=.82), at any timepoint.

**Discussion**

The single most important factor in the enhanced performance seen in these horses with MEA appeared to be improved environment, as manifest by the significantly decreased respirable particulate matter. Dexamethasone administration was not associated with any additional benefit over a reduction in inhaled particulate matter alone, on any measured or calculated variable. Treatment with reduced inhaled particulate matter and salbutamol administration, both with and without dexamethasone administration, was associated with a significant increase in \( \dot{V}O_2 \) peak. However, the addition of salbutamol administration did not result in a significant difference in \( \dot{V}O_2 \) peak when compared to the impact of decreased inhaled particulate matter from the month prior to T1, to T2 and T3, with or without dexamethasone administration. Horses’ mucus scores significantly improved by a mean of 1.27 in response to treatment. Treatment also reduced the proportion of eosinophils in BAL fluid by 0.79%; there was no change in the proportion of neutrophils or mast cells.
There were multiple challenges associated with the execution of the original study design, including uncontrollable factors requiring extension of the sampling timepoints; T1 was delayed after the initial BAL and mucus scoring due to ergospirometry mask repairs. An advantage of the study design was a longer treatment duration, which allowed additional time for clearance of inflammatory cells from the lungs, and improvement in respiratory performance.

A study of racing thoroughbreds found that moderate to severe tracheal mucus (Grades 2-4) (Gerber, Straub et al., 2004) is a risk factor for poor racing performance, based on race place closest to the time of sampling, and whether the horse was raced within 2 weeks of sampling (Holcombe, Robinson et al., 2006). Interestingly, tracheal mucus accumulation, and not an increased proportion of tracheal neutrophils, is associated with functionally significant airway inflammation (Holcombe, Robinson et al., 2006); it is important to note that tracheal mucus is positively correlated with BAL neutrophilia in some studies (Koblinger, Nicol et al., 2011), but not others (Gerber, Robinson et al., 2003; Richard, Fortier et al., 2010a; Depecker, Richard et al., 2014). Prior to treatment, the median mucus score of horses in the present study was 1-1.5, depending on treatment group. Whilst this was below the score previously associated with poor racing performance, this level of airway inflammation was associated with clinical signs of coughing and poor performance. The increased sensitivity of the methods employed in the present study, where performance was directly measured as the horses’ peak oxygen consumption, found a significant improvement in $\dot{V}O_{2\text{peak}}$ of 11.7% (SALINE) to 14.6% (DEX) from T1 to T3, with horses improving in mucus score by a mean of 1.27 grades.

Whilst there is strong evidence that corticosteroid therapy does not normalize airway neutrophilia without environmental modifications, even after treatment periods of up to 6 months (Lavoie, Leguillete et al., 2002; Lavoie, Pasloske et al., 2006; Leclere, Lavoie-Lamoureux et al., 2012; Bullone, Vargas et al., 2017; Leguillete, Tohver et al., 2017), when corticosteroid treatment is combined with measures to improve air quality an improvement in clinical signs, airway neutrophilia and inflammatory cytokines is observed in
horses with severe equine asthma (Couëtil, Chilcoat et al., 2005; Leclere, Lavoie-Lamoureux et al., 2012). It is possible that the lack of clearance of airway neutrophilia was due to the treatment timeframe; perhaps longer treatment periods are required for resolution of airway neutrophilia following prolonged exposure to high levels of inhalable particulate matter in the environment? Indeed, smoke inhalation is known to disrupt mucociliary clearance, with 5 minutes of cigarette smoke resulting in a marked loss of ciliated cells from the bronchial luminal surface (Sisson, Papi et al., 1994). The effects of chronic smoke inhalation on the equine respiratory tract have not been studied, however it is plausible that normalization of airway neutrophilia could be extended following damage to the mucociliary apparatus, and other deleterious effects of smoke exposure. Whilst the authors are unaware of a reported link between chronic smoke inhalation from bushfires and MEA, there is strong evidence linking development of airway inflammation with exposure to higher dust environs (Ferro, Ferrucci et al., 2000; Ivester, Couetil et al., 2014a; Ivester, Couetil et al., 2014b). Given that horses had no history of coughing, poor performance or respiratory disease in the polo season prior to the deterioration of air quality associated with bushfire smoke, and that exposure to higher levels of respirable particulate matter coincided with the onset of clinical signs, it is highly likely that the fact that 100% of horses in the study had both the clinical signs and pathology of MEA was associated with chronic smoke exposure.

In elite non-asthmatic human athletes (Meeuwisse, 1990) and mild asthmatics (Freeman, Packe et al., 1989), salbutamol administration does not significantly effect \( \dot{V}\text{O}_2\text{peak} \). However it does increase FEV\(_1\) (mean forced expiratory volume in 1s) both at baseline and post-exercise (Freeman, Packe et al., 1989; Meeuwisse, 1990). This agrees with post-exercise findings in horses with severe equine asthma after bronchodilator administration (ipratropium bromide) (Bayly, Duvivier et al., 2002); it would appear that maximal sympathetic drive associated with exercise overrides any pharmacologic benefits conferred at rest. Similarly, we found that salbutamol administration did not result in a significant difference in VO\(_2\)peak compared to an improvement in air quality, with and without dexamethasone administration. However, there was a strong trend for the VO\(_2\)peak to be greater following salbutamol administration, and this lack of statistical difference could be due to a type II error; it is possible that
we did not have enough power to detect a difference. Alternatively, horses might have had a greater understanding of what the jockey required of them; this improvement could represent a learned response to a repeated situation. Additional benefits of bronchodilators include increased mucociliary clearance (Norton, Jackson et al., 2013), anti-inflammatory properties (Barnes, 2003), and at higher doses, some human patients with chronic obstructive pulmonary disease exhibit improved exercise tolerance without concurrent improvement in airflow, attributed to increased diaphragmatic contractility (Jagers, Hawes et al., 2009). Effects of bronchodilator therapy on equine respiratory muscles have not been investigated.

This study highlights the importance of improved air quality on functionally significant airway inflammation. Dexamethasone administration was not associated with any additional benefit over a reduction in inhaled particulate matter alone (ie no difference between the control group and the group administered dexamethasone), on any measured or calculated variable. Improved air quality, both with and without dexamethasone, significantly increased VO\textsubscript{2}peak by individual improvements of an average 11.7% (SALINE) to 14.6% (DEX). Mild equine asthma affects up to 66% of horses at some time in their lives (Wasko, Barkema et al., 2011), with 100% of horses in the present study being affected by bushfire smoke, and yet owner compliance with veterinary recommendations, particularly regarding improving environmental management and limiting exposure to dust, is poor, with medical treatment being the preferred option for many clients. Therefore, the evidence regarding the corrective efficacy of treatment provided herein is central to increasing owner compliance with veterinary recommendations and thus improving not only the welfare, but also the performance of a large proportion of the equine population.
Chapter 7 - Conclusions

The overall aims of this thesis were: i) to characterize the respiratory microbiota and mycobiota of healthy horses, and those with mild equine asthma, and determine whether corticosteroid treatment is associated with changes in the local bacterial, fungal and viral communities of the equine respiratory tract; ii) to investigate possible endotypes of mild equine asthma, via exploring changes in relative inflammatory mRNA cytokine expression, and determine if corticosteroid treatment results in differences to gene expression; and iii) to determine the functional impact of airway inflammation on performance, and the efficacy of treatment.

We hypothesized that: i) airway inflammation alters the local bacterial, fungal and viral communities, and relative inflammatory cytokine expression in the lower respiratory tract compared to horses without airway inflammation; ii) treatment targeting lung inflammation alters local bacterial, fungal and viral communities and relative inflammatory cytokine gene expression in the lower respiratory tract; and iii) treatment designed to reduce lung inflammation improves $\dot{V}O_2$max in horses with mild equine asthma.

Overall, the objectives of this thesis have largely been achieved, and our hypotheses confirmed. We were unable to determine the functional impact of airway inflammation on performance, as to achieve this, airway inflammation must be induced in a healthy horse. Due to environmental conditions, we were unable to procure healthy horses for the trial outlined in Chapter 6; however, we were able to successfully assess the efficacy of treatment, thus confirming our hypothesis. The main findings of this thesis are summarized and discussed briefly below.

Differences between healthy horses and those with mild equine asthma

In the first clinical trial we conducted (described in Chapters 2 and 3), we investigated differences in the local bacterial communities, and relative inflammatory cytokine expression in the lower respiratory tract between healthy horses and those with MEA. This study provided the first report of the respiratory microbiota in horses. We found that the respiratory microbiome of horses is diverse, but was dominated
by four phyla, *Proteobacteria, Firmicutes, Bacteroidetes* and *Actinobacteria*. There was a clear separation between the bacterial community in the lower respiratory tract of healthy horses and those with mild equine asthma. This was consistent with the findings of Chapter 6, although this study was not designed to test differences between healthy horses and those with asthma (the results are not reported due to the extreme caution required in drawing reliable conclusions from them), as well as another recent study (Fillion-Bertrand, Dickson et al., 2018); environment and disease status both contribute to the bacterial communities present in the lung. It is interesting to question whether these observed differences in community structure lead to expression of disease, or is it perhaps the other way around? Amongst the 6 bacterial species that had different abundance with disease status, of importance is an increase in *Streptococcus* in horses with mild equine asthma; future studies investigating the role of *Streptococcus* in the development of lower airway inflammation are warranted. Whilst not overstating the findings of these studies, the long-held notion that mild equine asthma is a non-infectious disease is worth questioning; perhaps non-contagious is a more accurate term?

