NON-INVASIVE MONITORING OF CHANGES IN EXHALED MARKERS OF AIRWAY INFLAMMATION IN THOROUGHBRED RACEHORSES

by

Michael Peter Cathcart

BVMS BSc CertEM(Int Med) DipECEIM MRCVS

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Division of Equine Clinical Studies

School of Veterinary Medicine

College of Medical, Veterinary & Life Sciences

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ABSTRACT

Exhaled breath (EB) and exhaled breath condensate (EBC) contain numerous volatile gases and a wide-array of non-volatile compounds, several of which have been investigated as markers of lower airway inflammation in human and veterinary medicine and have been used to diagnose and monitor diseases associated with pulmonary inflammation. The identification of reliable biomarkers within EB and EBC is an active research focus with the common goal of establishing non-invasive and repeatable assessment of respiratory health and disease in mammals. The application of EB and EBC analysis holds considerable appeal in the investigation of respiratory disease in Thoroughbred racehorses, as inflammatory airway disease (IAD) is a common cause for poor performance in this population of animals.

This study documented that EB and EBC samples can be safely collected from Thoroughbred racehorses in their own environment, without adverse effect or interference with the horse's training regimen. The use of off-line collection and analysis of exhaled gases via chemiluminescence is suitable for the measurement of exhaled carbon monoxide, but is not appropriate for analyzing exhaled nitric oxide in horses.

Significant changes in the concentration of exhaled CO and the pH of EBC occurred in response to strenuous exercise and when exercising in different environmental temperatures. Exhaled CO was associated with tracheal mucus score (and the number of neutrophils in the mucus) and EBC pH was significantly different in horses with evidence of neutrophilic IAD compared to horses without IAD.

Numerous physiological and environmental variables were identified as confounding factors in the assessment of both exhaled CO and EBC pH, with respiratory rate prior to EB collection, and during EBC collection, consistently identified as an explanatory variable influencing the concentration of exhaled biomarkers. Further studies in EB and EBC analysis in horses need to focus on objectively accounting for key respiratory dynamics during sample collection.

AUTHOR'S DECLARATION

I, Michael Peter Cathcart, declare that the work contained within this thesis is original and was completed solely by myself, or with due acknowledgements. No part of this thesis has been submitted in any form for another degree or professional qualification.

Part of this thesis, or the work contained within, has been accepted for publication elsewhere:

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
BAL	bronchoalveolar lavage
BALF	bronchoalveolar lavage fluid
°C	degrees celsius
cm	centimetre
CO	carbon monoxide
CO_2	carbon dioxide
COPD	chronic obstructive pulmonary disease
CV	coefficient of variation
EB	exhaled breath
EBC	exhaled breath condensate
eCO	exhaled carbon monoxide
EDTA	Ethylenediaminetetraacetic acid
EIPH	exercise induced pulmonary haemorrhage
EHV	equine herpes virus
ELF	epithelial lining fluid
eNO	exhaled nitric oxide
$ETCO_2$	end tidal carbon dioxide
F_ECO	fraction of expired carbon monoxide
FOM	forced oscillatory mechanics
f_{res}	resonant frequency
H^+	hydrogen ion
HCO_3^-	bicarbonate anion
HO	haem-oxygenase
H_2O_2	hydrogen peroxide
IAD	inflammatory airway disease
Inc	incorporated
IgE	immunoglobulin E
IQR	inter-quartile range
kg	killogram
LAI	lower airway inflammation
LTB_4	leukotriene B_4
mg	milligram
min	minute
ml	millilitre
mm	millimetre
mm^2	millimetres squared
mmHg	millimetres of mercury
NADPH	nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
NOs	nitric oxide synthase
O_2^-	superoxide anion
P	p-value