In addition to differences observed in the local bacterial communities at the level of the lower respiratory tract, we also found that horses with mild equine asthma have a lower concentration of IL-10 in BAL fluid than healthy controls, a finding which is consistent with human asthmatics. IL-10 is the intrinsic physiologic mechanism that inhibits pro-inflammatory cytokine synthesis (Borish, Aarons et al., 1996). Therefore, the absence, or reduced concentration of IL-10, associated with asthma enables the continued secretion of pro-inflammatory cytokines that contribute to lower airway inflammation. There was also significant up-regulation of IL-17 in horses with mild asthma compared to control horses prior to treatment, suggesting these horses had a true tendency of “allergic” airway inflammation in response to environmental antigens.
Effects of treatment

Effects of treatment on relative gene expression

In a study where horses were housed outside, thus with good ventilation, on dirt paddocks, dexamethasone administration exerted anti-inflammatory effects, evidenced by down-regulation of TNF-α in all horses, and decreased levels of IL-5 mRNA expression in horses with mild equine asthma. TNF-α plays an important role in allergic inflammation of the bronchus, with increased levels of expression being reported in the serum of patients following an allergic asthma attack (Jiang, Yang et al., 2018), which decreases following a week of oral glucocorticoid administration (Guanghui, Rongfei et al., 2005). It is logical that there was a larger anti-inflammatory effect exerted on horses experiencing airway inflammation. The down-regulation of IL-5 observed in response to dexamethasone treatment in horses with MEA indicates a shift away from a dysregulated Th-2 response after allergen exposure. However, while dexamethasone administration can help to reduce airway hypersensitivity and hyperreactivity (Léguillette R., Tohver T. et al., 2017), there is strong evidence that without environmental modifications, corticosteroid therapy alone fails to normalize airway neutrophilia, as was observed in Chapter 3, even after treatment periods of up to 6 months (Lavoie, Leguillette et al., 2002; Lavoie, Pasloske et al., 2006; Leclere, Lavoie-Lamoureux et al., 2012; Bullone, Vargas et al., 2017; Leguillette, Tohver et al., 2017); maintained exposure to environmental antigens perpetuates airway inflammation.

This finding was strongly supported by the results of Chapter 4, where prolonged exposure to a dusty stable environment induced lower airway inflammation in 75% of healthy adult horses, with a Th1 immune response, observed via down-regulation of IL-4 and up-regulation of IL-12. The development of inflammatory BAL cytology is consistent with previous reports, where healthy mature horses develop airway neutrophilia in response to an organic dust challenge by exposure to straw and moldy hay (Pirie, Dixon et al., 2001; Gerber, Lindberg et al., 2004; Leclere, Lavoie-Lamoureux et al., 2012). However, this is the first report of a Th1 immune response in a group of horses not predisposed to equine asthma.
This is suggested by the fact that they retained a healthy BAL cytology after 7 days in an environment with a high concentration of respirable particulate matter. Therefore, their immune response might indicate how a “healthy” horse with no predisposing factors to lower airway inflammation responds to sustained exposure to airborne irritants. In this study, this subset of horses responded with a shift away from a Th2 cytokine response, with down-regulation of IL-4, towards a Th1 cytokine response, shown via up-regulation of IL-12, which plays as a key role in the differentiation of Th0 into Th1 cells. This is suggestive of a “non-allergic asthma” phenotype in response to sustained exposure to a sub-optimal environment.

Nebulized saline provided an ideal control; this group had no changes in relative expression of any inflammatory cytokine mRNA. Treatment with nebulized dexamethasone resulted in a non-specific immune response, which is harder to explain. That all horses with mild equine asthma showed up-regulation of IL-5 between day 0 and day 14 was contrary to what might be expected; typically, the production of eosinopoietic cytokines such as IL-5 is inhibited by glucocorticoid administration. IL-5 plays a role in asthma and other acute hypersensitivity manifestations. It is plausible that this result could indicate the overwhelming influence of the environment on the disease process, over and above the anti-inflammatory effects of dexamethasone administration, with an allergic Th2-weighted response. This could be supported by a non-significant decrease in neutrophil % in horses with only neutrophilic BAL inflammation, consistent with the down-regulation of IL-23 observed in this group. However, in addition to IL-5, IL-12 was also up-regulated in horses with both neutrophilic and mastocytic inflammation, which typically results in the differentiation of T progenitor cells to Th1 cells. Our supposition is that activation of both the Th1 and Th2 pathways was due to prolonged exposure to increased respirable particulate matter.

**Effects of treatment on local bacterial, fungal and viral communities**

In agreement with our hypothesis, treatment with injected dexamethasone had a significant effect on the lower respiratory tract microbiome in healthy horses and those with mild asthma. Numerous OTUs
increased in abundance, including *Streptococcus* spp. Interestingly, whilst treatment with nebulized saline had no significant effect on diversity, at this individual OTU level it was associated with a significant decrease in *Streptococcus* in the upper respiratory tract. Treatment with nebulized dexamethasone resulted in a significant decrease in microbiota diversity based on Chao1 (p=0.004) and Shannon (p=0.004) indexes in the upper respiratory tract; there were no significant differences in either beta or alpha diversity at the lower respiratory tract level with treatment. When treatment-specific effects were tested (via interaction between timepoint and treatment group), two genera (*Alysiella* and *Bordetella*) showing a differential effect between treatments. The interpretation of these results is provided with caution, as manual inspection of the counts for each group showed a few samples dominated by high counts suggesting the possibility of a false-positive result. Regardless, it appears that control of bacterial overgrowth in horses with mild asthma treated with dexamethasone could be further investigated for inclusion as a component of future treatment options.

Importantly, a strength of this thesis is the investigation and report of the respiratory mycobiota. Prior to the study conducted in Chapter 4, the contribution of fungi to the overall community structure of the equine airways via a large and non-targeted approach was unknown. The presence of fungi in respiratory samples of horses is associated with mild equine asthma; horses with fungi present in tracheal wash cytology are 2 times more likely to have mild equine asthma than those without (Dauvillier, ter Woort et al., 2018). We found that time (pre vs. post), rather than treatment, had the largest effect on the mycobiota. Interestingly, only one of the commonly isolated fungi from horses with mild equine asthma, *Aspergillus*, was found to increase following treatment with nebulized saline (Dauvillier, ter Woort et al., 2018). Perhaps more importantly, the genus *Alternaria*, a known opportunistic pathogen and allergen in humans which has been increasingly recognized as a risk factor for asthma, asthma severity and exacerbations (Bush and Prochnau, 2004), was significantly increased in both treatment groups. As a major aeroallergen, prolonged exposure to *Alternaria* has been recognized as a risk factor for the development of asthma (Bush and Prochnau, 2004). The mycobiota of the airways was overwhelmed by the effect of a sustained dusty environment.
A frequent observation made after cessation of corticosteroid treatment in horses with MEA is the re-emergence of clinical signs. Equine herpesviruses are ubiquitous in the equine population, establish lifelong latent infections and reactivate in times of immunosuppression or stress. It is logical to question whether the re-emergence of clinical signs is due to an insufficient improvement in exposure to inhalable particulate matter, or whether corticosteroid-derived immunosuppression leads to recrudescence of latent infection. Interestingly, relative expression of the equine gammaherpesviruses (EHV-2 and -5) were affected in response to treatment with both nebulized dexamethasone and saline, with EHV-2 being upregulated, and EHV-5 being downregulated. EHV-2 and EHV-5 are identified frequently in samples from horses with respiratory disease (Fortier, Van Erck et al., 2009; Hue, Fortier et al., 2014), and there is some evidence that EHV-2 is associated with poor performance and airway inflammation (Fortier, Van Erck et al., 2009). The role of EHV-2 in perpetuating clinical signs of MEA after completion of treatment warrants further investigation.

Effects of treatment on performance

One of the major aims of this thesis was to determine the functional impact of airway inflammation on performance, and the efficacy of treatment; our hypothesis was that treatment designed to reduce lung inflammation improves \( \dot{V}O_2 \text{max} \) in horses with mild equine asthma. However, performance is not just related to aerobic performance, with anaerobic energy contributing significantly to overall energy demands. Therefore, we successfully developed a technique (Chapter 5) which is able to describe the aerobic and anaerobic energy contributions in situations where the exercise intensity is unknown, or fluctuates, as is observed in the field. We were therefore able to utilize this technique to study the effects of treatment designed to reduce lung inflammation in horses with smoke-induced equine asthma (Chapter 6), which highlighted the importance of improved air quality. Interestingly, dexamethasone administration was not associated with any additional benefit over a reduction in inhaled particulate matter alone (ie no difference between the control group and the group administered dexamethasone), on any measured or calculated variable. Improved air quality, both with and without dexamethasone, significantly increased \( \dot{V}O_2 \text{peak} \) by individual improvements of an average 11.7% (in horses treated

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with a saline placebo) to 14.6% (in horses treated with dexamethasone). Whilst $\dot{V}O_2\text{max}$ is equated with the aerobic capacity of both horses and humans, it is characterized by demonstrating no increase in $\dot{V}O_2$ despite an increase in workload. Whilst we were able to demonstrate a plateau in $\dot{V}O_2$, we were unable to prove an increased workload, with the jockey attempting to elicit a maximal effort from the horses for the duration of the trial; thus, we felt $\dot{V}O_2\text{peak}$ was a more accurate descriptor for what we were able to measure. It is our sincere hope that the findings of this study provide sufficiently compelling evidence to improve owner compliance with veterinary recommendations regarding improving environmental management and limiting exposure to dust. Whilst we acknowledge the increased effort required to achieve this, compared to treatment with injected or inhaled corticosteroids, the strongest conclusion it is possible to draw from all the studies contained within this thesis is that the environment, and thus environmental management, is the single biggest contributing factor for the prevention and alleviation of the clinical signs of mild equine asthma.