PCR	polymerase chain reaction
$PaCO_2$	partial pressure of carbon dioxide in arterial blood
PaO_2	partial pressure of oxygen in arterial blood
ppb	parts per billion
ppm	parts per million
r	radius
r^2	coefficient of determination
RAO	recurrent airway obstruction
RBC	red blood cell
ROS	reactive oxygen species
RR	respiratory rate
rpm	revolutions per minute
s	seconds
SaO_2	oxygen saturation of haemoglobin
SD	standard deviation
TA	tracheal aspirate
TMS	tracheal mucus score
TCO_2	total carbon dioxide content of arterial blood
TW	tracheal wash
µg	microgram
µl	microlitre
UK	United Kingdom
USA	United States of America
V	volts
V_{CO}	total amount of carbon monoxide expired per minute ventilation
VO_{2max}	maximum volume of oxygen uptake
WBC	white blood cell
2D-gel	two dimensional gel

Chapter 1: Introduction

1.1 Inflammatory Airway Disease in Thoroughbred Racehorses

1.1.1 Definition

Lower airway inflammation (LAI) is common in horses, and encompasses diseases including recurrent airway obstruction (RAO), summer pasture-associated RAO, inflammatory airway disease (IAD) and exercise-induced pulmonary haemorrhage (EIPH) (Couëtil et al. 2007). IAD is particularly common in young racing Thoroughbreds, with a prevalence of 13.8% and an incidence of 8.9 cases/100 horses/month recorded in 2-year-old flat racehorses (Wood et al. 2005b). An age predilection for IAD has been documented with the condition being more common in younger racehorses (Ramzan et al. 2008).

Varying historical definitions of different forms of lower airway inflammation and a lack of understanding as to the pathophysiology of these different conditions has previously led to confusion relating to their diagnosis. Chronic Obstructive Pulmonary Disease (COPD), a term extrapolated from human medicine and incorrectly attributed to horses suffering with RAO ('heaves'), was also used to define young racehorses with excess tracheal mucus production and poor performance (Couëtil et al. 2007). In recognition of the disparities between COPD in humans and 'heaves' in horses, the term COPD has been replaced with RAO, and in 2001 the term inflammatory airway disease was coined to distinguish between this condition and RAO (Robinson 2001).

In a recent consensus statement from the American College of Veterinary Internal Medicine, Couëtil et al (2007) identified 3 main inclusion criteria for the definition of the IAD phenotype;

- Poor performance, exercise intolerance or coughing, with or without excess tracheal mucus.
- Non-septic inflammation as detected by cytological examination of bronchoalveolar lavage fluid (BALF)

- Pulmonary dysfunction based on evidence of lower airway obstruction, airway hyper-responsiveness, or impaired blood gas exchange at rest or during exercise.

Evidence of systemic infection (fever or haematological derangements) or signs of increased respiratory effort at rest (i.e. as is seen in cases of RAO) are considered exclusion criteria for IAD.

1.1.2 Aetiopathogenesis

Many different aetiological factors have been investigated in the pathogenesis of IAD, and current knowledge supports the concept that IAD is likely to be multifactorial, either through concomitant aetiological factors acting simultaneously to stimulate an inflammatory response, or different factors affecting different populations of horses, depending on environment, feeding, athletic use or geographical location (Couëtil et al. 2007).

Environmental factors are considered key in the development of IAD, with the simple act of stabling being associated with increase neutrophil percentages in BALF in yearlings (Holcombe et al. 2006). Stabling of horses increases their exposure to aerosolised particles such as stable and hay dusts, molds and endotoxin (McGorum et al. 1998, Whittaker et al. 2009a) as well as environmental pollutants, including ammonia (Whittaker et al. 2009b). Furthermore, ozone has been shown to increase indices of oxidative stress in the pulmonary epithelial lining fluid of healthy horses (Mills et al. 1996). Akin to the aetiopathogenesis of RAO, aeroallergens have also been implicated in IAD, based on the observation of high BALF eosinophil or mast cell percentages in some cases (Couëtil et al. 2007). BALF eosinophilia has been associated with airway hyper-responsiveness in young racehorses with a history of poor performance (Hare and Viel 1998).

The relationship between infectious agents and IAD has received considerable attention in the literature. A longitudinal study of 2 and 3-year-old race-

horses identified an association between lower airway disease and the isolation of *Streptococcus zooepidemicus* (Burrell et al. 1996), with those horses which were positive for *Streptococcus zooepidemicus* being 8 times more likely to develop lower airway disease, as defined by tracheal wash cytology. These findings were corroborated by a larger scale study of 724 thoroughbred race horses in training, which found tracheal inflammatory scores to be closely related to the isolation of bacteria from tracheal aspirates (Chapman et al. 2000). In the study by Chapman et al. (2000), *Streptococcus zooepidemicus* was the most common isolate, while *Streptococcus pneumonia, Actinobacillus equuli* and *Pasteurella* spp were also frequently identified. These studies clearly document the association between tracheal inflammation and the presence of bacteria, however; tracheal mucus cytology represents a global assessment of the pulmonary system and is not specific for lower airway inflammation, and these clinical findings could represent other disease processes such as primary bacterial tracheobronchitis, secondary bacterial involvement following a respiratory viral infection or reduced mucociliary clearance.

The association between IAD and viral infections of the respiratory system is more tenuous, with no association identified between the onset of IAD and seroconversion to any of the common respiratory viruses (Burrell et al. 1996; Christley et al. 2001). In one study, equine herpes virus 5 (EHV-5), as detected by polymerase chain reaction (PCR) assays, was found to be more prevalent in the tracheal mucus of clinical cases of airway inflammation compared to controls; the detection of EHV-5 correlated with the neutrophil counts on TW cytology, however EHV detection in BAL samples was not significantly different between the two groups (Fortier et al. 2009).

Exercise induced pulmonary haemorrhage (EIPH) is highly prevalent in racing Thoroughbreds with up to 75% of horses developing intra-tracheal haemorrhage immediately post-racing (Birks et al. 2002). EIPH is associated with strenuous exercise, which causes rupture of the pulmonary capillaries resulting in intra-alveolar haemorrhage, and has been associated with poor performance in racehorses (Hinchcliff et al. 2005).

The pulmonary haemorrhage primarily occurs in the caudodorsal lung fields (Birks et al. 2003). Numerous different mechanisms have been postulated in relation to the pathogenesis of EIPH, but of particular interest is the relationship between EIPH and IAD. Epidemiological investigations have identified an association between IAD and EIPH (Newton and Wood 2002) and *post mortem* studies have identified pulmonary inflammation and chronic inflammatory change (bronchiolitis and fibrosis) in areas of lung affected by EIPH (O'Callaghan et al. 1987). EIPH can, however, occur in young racehorses in the absence of IAD (McKane et al. 2010). Whilst no direct causality between IAD and EIPH has been documented, experimentally induced lower airway inflammation increased the risk of pulmonary haemorrhage during exercise (McKane and Slocombe 2010). In addition, instillation of autologous blood into pulmonary airspaces can cause morphometric and inflammatory changes consistent with chronic airway inflammation (McKane and Slocombe 2002) and exposure of primary bronchial epithelial cell cultures to autologous blood and serum results in increased chemokine expression (Ainsworth et al. 2012). It is therefore considered likely that EIPH can induce IAD, which can then perpetuate further episodes of EIPH leading to more chronic inflammatory changes in the affected lung fields (McKane and Slocombe 2010).

1.1.3 Clinical Signs

The clinical signs associated with IAD include chronic, intermittent cough, increased mucoid airway secretions and decreased performance (Couëtil et al. 2007). Coughing is an insensitive indicator of IAD (Robinson 2003) and many horses are asymptomatic at rest, in contrast to other forms of lower airway inflammation such as RAO where coughing and increased respiratory effort at rest are cardinal signs of the condition. Similarly, thoracic auscultation is invariably unremarkable in cases of IAD and pyrexia is not a feature of the condition (Ainsworth and Cheetham 2009).