**General conclusions**

Upon consideration of the shared factors between human asthma, IAD and RAO we conclude that adoption of the term equine asthma is appropriate, whilst acknowledging that significant heterogeneity exists within this broad disease category. We therefore support the proposal that the term mild/moderate equine asthma replace IAD and severe equine asthma replace RAO in the literature from this point onwards, whilst recognizing the need to preserve the spectrum of diseases which fall within the proposed new “equine asthma” classification. Furthermore, in addition to the sub-categorization of equine asthma based on severity, we propose that equine equivalents to specific human asthma phenotypes exist, based on shared clinical and pathophysiological characteristics. Finally, with the development of new biologic treatments in human asthma and the application of more targeted therapeutic approaches in the horse, it might be appropriate to further investigate and clarify the allergic (Th-2), non-allergic (non-Th-2) and late-onset phenotypes of equine asthma; further research is required to more fully determine the potential clinical utility of such a phenotypic classification exercise (Bond, Léguillette et al., 2018).
Chapter 8 - References


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Appendix A - Assessment of high-intensity over-ground conditioning and simulated racing on aerobic and anaerobic capacities in racehorses

Summary

A prospective, randomized study assessed the impact of high-intensity racetrack conditioning on aerobic and anaerobic capacities in seasoned Thoroughbred racehorses. The effect of 10 weeks race conditioning and two simulated races on $\dot{V}O_{2\text{max}}$ and maximum accumulated oxygen deficit (MAOD) were evaluated. An incremental treadmill test to determine $\dot{V}O_{2\text{max}}$, followed by three supramaximal runs to fatigue (at speeds (V105%, V115%, V125%) corresponding to oxygen requirements 105%, 115% and 125% of $\dot{V}O_{2\text{max}}$, in randomized order) were performed at each timepoint (T1 [pre-conditioning] and T2 [post-conditioning]). Prior to T1, racehorses were briefly de-trained for four-six weeks and given low-level treadmill conditioning to prepare them for the more strenuous race conditioning after T1. Paired variables between T1 and T2 were analysed using a paired t-test. A 2-way RM ANOVA compared variables with >1 measurement. Speed at $\dot{V}O_{2\text{max}}$ (p=0.04) and $\dot{V}O_{2\text{max}}$ (p=0.01) increased with conditioning. Calculated speeds for the supramaximal runs increased for V105% (p=0.02) and V115% (p=0.03) but not for V125% (p=0.08). There was no conditioning effect on time to fatigue (p=0.34), although it was different between all intensities (2.8, 2.2 and 1.4 mins at V105%, V115% and V125% respectively at T2). O\textsubscript{2} demand increased with conditioning (p=0.02) for each supramaximal intensity. On average, horses’ aerobic capacity improved 4.43% after conditioning. MAOD was unchanged with conditioning (p=0.25) and unaffected by exercise intensity. Fit racehorses that have undergone repeated intensive training programs, experience smaller, incremental improvement than completely unfit horses. The anaerobic capacity of previously trained racehorses is relatively stable, despite brief periods of de-training.
**Introduction**

Complex interactions between multiple major body systems including the cardiovascular, respiratory, and musculoskeletal systems are required for athletic performance. Performance achieved in many athletic activities is highly dependent on the amount of oxygen consumed by the body (\(\dot{V}O_2\)). Compared to other species, racehorses are considered elite athletes due to their large aerobic capacity (Poole, 2004) and physiological responses to exercise which increase oxygen transport within the body (McKeever, Hinchcliff et al., 1993). Specifically, maximal oxygen consumption (\(\dot{V}O_{2\text{max}}\)) in the average human is typically 50-60 ml.(kg.min\(^{-1}\)) (Helgerud, Høydal et al., 2007), with elite athletes having a \(\dot{V}O_{2\text{max}}\) of 80-97.5 ml.(kg.min\(^{-1}\)); the average racehorse typically has a \(\dot{V}O_{2\text{max}}\) greater than 150 ml.(kg.min\(^{-1}\)) (Rose, Hodgson et al., 1988). Furthermore, \(\dot{V}O_{2\text{max}}\) increases of 10-27% have been reported in previously unfit horses in response to exercise conditioning trials of 6-10 weeks duration and were associated with improved athletic performance (Evans and Rose, 1988a; Knight, Sinha et al., 1991; Evans, Rainger et al., 1995; Hinchcliff, Lauderdale et al., 2002). However, relative hypoventilation and the resultant inappropriate pulmonary gas exchange remains the limiting factor of performance in sound horses (Art and Lekeux, 1993; Roberts, Marlin et al., 1999; Ainsworth, 2008).

At very high exercise intensities, racehorses experience arterial hypoxemia and hypercapnia, as well as marked lactatemia. The arterial partial pressure of oxygen (PaO\(_2\)) can decrease from 92-99 mmHg at rest to 70 mmHg when running at maximal speeds and there is an associated desaturation of haemoglobin. Accompanying the hypoxemia, CO\(_2\) retention is observed during supramaximal exercise, with PaCO\(_2\) increasing from resting values of 44 mmHg to 49-54 mmHg (Bayly, Hodgson et al., 1989). It has been argued that this represents a ‘relative hypoventilation’, where gas exchange does not occur fast enough at the high blood flows and cardiac output that horses generate during exercise (HR\(_{\text{max}}\) = 220 beats/min; stroke volume (SV) ≈ 2.7 ml/kg; cardiac output ≈ 594 ml.(kg.min\(^{-1}\)) ) (Rose, Hodgson et al., 1988; Wagner, Gillespie et al., 1989; West and Mathieu-Costello, 1995). Blood lactate concentrations exceeding 20 mmol/L are commonly observed in Thoroughbred horses following
supramaximal exercise (Evans, Rainger et al., 1995). The anaerobic contributions to total energy expenditure are therefore also very important to athletic performance.

Whilst the aerobic capacity of a horse can be directly measured as the maximal rate of oxygen consumption, the anaerobic capacity is currently measured indirectly as a quantity which is referred to as the maximum accumulated oxygen deficit (MAOD) accrued during supramaximal exercise (Eaton, Evans et al., 1995). A single study investigating the effect of training on the anaerobic capacity of deconditioned Standardbred racehorses found a 27% increase in MAOD after 10 weeks of treadmill exercise, following 12 weeks of confined stall rest (Hinchcliff, Lauderdale et al., 2002). However, the effect of strenuous over-ground conditioning on aerobic and anaerobic capacities in Thoroughbred racehorses has not yet been reported.

Therefore, the purpose of this study was to investigate the effect of a standard race preparation and two competitive (simulated) races on the aerobic and anaerobic capacities in Thoroughbred racehorses.

Materials and Methods

Experimental design

This was a prospective, randomized study. The effect of 10 weeks of a standard race preparation, and two non-sanctioned races on aerobic and anaerobic variables were studied. Eight recently retired Thoroughbred racehorses (three mares, four geldings, one stallion) aged six to eight years were enrolled in the study; of these, three horses were unable to complete the post-training assessment due to lameness (n=1) and other reasons unrelated to the study (n=2) and were therefore excluded from all statistical analysis. Each horse completed two assessments on a high-speed treadmill, at baseline (before race training: T1) and after conditioning and simulated racing (T2). Horses were initially assessed following low-level conditioning, comparable to a horse that has undergone a pre-training program before beginning race training. Each assessment consisted of four runs: an initial incremental exercise test to
determine \( \dot{V}O_2_{\text{max}} \), followed by three supramaximal runs at speeds calculated to induce an oxygen demand of 105% (\( V_{105\%} \)), 115% (\( V_{115\%} \)) and 125% (\( V_{125\%} \)) of \( \dot{V}O_2_{\text{max}} \) for each horse. All horses were judged to be clinically healthy based on physical, lameness and endoscopic examinations, and had a history of exercise-induced pulmonary haemorrhage (EIPH). This study was approved by the Institutional Animal Care and Use Committee at Washington State University.

**Conditioning**

Racehorses were deconditioned in box stalls (3x4m) and outdoor pens for four to six weeks after a six to seven-month racing season which was followed by five months of regular treadmill based training and periodic (every two to three weeks) supramaximal treadmill exercise. Horses had a median \( \dot{V}O_2_{\text{max}} \) of 157 ml.(kg.min)\(^{-1} \) (IQR: 153.18-163.53) and a median bodyweight of 463.19kg (451.14- 484.88) prior to deconditioning. To simulate a light pre-conditioning program, as is routinely performed prior to beginning the more strenuous race training, horses underwent four weeks of light treadmill conditioning which involved trotting at 4m.s\(^{-1} \) on a treadmill for 10-15 mins two to three times a week prior to initial assessment (T1, see below). Race training commenced three to four days after the final supramaximal run of T1 for each horse, and the protocol is shown in Table A1. Horses were initially trained at the Hitchcock Research Racetrack facility at Washington State University for six weeks, prior to transportation to Emerald Downs Racetrack, Seattle WA where they completed another four weeks race-conditioning with a professional racetrack trainer. Horses then competed in two races of 1100m against each other 13 days apart with professional jockeys and starting gates simulating real racing conditions. Venous blood (jugular) was collected for lactate analysis within 15min of the second race. Horses were then transported back to Washington State University and commenced their second assessment (T2) 10 - 11 days after completing the second race.

**Husbandry details**

Horses were initially housed in small groups of two to three horses/paddock, and had ad libitum access to pasture, alfalfa and grass hay, and were fed Senior feed and beet pulp. At Emerald Downs Racetrack,
Seattle WA, they were housed in box stalls (3x4m) and fed alfalfa hay, Race Ready (Purina), salt, and electrolytes, and had ad libitum access to water. A lameness examination was performed each day, and treatments including phenylbutazone (1-2g PO SID) or flunixin meglumine (1.1mg/kg IV SID) were prescribed as required; several horses also received methocarbamol (25mg/kg PO BID).