Tracheal mucus accumulation is widely recognised in young Thoroughbred racehorses, which, together with neutrophilic inflammation in the tracheal

mucus, has been historically considered a diagnostic marker for IAD (Robinson 2003). The prevalence of tracheal mucus accumulation decreases with age in parallel with degree of neutrophilic inflammation (Wood et al. 2005a). Nasal discharge is also common in yearlings and 2-year-old racehorses (Wood et al. 2005b), however the association between the presence of nasal discharge and IAD, as defined by BAL cytology has not been examined (Couëtil et al. 2007). Poor racing performance has been associated with both increased tracheal mucus (Holcombe et al. 2006) and inflammatory changes in BALF (Fogarty and Buckley 1991). Horses with IAD are known to exhibit worsening of exercise-induced hypoxia, which will not only contribute to exercise intolerance, but also lead to other signs of IAD including delayed recovery of respiratory rate after exercise and increased respiratory effort during and after exercise (Couëtil et al. 2007).

1.1.4 Diagnosis

Due to the non-specific nature of many of the clinical signs associated with IAD, and the sub-clinical presentation of the condition at rest, achieving a definitive diagnosis can be challenging. Additional diagnostic tests in which respiratory secretions are assessed and submitted for cytological analysis remain the gold standard for the diagnosis of IAD.

1.1.4.1 Tracheal Wash

Endoscopic examination of the upper respiratory tract allows for the examination of the trachea, assessment of the degree of tracheal mucus accumulation and also facilitates the collection of a tracheal wash sample. Tracheal mucus accumulation is graded on a six-point scale (0-5), as assessed at the level of the thoracic inlet (Table 1.1). Historically, lower airway inflammation was defined by tracheal mucus accumulation and tracheal wash (TW) cytology (Burrell 1985; Burrell et al. 1996; Christley et al. 2001; Wood et al. 2005b), however; tracheal mucus represents respiratory secretions from the entire lung field (trachea, main stem bronchi and bronchioles) and is not specific for lower airway inflammation.

Table 1.1: Trans-endoscopic tracheal mucus scoring (Dixon et al. 1995; Saulez et al. 2009)

Grade	Endoscopic Findings
0	No visible mucus present
I	Single droplets of mucus
II	Multiple droplets of mucus which are partially confluent
III	Mucus which is ventrally confluent
IV	Large ventral pool of mucus
V	Profuse amounts of mucus covering >25% of the tracheal lumen

Absolute nucleated cell counts are of limited value in tracheal wash cytology due to the variable dilutional effect of the fluid used to obtain the sample, and most studies in which IAD is defined by tracheal cytology use differential nucleated cell counts, in particular the neutrophil percentage of the tracheal wash. A cut-off value of >20% neutrophils in tracheal wash samples is widely published as diagnostic for lower airway inflammation (Malikides et al. 2003), although as discussed previously, this will represent inflammation at any level of the respiratory tract. Furthermore, the amount of tracheal mucus accumulation (Burrell et al. 1985) and the degree of neutrophilic infiltration in tracheal wash samples (Malikides et al. 2007) both increase following strenuous exercise, which can confound interpretation of these diagnostic modalities.

The use of tracheal wash cytology alone is now not considered appropriate to diagnose IAD (Couëtil et al. 2007) with increased neutrophil percentages with/without bacteria used, instead, to define the syndrome of tracheal inflammation (Cardwell et al. 2011)

1.1.4.2 Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) collection allows for specific cytological assessment of the lower airways. The procedure isolates and occludes a single 4th or 5th branch terminal bronchi (Hoffman et al. 2008) which, following lavage

of large volumes of sterile saline (180-300mls), results in retrieval of the epithelial lining fluid and associated cellular component from isolated bronchiolar and alveolar segments. As a result, BAL cytology is not representative of the entire lung field (in comparison to tracheal wash analysis), and is only of value when a generalised pulmonary condition, such as lower airway inflammation, is being investigated. The differential nucleated cell counts for BALF obtained from normal horses are summarised in Table 1.2.

Table 1.2: Differential nucleated cell count in bronchoalveolar lavage fluid (BALF) from normal horses. (Hoffman et al. 2008)

Leukocyte	Differential count in BALF (%)
Macrophages	50-70
Lymphocytes	30-50
Neutrophils	<5
Mast Cells	<2
Eosinophils	<0.1

Mild neutrophilia, lymphocytosis and monocytosis of the BALF are characteristic of IAD in horses (Couëtil et al. 2007). Therefore, any BALF sample with >5% neutrophils would be considered abnormal and representative of lower airway inflammation. This however, is not specific for IAD as other forms of lower airway inflammation, in particular RAO, can result in increased number of neutrophils in the BALF. In general, the neutrophilic infiltration in RAO is considered to be more severe (>20%, often in excess of 70%) in comparison to the mild BALF neutrophilia noted in cases of IAD (<20%), however; no definitive cut-off point for BALF neutrophilia between the two forms of LAI has been published, and the distinction between IAD and RAO requires consideration of clinical data (history, signalment and clinical signs).

The quantification of the mast cell and eosinophil populations on BALF cytology confers a further advantage of BAL over TW (where these cells are invariably absent), as increased numbers of these cells has also been associated with the IAD phenotype (Hare and Viel 1998; Hoffman 1998). In a population of performance horses presenting for evaluation of suspected IAD, the degree of

BAL neutrophilic inflammation was associated with the presence of a cough, but not with changes in respiratory mechanical function (Bedenice et al. 2008). In the same population, however, mast cell release in BALF was associated with increased airway hyper-responsiveness.

The concept of defining IAD by BALF cytology alone raises controversy among equine clinicians. Poor correlations between TW cytology and BAL cytology have been documented (Derksen et al. 1989; Malikides et al. 2003) which stand in favour of the use of BAL cytology for the diagnosis of lower airway conditions such as IAD. Despite this, many clinicians still use TW cytology to diagnose IAD, largely due to the simplicity and owner/trainer compliance of this procedure in Thoroughbred veterinary work. Obtaining a BAL is considered more invasive, time consuming and non-compliant for investigations in horses which are actively in training, due to the requirement for a short period of rest after the procedure and the recognition that BAL can induce transient airway inflammation (Sweeney et al. 1994). In acknowledgement of this clinical bias toward TW cytology, some investigators continue to use TW cytology in studies of IAD, and a recent proposal has been put forward that, when defining the IAD phenotype in a study population, the terms brIAD and trIAD should be adopted to representing IAD diagnoses by BAL cytology and TW cytology, respectively (Cardwell et al. 2011).

Regardless of the differences between TW and BAL techniques, both are invasive and the latter should not be conducted without appropriate sedation of the animal. As a result, neither procedure is optimal for serial monitoring of disease progression or response to treatment in a clinical setting.

1.1.4.3 Thoracic Radiography

Thoracic radiographs are considered a low-yield diagnostic modality in horses with IAD, with no correlations found between BAL cytology, lung function testing, airway hyper-responsiveness and radiographic pulmonary changes (Mazan et al. 2005). At best, thoracic radiography can be employed to exclude other differential diagnoses of respiratory disease.

1.1.4.4 Non-invasive diagnostics

In the absence of non-septic inflammation (documented on BALF cytology), evidence of pulmonary dysfunction must be identified in order to definitively diagnose IAD (Couëtil et al. 2007). This can be achieved by documenting lower airway obstruction, airway hyper-responsiveness, or impaired blood gas exchange, either at rest, or during exercise, all of which require physiological lung function testing.