Instrumentation for high-speed treadmill assessment

Horses were weighed using a digital weigh scale (Brecknell, model PS-3000HD, USA) immediately before or after instrumentation for T1 and T2. Catheterization of the jugular vein (14-gauge, 5½” Abbocath-T, Hospira Inc., Lake Forest, IL, USA) was performed on alternating sides to facilitate blood sampling during exercise. A digital-display polar cardiotachometer (Polar Equine RS800CX G3, WA, USA) was attached to a surcingle to record the heart rate (HR). Horses were hand walked for a minimum of 10 minutes prior to application of a mask fitted with an ergospirometer (Sides, Bayly et al., 2014); the horse was then walked immediately onto the high-speed treadmill (SATO, USA).
Table A1: Conditioning protocol for five Thoroughbred racehorses. Conditioning was modified from this general protocol based on ability and lameness. Each sentence indicates training for one day unless otherwise indicated. WSU = Washington State University, Pullman

<table>
<thead>
<tr>
<th>Treadmill light pre-conditioning</th>
<th>Trot at 4m/s on a treadmill for 10-15 mins 2-3 times a week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial assessment (T1)</td>
<td>Incremental treadmill test, followed by 3 supramaximal runs (105%, 115%, 125% of VO₂max) in randomized order</td>
</tr>
<tr>
<td>Track conditioning 6 weeks</td>
<td></td>
</tr>
<tr>
<td>Week 1</td>
<td>Lunge. Trot on treadmill 4m/s for 15 mins. Trot 1 mile +/- gallop 1 mile for 2-3 days. Trot 1 mile, breeze ¼ mile. 1-2 rest days.</td>
</tr>
<tr>
<td>Week 2</td>
<td>Trot 1- 1.5 miles, gallop 1-1.5 miles, 5 days. One day breeze ¼ - 3/8 mile. One rest day.</td>
</tr>
<tr>
<td>Week 3</td>
<td>Trot 2-2.5 miles, gallop 1-1.5 miles, 5 days. One day breeze ½ mile. One rest day.</td>
</tr>
<tr>
<td>Week 4</td>
<td>Trot 2-2.5 miles, gallop 1.5 miles, 2-5 days. Match race ½ mile. One - 4 rest days.</td>
</tr>
<tr>
<td>Week 5</td>
<td>Trot 2-2.5 miles, gallop 1.5 miles, 5 days. Match race ½ mile. One rest day.</td>
</tr>
<tr>
<td>Week 6</td>
<td>Trot 2 miles, gallop 1.5 miles, 2 days. Match race ½ mile. Transport to racetrack. Trot 2 miles. Trot 1 mile, gallop 1 mile. Gallop 1 mile.</td>
</tr>
<tr>
<td>Racetrack 4 weeks</td>
<td></td>
</tr>
<tr>
<td>Week 1</td>
<td>Gallop 1 mile. Hot walker, 2 days. Trot 1 mile, 3 days. Breeze ½ mile.</td>
</tr>
<tr>
<td>Week 2</td>
<td>Breeze 5/8 mile. Hot walker, 4 days. Gate training. Race 1100m.</td>
</tr>
<tr>
<td>Week 3</td>
<td>Hot walker, 4 days. Trot 1-2 miles, 3 days.</td>
</tr>
<tr>
<td>Week 4</td>
<td>Trot 1-2 miles, 2 days. Hot walker, 2 days. Race 1100m. Transport to WSU.</td>
</tr>
<tr>
<td>Post-conditioning assessment (T2)</td>
<td>Incremental treadmill test, followed by 3 supramaximal runs (105%, 115%, 125% of VO₂max) in random order</td>
</tr>
</tbody>
</table>
Incremental exercise test: measurement of maximum O$_2$ consumption ($\dot{V}O_{2\text{max}}$)

$\dot{V}O_{2\text{max}}$ measurements were performed on the high-speed treadmill using a full mask ergospirometry system (Sides, Bayly et al., 2014). Horses were acclimatised to exercise on the treadmill whilst wearing the mask prior to commencement of the study, through participation in a previous study that also involved supramaximal exercise. The mask was internally padded to minimise dead space. The system was calibrated (flowmeter and gas analyser) as previously reported (Sides, Bayly et al., 2014) prior to application on the horse. All tests were performed with the treadmill set at a 10% incline. The incremental exercise test consisted of a four-minute warm-up period at 4 m.s$^{-1}$; thereafter the treadmill speed was increased to 6 m.s$^{-1}$ for 60s, followed by 1 m.s$^{-1}$ increments in speed every 60s until the horse could not maintain speed on the treadmill despite strong verbal encouragement. The treadmill was then rapidly slowed and stopped (<10s). The same person (RS) judged when to stop all exercise tests (both the incremental and supramaximal intensity tests) for both T1 and T2. Oxygen consumption ($\dot{V}O_2$) was calculated based on data acquired in the last 15s of each speed, or the last 15s before the incremental test terminated for the final speed as previously reported (Sides, Bayly et al., 2014). Venous blood was collected for lactate analysis and a HR measurement was obtained concurrently at the following timepoints: before exercise, at the end of the warm-up period, in the last 10s of each speed, within 10s of the end of the test, two, five and 10 minutes post exercise, and then every 10 minutes until 60 minutes post exercise. The treadmill speed at which the HR reached 200bpm ($V_{200}$), and the speed at which blood lactate concentration (BL) reached 4 mmol/L ($V_{La_{4}}$) and 10 mmol/L ($V_{La_{10}}$) were interpolated from the respective HR-speed and BL-speed curves.

Supramaximal exercise tests

After completion of the incremental exercise test and identification of the $\dot{V}O_{2\text{max}}$ plateau, a $\dot{V}O_2$ versus speed regression equation for the linear portion of the curve was determined for each horse, as previously described (Rose, Hodgson et al., 1988). From this equation, speeds were calculated that would theoretically induce an oxygen demand of 105% ($V_{105\%}$), 115% ($V_{115\%}$) and 125% ($V_{125\%}$) of $\dot{V}O_{2\text{max}}$ for each horse. High intensity exercise tests consisted of the horse trotting on the inclined
treadmill at 4m/s for 4 minutes, after which the speed was rapidly increased (<12s) to V105%, V115% or V125%. Horses ran at this speed until they were unable to maintain speed on the treadmill despite strong verbal encouragement; the treadmill was then rapidly slowed and stopped (<10s). Horses had a minimum of 48 hours to recover between high intensity exercise tests, and the order of the intensity of the tests was randomized. \( \dot{V}O_2 \) was measured using the same mask system as for the incremental exercise test, recorded and was later calculated every 15s during the run. Venous blood was collected for lactate analysis, and a HR obtained concurrently at the following timepoints: before exercise, at the end of the warm-up period, every 15s during the test, two, five and 10 minutes post exercise, and then every 10 minutes until 60 minutes post exercise. Maximum heart rate (HR_{max}) and maximum blood lactate concentration were recorded.

**Assessment of anaerobic capacity: maximum accumulated \( O_2 \) deficit (MAOD)**

Anaerobic capacity was indirectly calculated from the MAOD during the supramaximal exercise tests. Oxygen demand for each supramaximal exercise test was extrapolated from the \( \dot{V}O_2 \) versus speed regression equation calculated for each horse after the incremental exercise test, as previously described (Rose, Hodgson et al., 1988). The MAOD was calculated as the difference between the measured \( \dot{V}O_2 \) (subtracting resting \( \dot{V}O_2 \) values) and the estimated oxygen demand over the duration of each high intensity exercise test, starting from the moment the speed was rapidly increased from 4m/s to V105%, V115% or V125% (Hinchcliff, McKeever et al., 1996; Geor, McCutcheon et al., 2000).

**Statistical analysis**

All measures are reported where appropriate as median (interquartile range [IQR]). A commercially available statistics program was used for all analysis (SigmaPlot 12.5). Paired variables including body weight, the speed at which each horse reached \( \dot{V}O_2\text{max} \), \( V_{200} \), peak lactate, peak HR, \( VL_a \), \( VL_{a10} \) and oxygen demand were analysed using a paired t-test (De Winter, 2013). A Shapiro-Wilk test was used to test for normality. A 2-way repeated measures ANOVA, controlling for training and exercise intensity, followed by a Holm-Sidak post-hoc test compared variables with >1 measurement at each
timepoint, including the speed of high intensity trials, time to fatigue, \( \dot{V}O_{2\text{max}} \) and MAOD. \( P \) values \( \leq 0.05 \) were considered significant.

**Results**

Values for physiologic variables including body weight, \( \dot{V}O_{2\text{max}} \) speed, speed of runs, time to fatigue, peak heart rate, peak lactate, \( V_{200} \), \( VL_a4 \), \( VL_{a10} \), oxygen demand, \( V\dot{O}_{2\text{max}} \), and MAOD are reported in Table A2. There was no significant effect of high-intensity race conditioning on body weight, peak HR or \( V_{200} \) (Table A2).

There was no significant effect of high-intensity race conditioning on blood lactate concentration at rest, during the incremental exercise test (Figure A1), or during recovery (Figure A2).

The speeds corresponding to blood lactate concentrations of 4mmol/L (\( VL_a4 \)) or 10mmol/L (\( VL_{a10} \)) were not significantly different after race conditioning (Table A2). The peak blood lactate concentration obtained after the supramaximal runs was not significantly affected by race conditioning (Table A2), nor was the post-exercise blood lactate concentration during recovery (Figure A3). Furthermore, there was no change with the rate of blood lactate clearance with race-conditioning; at both time-points there was no significant decrease in blood lactate concentration from the peak blood value until 20 mins post exercise (T1, \( p=0.03 \); T2, \( p=0.047 \)) (Figure A3). Blood lactate concentrations measured post-race had a median value of 21.05 (19.28-23.83) mmol/l, which was significantly lower than the peak blood lactate concentration obtained after the post-conditioning supramaximal runs on the treadmill (23.1 [20.9- 24.2] mmol/l, \( p=0.039 \)).
Figure A1: Blood lactate concentrations of five horses before and during an incremental treadmill test, before and after eight weeks of high intensity over-ground conditioning. Mean±SD. Pre-conditioning error bars go down; post-conditioning error bars go up.
Table A2: Values of physiologic variables in five Thoroughbred horses before and after eight weeks of high-intensity conditioning and two competitive races