In its simplest form, lung function can be evaluated using arterial blood gas analysis. The oxygen (PaO_2) and carbon dioxide ($PaCO_2$) tensions, total saturation of haemoglobin with oxygen (SaO_2) and total carbon dioxide content (TCO_2) of arterial blood provide information relating to the efficiency of gas exchange at the alveolar level, whilst the plasma pH and total bicarbonate (HCO_3^-) content can be used to evaluate any acid-base derangements such as respiratory alkalosis. Physiological hypoxia (decreased PaO_2) and hypercapnia (increased $PaCO_2$) occur in horses undergoing strenuous exercise (Christley et al. 1999), however further impairment of gas exchange has been documented in horses with IAD (Couëtil et al. 1999; Couroucé-Malblanc et al. 2002; Sánchez et al. 2005). Again, this alteration in gas exchange efficiency is not specific for IAD, and in Thoroughbred race- horses conditions causing upper respiratory tract obstruction (e.g. left laryngeal hemiplegia, dorsal displacement of the soft palate or dynamic pharyngeal collapse) or EIPH can, in isolation or in combination with IAD, cause hypoxia and hypercapnia (Sánchez et al. 2005). Indeed the most severe impairment of gas exchange has been documented in horses affected by both upper airway obstruction and EIPH in the absence of IAD (Sánchez et al. 2005). Arterial blood gas data are not solely dependent upon pulmonary gas exchange; maximum oxygen uptake (VO_{2max}) significantly effects the oxygen tension of arterial blood (Art and Lekeux 1995). In an individual horse, VO_{2max} depends on the inherent capacity of the respiratory, cardiac and haematological systems, as well as the rate of extraction for aerobic metabolism (Evans 2007), therefore arterial blood gas analysis alone is insufficient for assessing airway function.

Respiratory dynamics such as pulmonary airflow, tidal volumes and minute ventilation can be measured either during or after exercise tests using pneumotachographs and respiratory spirometry (Evans 2007). Trans-pulmonary or pleural pressures can be measured directly via placement of a pressure transducer into the thoracic cavity (Jones et al. 2002), or indirectly via the placement of an oesophageal balloon catheter at the level of the mid-thoracic cavity (Hare and Veil 1998; Sleutjens et al. 2012). Horses with severe RAO have raised maximal intrapleural pressure changes at rest, (Evans 2007) and respiratory mechanics, in conjunction with re-breathing techniques, have been used to document sub-clinical IAD in Standardbred horses (Pirrone et al. 2007)

Forced expiratory (FE) manoeuvres, which provide peak inspiratory and expiratory volumes and flow rates, are routinely used in human medicine to detect early stages of lower airway disease. A technique for measuring FE data in sedated, intubated horses has been described (Couëtil et al. 2000). This FE technique has been shown to specifically detect early stages of airway obstruction in horses with RAO and IAD, with a higher sensitivity for detecting RAO compared to clinical examination or standard lung mechanics tests (Couëtil et al. 2001)

Forced oscillatory mechanics (FOM) are non-invasive techniques that provide a comprehensive measure of lung mechanical function and have been used to document lower airway obstruction in horses with IAD or RAO (Hoffman et al. 1999). By measuring the amplitude and frequency of resonant oscillatory signals transmitted through the lung field, investigators can calculate the total impedance across the respiratory tract and the resonant frequency (f_{res}), both of which are affected by airway resistance, elastance (lung compliance) and inertance. These three factors are significantly altered in conditions which cause airway obstruction, airway narrowing (bronchoconstriction) or in cases of reduced lung compliance (e.g. fibrosis), and as a result the measured respiratory impedance and f_{res} increase (Hoffman et al. 1999). The technical methodologies involved in FOM negate its use in clinical practice, and currently the techniques are restricted to the research environment. Impulse oscillometery system is a

non-invasive technique based on the forced oscillometery technique which can be performed in conscious horses, and has been shown to differentiate between IAD-affected and control Standardbred horses (Richard et al. 2009).

Airway hyper-responsiveness is a key feature of IAD in racehorses (Couëtil et al. 2007). The basis for this response is not completely understood, however, airway inflammation, airway wall thickening and dysfunction of the autonomic nervous system are all likely contributory factors (Hoffman et al 1999). Airway hyper-responsiveness can be experimentally induced using bronchoprovocation tests, which commonly involve nebulisation of histamine (Hoffman et al. 1998; Hare and Viel 1998), with the resultant bronchoconstriction and pulmonary dysfunction measured using a combination of lung function tests. These techniques are therefore useful for detecting mild or sub-clinical IAD, and the degree of airway hyper-responsiveness has been correlated to both the percentage of eosinophils and mast cells in BALF (Hare and Viel 1998; Bedenice et al. 2008).

Recent investigations have identified various serum proteins that may serve as biomarkers for LAI. Surfactant protein D, expressed by type-II alveolar cells has been documented to be significantly higher in IAD-affected horses compared to controls, although no correlations with BALF cytology were noted (Richard et al. 2012). Further, by combining surfactant protein D and serum amyloid A concentrations, Bullone et al. (2015) were able to differentiate between IAD cases and controls and define diagnostic cut-off values (Bullone et al. 2015).

1.1.5 Summary

The adoption of BALF analysis and pulmonary function testing in IAD studies has allowed for a better understanding of IAD and a more concise definition of the IAD phenotype, particularly in young racehorses, which already confers significant advantages in the recognition and awareness of this condition and its impact on the racing Thoroughbred. Despite the advantages of these diagnostic techniques, their application in the clinical setting will always be restricted by their invasive nature and technical complexity. The use of tracheal wash

cytology does allow for the identification of those individuals which are severely affected by lower airway inflammation, but as previously discussed, this technique lacks sensitivity and specificity for IAD, and is not applicable for routine monitoring of airway inflammation. The development of further non-invasive techniques which could be used in the field to detect low grade IAD without detriment to the animal would confer significant advantages in the diagnosis, treatment and monitoring of IAD and provide economic and welfare benefits to the Thoroughbred industry.

1.2 Exhaled Breath and Exhaled Breath Condensate Analysis

Over the last two decades, interest has developed in the analysis of volatile gases in exhaled breath (EB) and non-volatile molecules within exhaled breath condensate (EBC) of animals for research of respiratory physiology (Mills et al. 1996), gastrointestinal physiology (Sutton et al. 2003) and for diagnosis of respiratory disease (Wyse et al. 2004a, b). In humans, increased concentrations of several biomarkers in EB/EBC have been found in asthma (Horvath et al. 1998a), chronic obstructive pulmonary disease (COPD) (Kostikas et al. 2003) and cystic fibrosis (Tate et al. 2002). These investigations have shared a common goal of establishing a non-invasive, reliable and repeatable modality for investigating and monitoring lower airway inflammation (LAI).

Collection and analysis of EB/EBC is simple, however, its application and interpretation in medical research has been confounded by methodological and analytical differences between studies (Horvath et al. 2005). Additionally, EB/EBC may be influenced by physiological variables (Effros et al. 2002. Effros et al. 2003), respiratory dynamics (Schleiss et al. 2000) and environmental factors (Kullmann et al. 2008), which may result in large intra- and inter-subject variability for many biomarkers. In 2005 the American Thoracic Society and the European Respiratory Society set up task forces with the aim of standardising procedures for the collection and analysis of EB (Silkoff et al. 2005) and EBC (Horvath et al. 2005) from people. To date, similar recommendations do not exist in veterinary medicine.

1.2.1 Gaseous Exhaled Breath

The gaseous phase of EB represents a mixture of volatiles within the airways, which may exceed 3000 in number (Phillips et al. 1999). Most studies of EB in man and animals have investigated the detection of nitric oxide (NO), carbon monoxide (CO), and the hydrocarbon gases ethane and pentane (tables 1.3 and 1.4).