<table>
<thead>
<tr>
<th>Variable</th>
<th>Before training</th>
<th>After training</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (kg)</td>
<td>497 (483-502)</td>
<td>496 (489-505)</td>
<td>0.5</td>
</tr>
<tr>
<td>( \text{VO}_{2\text{max}} ) (100%) speed(^\text{a} ) (m/s)</td>
<td>9.0 (8.8-9.3)</td>
<td>9.5 (9.0-9.8)</td>
<td>0.04</td>
</tr>
<tr>
<td>Speed of 105% run (m/s)</td>
<td>9.5 (9.2-9.7)</td>
<td>10.0 (9.4-10.2)</td>
<td>0.02</td>
</tr>
<tr>
<td>Speed of 115% run (m/s)</td>
<td>10.4 (9.9-10.7)</td>
<td>10.8 (10.1-11.1)</td>
<td>0.03</td>
</tr>
<tr>
<td>Speed of 125% run (m/s)</td>
<td>11.2 (10.7-11.5)</td>
<td>11.5 (10.8-12.0)</td>
<td>0.08</td>
</tr>
<tr>
<td>Time to fatigue (105%) (mins)</td>
<td>2.4 (1.9- 4.0)</td>
<td>2.8 (1.9- 2.8)</td>
<td>0.34</td>
</tr>
<tr>
<td>Time to fatigue (115%) (mins)</td>
<td>1.8 (1.8- 2.4)</td>
<td>2.2 (1.4- 2.4)</td>
<td>0.34</td>
</tr>
<tr>
<td>Time to fatigue (125%) (mins)</td>
<td>1.5 (1.4- 1.7)</td>
<td>1.4 (1.1- 1.7)</td>
<td>0.34</td>
</tr>
<tr>
<td>Peak heart rate (bpm)</td>
<td>221 (218- 227)</td>
<td>221 (215- 227)</td>
<td>0.7</td>
</tr>
<tr>
<td>( V_{200} ) (m/s)</td>
<td>6.9 (6.4- 7.5)</td>
<td>6.8 (5.8- 7.4)</td>
<td>0.6</td>
</tr>
<tr>
<td>Peak lactate (mmol/L)</td>
<td>22.9 (20.6- 24.3)</td>
<td>23.1 (20.9- 24.2)</td>
<td>0.7</td>
</tr>
<tr>
<td>VL\text{at} (m/s)</td>
<td>6.0 (5.7- 6.7)</td>
<td>6.4 (5.1- 6.5)</td>
<td>0.44</td>
</tr>
<tr>
<td>VL\text{at} (m/s)</td>
<td>8.7 (8.2- 9.4)</td>
<td>9.0 (7.7- 10.2)</td>
<td>0.75</td>
</tr>
<tr>
<td>( \text{O}_2 ) demand 105% (ml.kg(^{-1}).min(^{-1}))</td>
<td>150.8 (148.2-159.0)</td>
<td>155.6 (153.9-162.3)</td>
<td>0.017</td>
</tr>
<tr>
<td>( \text{O}_2 ) demand 115% (ml.kg(^{-1}).min(^{-1}))</td>
<td>165.1 (162.3- 174.2)</td>
<td>170.4 (168.6- 177.7)</td>
<td>0.017</td>
</tr>
<tr>
<td>( \text{O}_2 ) demand 125% (ml.kg(^{-1}).min(^{-1}))</td>
<td>179.5 (176.4- 189.3)</td>
<td>185.3 (183.3- 193.2)</td>
<td>0.017</td>
</tr>
<tr>
<td>( \text{VO}_{2\text{max}} ) (ml.kg(^{-1}).min(^{-1}))</td>
<td>147.5 (141.4-153.8)</td>
<td>150.1 (147.9- 158.1)</td>
<td>0.033</td>
</tr>
<tr>
<td>MAOD (mlO\text{2eq} kg(^{-1}))</td>
<td>78.0 (75.4- 93.4)</td>
<td>69.1 (61.1- 104.6)</td>
<td>0.25</td>
</tr>
</tbody>
</table>

Values are reported as Median (IQR). MAOD = Maximum Accumulated Oxygen Deficit. \(^\text{a}\) Treadmill inclined on a 10% slope.
Figure A2: Blood lactate concentrations of five horses after an incremental treadmill test, before and after eight weeks of high intensity over-ground conditioning. Mean±SD. Pre-conditioning error bars go down; post-conditioning error bars go up.
Figure A3: Blood lactate concentrations of five horses after supramaximal runs performed on a high-speed treadmill, before and after eight weeks of high intensity over-ground conditioning. Mean±SD. Pre-conditioning error bars go down; post-conditioning error bars go up.
The speed at which horses reached $\dot{V}O_2_{\text{max}}$ increased with race conditioning, from 9.0m/s to 9.5m/s (p=0.04) (Table A2). There was a significant difference in the speeds of the 105%, 115% and 125% intensity runs both before (all p<0.001), and after race conditioning (all p<0.001) (Table A2). The speeds of the 105% (p=0.02) and 115% (p=0.03) runs increased significantly with race conditioning (Table A2). Whilst the speed of the 125% $\dot{V}O_2_{\text{max}}$ trial did increase at T2 (Table A2), this was not significant (p=0.08). There was no race conditioning effect on time to fatigue (p=0.34), although it was different between all supramaximal intensities (105% vs. 125% p=0.001; 105% vs. 115% p=0.03; 115% vs. 125% p=0.03) (Table A2). $\dot{V}O_2$ increased with race conditioning for each supramaximal intensity (all p=0.02) (Table A2).

Horses’ aerobic capacities improved by an average of 4.43% (Range: 1.16-7.73%) in response to race conditioning with $\dot{V}O_2_{\text{max}}$ after race conditioning (150.11 ml.(kg.min)$^{-1}$) being higher than after the baseline light pre-conditioning (147.3 ml.(kg.min)$^{-1}$) (p=0.014) (Table A2). Individual aerobic capacity was constant within each timepoint irrespective of supramaximal exercise intensity.

MAOD was unchanged with race conditioning (p=0.25), with a median pre-training value of 78.0 ml O$_2$Eq.kg$^{-1}$ [75.35- 93.44], and a post-conditioning median of 69.05 ml O$_2$Eq.kg$^{-1}$ [61.05- 104.62]. Individual anaerobic capacity was constant at each of the exercise intensities used at T1 and T2.

**Discussion**

This study reports the effects of a strenuous over-ground race conditioning protocol on the aerobic and anaerobic capacities of Thoroughbred racehorses. Despite its comparative brevity, the deconditioning period prior to the initial assessment was sufficient; the $\dot{V}O_2_{\text{max}}$ reduced by 6.05% from values obtained. Previous studies have reported a large increase in aerobic and anaerobic capacities in response to treadmill conditioning protocols on unfit research horses, however, those studies were not performed with high calibre racehorses, as enrolled in this study. We observed a lesser, but still significant, increase in the $\dot{V}O_2_{\text{max}}$ and no change in the MAOD after an eight-week conditioning protocol, and two competitive, non-sanctioned races in professional racehorses. Once racehorses are fit and have
undergone repeated intensive training programs, the horses tend to maintain their condition and improvement occurs in much smaller increments than reported in studies with completely unfit or juvenile horses (Evans and Rose, 1988b; Ohmura, Matsui et al., 2013). However, these smaller increments are still important and significant when considered in terms of performance.

Conditioning effects of both low and high intensity treadmill training have previously been studied in humans and horses (Evans and Rose, 1988b; Knight, Sinha et al., 1991; Evans, Rainger et al., 1995; Hinchcliff, Lauderdale et al., 2002; Helgerud, Høydal et al., 2007). The advantage of conditioning on a treadmill is that it provides a highly controlled, repeatable environment, where exercise intensity may be calculated. Conditioning protocols can be based on the intensity calculated to maintain a set blood lactate concentration (Evans, Rainger et al., 1995; Hinchcliff, Lauderdale et al., 2002), a targeted percentage of the maximum heart rate (Evans and Rose, 1988b; Helgerud, Høydal et al., 2007), or a percentage of pre-conditioning \( \dot{V}O_{2\text{max}} \) (Knight, Sinha et al., 1991). A major limitation regarding treadmill exercise is the fact that it does not reflect over-ground exercise. The locomotion biomechanics of running on a treadmill are different (Barrey E, Galloux P et al., 1993), and the workload is altered (Sloet and Barneveld, 1995) as trials are typically conducted on a 5-10% slope. Furthermore, in horses, a major disadvantage is that the artificial nature of the setting does not inspire the competitive running behaviour observed on a track, and horses can learn behaviour required to stop both training and assessment treadmill protocols. Therefore, a strength of our study design was to examine the effect of over-ground conditioning, as well as real-life racing environment, where horses were competing against one another on a track.

In response to strenuous over-ground race conditioning, horses in this study improved their aerobic capacity by an average 4.43%. Regarding performance, this represents an improvement of ~5 seconds over a mile, or greater than 30 lengths; over 1100m it would be ~3.5 seconds (20-25 lengths). When considering the frequent occurrence of photo-finishes, where the distance between first and second
place is indiscernible to the naked eye, this improvement is marked. In contrast, rapid increases in aerobic capacity of 9.4-25% and 10.7%-11.7% have been reported in horses and ponies respectively, in response to as little as two to seven weeks training (Evans and Rose, 1988b; Knight, Sinha et al., 1991; Art and Lekeux, 1993; Katz, Bayly et al., 2000), with increases attributed to increased stroke volume and cardiac output (Evans and Rose, 1988b; Art and Lekeux, 1993), or an increase in the arteriovenous oxygen concentration difference (Knight, Sinha et al., 1991). Furthermore, both low and high intensity treadmill training for six to 10 weeks have resulted in $\dot{V}O_{2max}$ increases of 10-27% (Evans and Rose, 1988b; Knight, Sinha et al., 1991; Evans, Rainger et al., 1995; Hinchcliff, Lauderdale et al., 2002).

However, the greater magnitude of improvement can be attributed to complete detraining prior to study commencement, the fact that these horses and ponies had not undergone repeated intensive training programs, and, in several studies, a decreased body weight. Improvement in mass specific $\dot{V}O_{2max}$ can be related to either a weight decrease, an increase in absolute $\dot{V}O_{2max}$, or both. Whilst not statistically significant, three of the studies reported weight loss of 3kg (0.6%) (Evans, Rainger et al., 1995), 9kg (2.1%) (Hinchcliff, Lauderdale et al., 2002) and 26kg (5.5%) (Evans and Rose, 1988b) from pre-training to post-training which could have impacted mass specific $\dot{V}O_{2max}$; the other study investigating the effect of training on $\dot{V}O_{2max}$ did not report bodyweight (Knight, Sinha et al., 1991). In comparison, the horses in the present study experienced virtually no weight loss from T1 to T2 (1kg). Therefore, the 4.43% increase in $\dot{V}O_{2max}$ observed can be attributed entirely to an increase in absolute $\dot{V}O_{2max}$.

Multiple equine treadmill studies have found no difference in responses to training between low and high intensity protocols (Knight, Sinha et al., 1991; Evans, Rainger et al., 1995). In contrast, in humans it has been shown that high-intensity interval training on a treadmill is more effective at improving $\dot{V}O_{2max}$ than moderate aerobic training, with increases in $\dot{V}O_{2max}$ of 5.5% and 7.2% being reported in response to high-intensity training for eight weeks (Helgerud, Høydal et al., 2007). Therefore, the 4.43% increase in $\dot{V}O_{2max}$ that we observed is reasonable, particularly considering that, of the previous equine studies, three reported pre-conditioning $\dot{V}O_{2max}$ values between 116 and 129.7 ml.(kg.min)$^{-1}$ (Evans and Rose, 1988b; Evans, Rainger et al., 1995; Hinchcliff, Lauderdale et al., 2002), which are substantially
lower than we observed (147.5 ml.(kg.min)\(^{-1}\)). Furthermore, of these studies, two reported post-conditioning values of 135 to 148 ml.(kg.min)\(^{-1}\) (Evans, Rainger et al., 1995; Hinchcliff, Lauderdale et al., 2002), which are comparable to our pre-conditioning value of 147.5 ml.(kg.min)\(^{-1}\), which is further indication of the quality of the racehorses enrolled in the present study.

A study with low- and high-intensity conditioning groups reporting comparable pre-conditioning aerobic capacities (149 and 142 ml.(kg.min)\(^{-1}\) respectively) found that adaptions of the aerobic capacity of a horse are rapid regardless of exercise intensity (reporting no difference between two groups training at 40% and 80% of \(\dot{V}O_{2\text{max}}\) over 3000m, six days a week), with the \(\dot{V}O_{2\text{max}}\) increasing 10% in the first two weeks, and no further increase being observed by the beginning of week seven (Knight, Sinha et al., 1991). Another study found a 16% increase in \(\dot{V}O_{2\text{max}}\) with only water treadmill conditioning for three weeks (Greco-Otto, Bond et al., 2018). It is therefore possible that the pre-conditioning protocol that was used in this study, where horses were trotted at 4m/s on a treadmill for 10-15 mins two to three times a week for four weeks led to some increase in \(\dot{V}O_{2\text{max}}\) values prior to T1. Nevertheless, horses were fitter at T2 than T1. Objective evidence of this includes the higher speeds at which \(\dot{V}O_{2\text{max}}\) was reached, as well as at 105%, 115% and 125% \(\dot{V}O_{2\text{max}}\); i.e. the same relative intensity was associated with significantly higher speed. The ~5.5% increase in these speeds, combined with the 4.43% increase in \(\dot{V}O_{2\text{max}}\) represents significantly improved performance.

Whilst significant and meaningful in terms of performance, the magnitude of the increase in \(\dot{V}O_{2\text{max}}\) might have been expected to be larger based on previous treadmill studies. That it was not could be indicative of overtraining or functional overreaching. Overtraining in humans is where an athlete is unable to perform at their optimal level after a normal rest period, due to training load, and is therefore associated with a decrease in performance (Kuipers and Keizer, 1988). Overtraining in horses is based on the measurement of reduced performance capacity and has been defined as a decrease (p<0.05) in run time to fatigue during an incremental exercise test on a high-speed treadmill, and a significant
decrease in bodyweight (with no reduction in feed intake) (Tyler, Golland et al., 1996). In contrast, functional overreaching is defined as a short-term reduction in performance as a result of increased training stress and is reversible over a few days with a lightened training load, or a brief period of no training (Kuipers and Keizer, 1988; Bayly, 2002). It is important to note that in humans, adaptations and maladaptations to training load are observed in blood biomarkers before behavioural modification, prior to any negative impact on performance (Arent, 2018). In this study, we did not observe a significant decrease in bodyweight, nor was there any decrease in the run to fatigue time at any supramaximal exercise intensity, nor during the incremental exercise test. However, empirical observations of the horses after conditioning revealed that their behaviour was altered from before conditioning: horses were generally less willing to accept the mask, enter the treadmill, would shake their heads and paw whilst on the treadmill, and the effort exerted during several runs at T2 were out of character. This concurs with previous equine overtraining studies, where a noticeable decrease in horses’ willingness to perform was observed (Persson, Larsson et al., 1980; Tyler, Golland et al., 1996). When combined with the fact that the aerobic capacity of horses – used to assess “performance” in overtraining studies - was improved at T2, this could be an indication that functional overreaching occurred in some of the horses, with the 10-11- day rest period after the second race providing sufficient recovery time. In elite athletes, utilization of monitoring techniques provides the ability to prevent possible injury, as well as assess responses to accumulated training load, providing early indicators of non-functional overreaching and overtraining syndrome (Arent, 2018). The implementation of similar monitoring strategies in elite equine athletes is worthy of careful consideration, to detect horses that are overreaching due to their accumulated training load, before non-functional overreaching or overtraining syndrome occurs.

In order to accurately assess the anaerobic capacity, it is essential to ensure that a maximal effort is provided, and of sufficient duration to utilise all anaerobic capacity. Whilst our findings concurred with Eaton et al (Eaton, Evans et al., 1995) regarding the reproducibility of MAOD, irrespective of supramaximal exercise intensity, we found that 125% of VO2max more accurately corresponded to
exercise being sustained for 1.5 minutes, rather than 1 minute as was previously reported (Eaton, Evans et al., 1995). In contrast, trained humans are able to sustain exercise intensities of 171% of VO$_{2\text{max}}$ for 1 minute (Hermansen and Medbø, 1984). Unlike in humans, where the importance of giving a maximal effort can be impressed upon subjects, in equine research the judgement regarding when a horse has fatigued, thus ending the trial, is of vital importance. At 105% of VO$_{2\text{max}}$, similar to Eaton et al (Eaton, Evans et al., 1995), there were several runs where the measured oxygen consumption exceeded the calculated oxygen demand, therefore these runs were excluded from analysis. This could indicate that whilst the VO$_{2\text{max}}$ of a horse is reproducible, thus termed the aerobic ‘capacity’, there is some day-to-day variability in performance, leading to challenges in extrapolating the oxygen demand at supramaximal exercise intensities. The effect of 10 weeks of “high intensity” treadmill conditioning has previously been reported to result in a 27% increase in anaerobic capacity in unfit Standardbred research horses, with the MAOD increasing from 64.7 to 82.2 mLO$_2$E kg$^{-1}$ after conditioning (Hinchcliff, Lauderdale et al., 2002). However, there was no racing or training history available to investigators; horses were purchased from a commercial vendor. In comparison, whilst we found no conditioning effect on the anaerobic capacity (p=0.25) of our racehorses, the horses enrolled in the present study were professional athletes that had been training and racing regularly for 3-5 years and were still capable of competitive times (several horses could run 800m in <48 sec; all horses were <49 sec). As discussed for the aerobic capacity, once fit racehorses have undergone repeated, intensive training programs, improvement occurs in much smaller increments than reported in studies with completely unfit horses. It is therefore plausible that the anaerobic capacity of previously trained racehorses is relatively stable, despite brief periods of de-training.

The effects of conditioning on blood lactate have been well described in both horses and humans. VLa$_{10}$ was assessed in addition to VLa$_{4}$ because in racehorses, which frequently have a maximum blood lactate greater than 20mmol/L, the use of 4mmol/L as a threshold is arbitrary and is probably irrelevant in terms of the level of exertion manifest by Thoroughbred racehorses. In a study assessing the effects of both the intensity and duration of exercise conditioning on blood lactate concentration, it was reported
that whilst there was significant effect on VLa₄, which increased from 7.0 to 9.2m/s, intensity of exercise during conditioning had no effect (Evans, Rainger et al., 1995). Furthermore, conditioning had no significant effects on either the peak or 10 minute post-exercise blood lactate concentrations, or on lactate clearance after supramaximal runs performed at 115% of \( \text{VO}_{2\text{max}} \) (Evans, Rainger et al., 1995). Similarly, we found there was no significant conditioning effect on blood lactate at rest, peak lactate concentration, or on the post-exercise blood lactate recovery kinetics. In comparison, however, the increase in speeds corresponding to blood lactate concentrations of 4mmol/L (VLa₄) or 10mmol/L (VLa₁₀) after the race conditioning protocol were not significant. VLa₄ is an arbitrary parameter, the physiologic significance of which is widely debatable, and its relevance in racehorses which routinely generate lactates > 20mmol/l is questionable. Given the exponential nature of the La-V relationship and the considerable individual variation in the slope of the exponent, VLa₁₀ was assessed in this study, as it is probably more relevant in the context of strenuously exercise racehorses. When assessed in conjunction with the absence of a significant conditioning-associated change in the MAOD, the absence of a significant change in either VLa₄ or VLa₁₀ could provide evidence of the relative stability of the anaerobic metabolic capacity of horses.

The findings of the present study indicate that once fit racehorses have undergone repeated, intensive training programs, improvement to both the aerobic and anaerobic capacity occurs in much smaller increments than is observed in completely unfit horses. Horses in this study improved their absolute and mass specific aerobic capacities by an average 4.43% in response to a strenuous over-ground race conditioning protocol, with no change being observed in the anaerobic capacity. Whilst the magnitude of the increase in \( \text{VO}_{2\text{max}} \) might have been expected to be larger based on findings in other training studies, a principal reason for this difference could be that our horses did not lose weight whereas, the larger increase in \( \text{VO}_{2\text{max}} \) in these other studies was at least partly due to weight loss. A role of functional overreaching in this smaller increase in \( \text{VO}_{2\text{max}} \) could not be ruled out although the horses did not exhibit signs of overtraining. Furthermore, it is plausible that the anaerobic capacity of previously trained racehorses is relatively stable, despite brief periods of de-training. Considering there
was a significant increase in both the aerobic capacity, and the calculated oxygen demand at every supramaximal exercise intensity, the lack of improvement of MAOD could also represent a shift in the aerobic and anaerobic contributions to total energy production in response to conditioning, with the aerobic contribution being greater after conditioning.
Appendix B – Supplementary figures

Figure B1: Abundance of contaminating OTUs identified in the blank samples (n = 20). Blank negative controls did not include water (kit only)
Figure B2: Top 10 genera (by relative abundance) in each of the major phyla identified in the upper and lower respiratory tract of 6 healthy horses (H1 to H6)
Figure B3: Abundance of 2 OTUs (labelled with genus) that differed between the upper respiratory tract (URT) and lower respiratory tract (LRT) of healthy horses (n=6)
Figure B4: Dexamethasone treatment (10 days) effect (Pre: day 0; green bars and Post: day 11; purple bars) in the lower respiratory tract of both healthy (n = 6) and Inflammatory Airway Disease (IAD, n = 7) horses. Each panel shows the abundance for an individual OTU and is labelled with the taxa and taxa rank (p: Phylum or g: Genus) that was assigned to it.
Figure B5: Alpha diversity measures (Chao1 and Shannon) of dexamethasone treatment (10 days) effect (Pre: day 0; green bars and Post: day 11; purple bars) in both upper respiratory tract and lower respiratory tract samples. There was no significant decrease in the evenness in the lower airways of both healthy (Normal) and Inflammatory Airway Disease (IAD) horses after p-value adjustment for multiple comparisons (p = 0.071, Wilcoxon test)
Appendix C – Supplementary data

Originally an online data supplement containing extended Materials and Methods for Chapter 4 - Effects of treatment on respiratory cytokine mRNA and micro/mycobiota in an equine model of asthma

Materials and Methods

Animal care statement

This study was conducted in accordance with the recommendations of the Canadian Council of Animal Care. The research protocol was reviewed and approved by the University of Calgary Veterinary Sciences Animal Care Committee (AC17-0097).

Animals and study design

This was a prospective, randomized, controlled, blinded clinical trial. Geldings (n=20, 435 - 612kg) were enrolled in the study based on an external veterinarian’s diagnosis of mild equine asthma (MEA); horses had a history of nasal mucus and coughing. Inclusion criteria were a clinical examination within normal limits, aside from nasal mucus and coughing, and no signs of systemic illness on complete blood count or chemistry analysis. Horses resided on 2 properties (Lake Louise and Cochrane, AB, Canada), and were transferred to an indoor stable on Day -7. Individual stalls with open tops, enabling free movement of air between stalls, with straw bedding were used. Horses were fed grass hay suspended in hay nets for the duration of the trial and were given free access to water. Horses were lightly exercised every second day, and cough scoring was performed during exercise and stall cleaning (data not shown). Respirable dust concentrations were measured for 4-minute sampling periods every 4 hours throughout the trial (SidePak AM520, TSI®, MN, USA) using a PM$_4$ impacter (4μm); the flow rate (1.7L/min) was verified using a flow calibrator (4140 Flowmeter, TSI®, MN, USA). Dust concentration data were analyzed using commercially available software (TrakPro 5, TSI®). Nasal swabs, transendoscopic tracheal washes and bronchoalveolar lavages (BAL) were performed on all horses (n = 20) on day 0.
(Figure 1). On day 1, horses were allocated into one of three treatment groups (A, B, C) based on their BAL cytology (MEA versus horses with a non-inflammatory BAL profile [healthy]) and random selection (within horses with MEA) (Figure 1). Horses were considered to have MEA based on the following inclusion criteria: 1. inflammatory BAL with an increased percentage of mast cells (> 2%) or/and eosinophils (> .5%) or/and neutrophils (> 5%); 2. History of nasal mucus, coughing or both; 3. absence of labored breathing at rest (Couëtil, Cardwell et al., 2016). Group A was treated with 15mg (5mg/mL) nebulized dexamethasone sodium phosphate SID (horses with MEA; n = 8), Group B was treated with 3mL of nebulized saline SID (horses with MEA; n = 8) and Group C was a no treatment environmental control (healthy horses; n = 4) (Figure 1). All horses were treated for 13 days, and the nasal swab, transendoscopic tracheal wash and BAL procedures were repeated on Day 14 (Figure 1).

No other medications were given to horses for the duration of the trial. Those administering treatments (UCVM class) were blinded to the treatment provided to Groups A and B, as were the specialists (RL, AG) who reported the BAL results.

**Sampling procedures**

Horses were pre-medicated with acepromazine maleate (0.07-0.08 mg/kg, IM/IV), and sedated with xylazine hydrochloride (0.4 – 0.5 mg/kg, IV) and butorphanol tartrate (0.05-0.1 mg/kg). Following sedation, nasal swab samples were collected first, then transtracheal samples were collected prior to the bronchoalveolar lavage (BAL).

Nasal swab samples were collected using sterile swabs with a cotton tip. Two nasal swabs were obtained per horse (one per nasal cavity). Control swabs were also collected on each collection day (n=2) with the tip of the swab being exposed to the stable air. Immediately after collection, NS were frozen at -20°C and were transferred within 4 hours to −80 °C storage pending DNA extraction.

A transendoscopic tracheal wash was then performed as previously described (Mathieson, 2012), with the following modifications. A self-manufactured sterilized plastic catheter was introduced into the sterilized biopsy channel of a 1.3m videoendoscope (GIF-130, Olympus, Canada) until 2-3cm emerged
from the distal end. Sterile saline (~3 ml) was injected through the catheter into a sterile 10mL plain tube and stored at -20°C, to be used as a negative control. The tubing was then retracted until shielded in the endoscope; the endoscope was then introduced into the ventral meatus, passed into the trachea, advancing to approximately 90cm from the nares. The catheter was then advanced until observed to be protruding from the distal end of the endoscope, and the walls of the tracheal lumen were lavaged with ~10ml sterile saline; the fluid was then aspirated. The aspirate was immediately transferred into a sterile 10 mL plain tube and stored at -20°C; samples were transferred within 4 hours and stored at −80°C pending DNA extraction.

A BAL was then performed as previously described (Bond, Timsit et al., 2017). Briefly, a balloon-tipped BAL tube (Mila International, SKU: BAL300) was inserted until wedged against the wall of a bronchus, and 2 boluses (250 ml/bolus) of sterile isotonic saline (0.9% NaCl) solution were sequentially instilled. Lavage fluid was recovered, and 2 10 mL aliquots were immediately stored at 4°C. A differential count of BALF was performed on a minimum of 400 cells for allocation of treatment groups; epithelial cells were not included in the differential count (Fernandez, Hecker et al., 2013). Preparation of slides was performed with 400 μl of BAL fluid within 4h of sample collection, which was centrifuged using a Cytospin (90 X g for 5 min) and stained with modified Wright-Giemsa stain. A differential cell count of BALF obtained on Day 0 and Day 14 was later performed on a minimum of 2000 cells, excluding epithelial cells. Normality of the distribution of the BALF differential cell counts were tested by a Shapiro-Wilk normality test. A two-way repeated measures ANOVA (controlling for treatment group and timepoint [Day 0 versus Day 14]) was used to assess differences in cell counts between groups. A p-value ≤ 0.05 was considered significant. Two 50 mL aliquots of BALF were centrifuged at 700 × g for 10 minutes; the supernatant was then discarded, and the cell pellets resuspended in 1.5 mL of RNAlater (Qiagen, Mississauga, Ontario, Canada). Samples were stored at −80°C until extraction.

**RNA extraction, cDNA synthesis and qPCR analysis**

Total RNA was extracted from BAL samples using the RNeasy Mini Kit (Qiagen, Mississauga, Ontario, Canada), as per manufacturer instructions, using 40μL RNase-free water to elute samples. The initial
eluate was reapplied directly to the spin column membrane and centrifuged at 8000g for 1 min. The quantity and quality of the extracted RNA were measured using the Nanodrop (ND-1000) spectrophotometer. Contaminating genomic DNA was removed prior to cDNA synthesis using dsDNase (Thermo Scientific, #EN0771, Wilmington, DE, USA). Approximately 500 ng total RNA was retro-transcribed with the Omniscript® Reverse Transcription Kit (Qiagen, Mississauga, Ontario, Canada), as per manufacturer instructions, with RNaseOUT (Thermo Scientific, Wilmington, DE, USA) and Oligo(dT) primers (Invitrogen, Burlington, Ontario, Canada) included in the reaction mixture. Amplification of target RNA (IL-1β, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-17, IL-23, IFN-γ, Eotaxin-2 and TNF-α) was performed using previously optimized reaction conditions (Giguère and Prescott, 1999; Hughes, Nicolson et al., 2011; Beekman, Tohver et al., 2012). Reference genes included GAPDH, SDHA, HPRT and RPL-32, which have been shown to provide accurate normalization for gene expression studies in BALF from horses with mild equine asthma, treated with dexamethasone (Beekman, 2011). Amplification of target RNA was in 25μL total reaction volume containing 13μL PerfeCTa® SYBR® Green SuperMix, Low ROX™ (Quanta Biosciences), 50nM (Eotaxin-2 and IFN-γ) and 100nM (all other genes) forward and reverse gene-specific primers, 4μL nuclease-free H₂O, and was completed by adding 4μL of cDNA template. Amplification was performed in 96-well skirted qPCR plates (VWR 82006-704) in a thermal cycler (BioRad CFX96 Touch™ Real-Time PCR Detection System). The reaction was initially denatured at 95°C for 3 min, which was followed by 45 cycles of 15s denaturation at 95°C, and 30s annealing at 62 or 64°C (gene specific; Table 1). Fluorescence data acquisitions occurred at the end of each annealing cycle. A final melt curve analysis was run from 60-90°C at .5°C increments for 5s, with a fluorescence data acquisition after each step. Reactions were executed in triplicate, with template from samples collected on day 0 and day 14 from the same horse included on the same plate. No RT and negative controls were included on each plate. Cycle threshold (Ct) values were generated from Bio-Rad CFX Manager 3.1 software, with a user defined baseline threshold of 1146.22 (genes with 62°C annealing temperature) and 984.66 (genes with 64°C annealing temperature). Cycle threshold (Cₜ) results < 45 were classified as positive.
**DNA extraction**

Total DNA was extracted from nasal swab and transendoscopic tracheal wash samples using a Qiagen DNEasy Tissue kit (Qiagen Inc., Mississauga, ON, Canada) with the following modifications; briefly, after thawing, 2ml of TTW fluid was centrifuged (for samples <2ml, PBS pH7.2 added to 2ml) (13,000×g for 15 min) prior to the DNA extraction procedure. The supernatant was discarded. The tip of the NPS was removed from the applicator and then placed in a microfuge tube. Enzymatic buffer (250 µl) containing mutanolysin (300 U ml−1) and lysozyme (20 mg ml−1) was added to each sample. The mixtures were vortexed and then incubated for 1 hour at 37 °C. Twenty-five µl of proteinase K and 200 µl Buffer AL (without ethanol) were added; samples were then vortexed and incubated at 56 °C for 30 min. Approximately 300 mg of 0.1 mm zircon/ silica beads were added and mixed using a Tissue Lyser II (Qiagen) at 30 Hz for 5 min. The mixtures were centrifuged (13,000×g for 5 min) and 200 µl of ethanol was added to the supernatants, which were then vortexed. The remainder of the protocol of the DNEasy Tissue Kit was followed as per manufacturer instructions. Extracted DNA was stored at −80 °C until amplification and sequencing. Blank negative controls (kit only) were included in triplicate during DNA extraction.

**EHV qPCR analysis**

Primer sequences used for EHV-1, EHV-2, EHV-4 and EHV-5 have been previously described, and reaction conditions optimized (Dynon, 2010). To provide normalization, β2M was used as a reference gene (Dynon, 2010). Amplification of target DNA was in 25µL total reaction volume containing 12.5µL PerfeCta® SYBR® Green SuperMix, Low ROX™ (Quanta Biosciences), 100µM (EHV-1, EHV-2, EHV-5) and 2µM (EHV-4) forward and reverse gene-specific primers, 6.5µL nuclease-free H2O, and was completed by adding 4µL of DNA template. Reactions were executed in duplicate, using extracted DNA from nasal swab samples as the template. Samples collected on day 0 and day 14 from the same horse were included on the same plate. Negative controls were included on each plate. A lung sample that tested positive by quantitative PCR for EHV-1 from Ontario Veterinary College, The University
of Guelph was run in duplicate as a positive control. Cycle threshold (Ct) values were generated using Bio-Rad CFX Manager 3.1 software. Cycle threshold (C_t) results < 40 were classified as positive.

**qPCR statistical analysis**

The relative expression software tool (REST), which allows for correction for PCR efficiency and normalization with multiple reference genes, was used for analysis, which has been previously validated (Pfaffl, Horgan et al., 2002; Léguillette, Laviolette et al., 2009) and shown to be a powerful tool in the investigation of relative gene expression in BALF from horses with mild equine asthma (Beekman, Tohver et al., 2012). Briefly, the REST software uses a P(H1) test for statistical analysis which represents the probability of the alternate hypothesis; that the difference between the “sample” and the “control” group is due only to chance. The hypothesis test performs 2,000 random reallocations (“Iterations”) of “samples” and “controls” between the 2 groups and counts the number of times the relative expression on the randomly assigned group is greater than that of the sample data. Subsequently the expression ratio results of the investigated genes are tested for significance by a randomisation test, which accounts for multiple comparisons. In this study, “samples” referred to the post-treatment samples collected on day 14, and “control” referred to the pre-treatment samples collected on day 0; analysis was performed with horses separated by treatment group (A, B and C).

**16S and ITS amplification and sequencing**

The 16S amplicon PCR forward primer (5’GTGYCAGCMGCGCGGTAA) and reverse primer (5’GGACTACNVGGGTWTCTAAT) with forward-primer barcodes were used to amplify the V4 variable region. The ITS2 amplicon PCR ITS1F forward primer (5’CTTGGTCATTAGGAGGAAGTAA) and ITS2 reverse primer (5’GCTGCCTTCTCAGCATCGATGC) with forward-primer barcodes were used. A 30-35 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) using the following conditions: 94°C for 3 minutes, followed by 30 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed. After amplification, PCR
products were checked in 2% agarose gel to determine the success of amplification and the relative intensity of bands. Amplicons were then purified using calibrated Ampure XP beads. Sequencing was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on an Illumina MiSeq system using the 2 x 300 bp paired-end sequencing kit following the manufacturer’s guidelines.

Sequence processing

After quality check with FastQC 0.11.5 and MultiQC 1.0 (Ewels, Magnusson et al., 2016) primers and low quality sequence were trimmed off the raw sequence reads using Cutadapt 1.14 (Martin, 2011a). The trimmed reads were used to construct amplicon sequence variants (ASVs) using dada2 1.4.0 (Callahan, McMurdie et al., 2016b) in R 3.4.1 (Team, 2016). Unless otherwise stated all dada2 functions were used with default parameters. Reads were first filtered with dada2::filterAndTrim with a max expected error of 1. Error rates were learned using 2 million sequences for the forward and reverse reads separately and these error rates were used to infer exact sequences (error correct) for each sample from dereplicated, trimmed reads. Following this the forward and reverse reads were merged using dada2::mergePairs. Chimeras were removed with dada2::removeBimeraDenovo and taxonomy was assigned using the naïve Bayesian classifier (Wang, Garrity et al., 2007b) as implemented in dada2::assignTaxonomy trained with the RDP training set version 16. Species level assignment was done with dada2::addSpecies which uses exact matching to assign species where possible. ASVs were aligned with ssu-align 0.1.1 (Nawrocki, 2009a) and a phylogenetic tree constructed with FastTree 2.1.9 (Price, Dehal et al., 2010).

Statistical analysis

All statistical analysis was done with R 3.5.1 primarily with phyloseq 1.26.0 (McMurdie and Holmes, 2013), and vegan 2.5.3 (Oksanen, Blanchet et al., 2011). Plots were created with ggplot2 3.1.0. Sequences matching mitochondria or chloroplast were removed along with any sequences that weren’t assigned to Bacteria. Contaminant sequences present in the blank controls were removed from further analysis. A filtered copy of the ASV sequence table was created that retained ASVs present (count >=
in at least 2% of the samples. This served to reduce noise for downstream analysis. The full version of the sequence table was used for alpha diversity, which was assessed with the Shannon diversity index and Chao1 species richness estimator. Group means were compared using the Wilcoxon Rank Sum test ($\alpha < 0.05$). To calculate beta diversity the ASV counts (filtered table) were normalized by relative abundance (total-sum scaling) and sample-sample distance were determined with the Bray-Curtis distance and visualized with principal coordinates analysis (PCoA).

Differentially abundant ASVs were identified using generalized linear models as implemented in DESeq2 1.22.1 (Love, Huber et al., 2014a). Prior to testing the ASV table was filtered to retain ASVs present (count $\geq 2$) in at least 5% of the samples to remove low-abundant noise. A 2x2 factorial model was used for testing, including an interaction term and a p-value cutoff of 0.05 was used to identify significant ASVs.
Appendix D – Letters of Permission

Letter of Permission


I give permission to include the above paper, of which I am a co-author, in Stephanie Bond’s thesis: Mild Equine Asthma: Effects of Commonly Used Treatments on the Respiratory Microbiota, Inflammatory Gene Expression, and Aerobic Performance during High-Intensity Exercise

I understand that this thesis will be added to the institutional repository at the University of Calgary and the Library and Archives Canada.

Renaud Léguillette

Eric A. Richard

Laurent Couetil 3/21/19

Jean-Pierre Lavoie
James G. Martin

R. Scott Pirie 21st March 2019
Letter of Permission


I give permission to include the above paper, of which I am a co-author, in Stephanie Bond’s thesis: 
Mild Equine Asthma: Effects of Commonly Used Treatments on the Respiratory Microbiota, Inflammatory Gene Expression, and Aerobic Performance during High-Intensity Exercise

I understand that this thesis will be added to the institutional repository at the University of Calgary and the Library and Archives Canada.

Edouard Timsit

Matthew Workentine

Trevor Alexander

Renaud Léguillette
Letter of Permission

RE: Bond, S; Hundt, J; Léguillette, R. Effect of injected dexamethasone on relative cytokine mRNA expression in bronchoalveolar lavage fluid in horses with mild equine asthma. Submitted to BMC Veterinary Research.

I give permission to include the above paper, of which I am a co-author, in Stephanie Bond’s thesis: Mild Equine Asthma: Effects of Commonly Used Treatments on the Respiratory Microbiota, Inflammatory Gene Expression, and Aerobic Performance during High-Intensity Exercise.

I understand that this thesis will be added to the institutional repository at the University of Calgary and the Library and Archives Canada.

Jana Hundt

Renaud Léguillette
Letter of Permission

RE: Bond, S; Workentine, M; Hundt, J; UCVM Class of 2019; Gilkerson, JR; Léguillette, R. Effects of treatment on respiratory cytokine mRNA and micro/mycobiota in an equine model of asthma. Submitted to the American Journal of Respiratory Cell and Molecular Biology.

I give permission to include the above paper, of which I am a co-author, in Stephanie Bond’s thesis: Mild Equine Asthma: Effects of Commonly Used Treatments on the Respiratory Microbiota, Inflammatory Gene Expression, and Aerobic Performance during High-Intensity Exercise.

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Matthew Workentine

Jana Hundt

UCVM Class of 2019

James Gilkerson

Renaud Léguillette
Letter of Permission


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Persephone Greco-Otto

Raymond Sides

Grace Kwong

Renaud Léguillette

Warwick Bayly
Letter of Permission


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Persephone Greco-Otto

Raymond Sides

Renaud Léguillette

Warwick Bayly
Letter of Permission

RE: Bond, S; Greco-Otto, P; MacLeod, J; Galezowski, A; Bayly, WM; Léguillette, R. Efficacy of injected dexamethasone, aerosolized salbutamol and reduced respirable particulate concentration on respiratory performance in horses with smoke-induced mild equine asthma. Submitted to Journal of Veterinary Internal Medicine.

I give permission to include the above paper, of which I am a co-author, in Stephanie Bond’s thesis: Mild Equine Asthma: Effects of Commonly Used Treatments on the Respiratory Microbiota, Inflammatory Gene Expression, and Aerobic Performance during High-Intensity Exercise

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Persephone Greco-Otto

Jacqueline MacLeod

Angelica Galezowski

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Renaud Léguillette