Table 1.3: Reported values for concentrations of gases in exhaled breath collected from healthy animals of various mammalian species. ppb = parts per billion

BIOMARKER	SPECIES	COLLECTION METHOD	ANALYTICAL METHOD	REPORTED VALUES	Reference
Nitric Oxide	Horse	Extraction of exhaled air into reservoir bag	Chemiluminescence	3.25 ± 0.7h ppb	Mills et al. 1996
	Rabbit	Online measurement during mechanical ventilation under general anaesthesia	Chemiluminescence	15 ± 0.8 ppb	Gustafsson et al. 1991
	Rabbit	Online measurement during mechanical ventilation under general anaesthesia	Chemiluminesence	12.9 ± 1.0 ppb	Bernareggi et al. 1998
	Guinea Pig			6.2 ± 0.70 ppb	
	Rat			0.9 ± 0.01 ppb	
	Baboon	Online measurement during spontaneous breathing under general anaesthesia	Chemiluminesence	1.0 ± 0.59 ppb	Lewandowski et al. 1998
	Cow	Offline during conscious breathing	Laser absorption spectroscopy	0.31 ± 0.41 ppb	Roller et al. 2007
Ethane	Horse	Offline during conscious breathing	Laser absorption spectroscopy	4.7 ± 2.5 pmol/min/kg	Wyse et al. 2005a
	Dog			14.8 ± 11.6 pmol/min/kg	
Pentane	Horse	Offline during conscious breathing	Gas chromatography	0.3 – 3.0 ppb	Wyse et al. 2004b

Collection of EB is technically very simple (Figure 1.1) and is amenable to both direct 'on-line' measurement of gases (EB is sampled continually from the patient and analysed directly by chemiluminescence) and also 'off-line' measurement, where a single exhalation is collected in a gas-impermeable reservoir for subsequent analysis by chemiluminescence or mass spectrometry (Kharitonov and Barnes, 2000). Controlling expiratory flow rate during on-line measurement of EB is desirable for standardisation and to obtain repeatable and reproducible biomarker measurements (Horvath et al. 2005). This is achieved in humans by the placement of resistors within the collecting apparatus (Horvath et al. 2005) or utilising slow vital capacity manoeuvres (Kharitonov et al. 1994). These techniques are not practical in animals, as resistors would create unacceptable resistance during expiration and it is not feasible to interfere with normal conscious breathing patterns in animals without causing undue distress.

Regardless of the method of EB collection, the exogenous contribution toward EB gas concentrations, arising from the presence of volatile gases in ambient air (i.e. that which is inspired) must be accounted for. This is usually achieved by analysing samples of ambient air, collected from the subject's immediate breathing environment, in parallel with each EB sample.

Table 1.4: Influences of pulmonary diseases on exhaled breath (EB) biomarkers reported in human and veterinary medicine (BAL, bronchoalveolar lavage; BRD, bovine respiratory disease; CF, cystic fibrosis; CO, carbon monoxide; COPD, chronic obstructive pulmonary disease; NO, nitric oxide; NOS, nitric oxide synthase; RAO, recurrent airway obstruction)

Pulmonary condition	Biomarker	Effect	Comments	References
Human medicine				
Asthma	Ethane	↑	Concentrations increased in non-treated asthmatics in comparison to treated asthmatics and normal controls	Paredi et al. 2000a
	NO	↑		Kharitonov et al. 1994, Horvath et al. 1998a
	CO	↑	Increased in treated and non-treated asthmatics	Horvath et al. 1998a
COPD	Ethane	↑	Concentrations increased in smokers with COPD	Paredi et al. 2000b
	NO	↑	Increased concentrations in unstable COPD patients compared to controls.	Maziak et al. 1998
		↓	Concentrations decrease in smokers	Kharitinov et al. 1995a
Cystic fibrosis	Ethane	↑	Increased concentrations in CF are significantly correlated with exhaled CO	Paredi et al. 2000c
	NO	↓	Decreased expired NO concentrations in patients with CF, associated with decreased NOS expression	Ojoo et al. 2009; Thomas et al. 2000; Downey et al. 2000
	CO	↑	Concentrations increased in CF patients and increase further during exacerbations of disease	Antuni et al. 2000
Bronchiectasis	NO	↑	Increases in concentration are related to severity of disease	Kharitonov et al. 1995b
	CO	↑		Horvath et al. 1998b
Interstitial lung disease	NO	↑/↓	Variable NOS expression and exhaled NO concentrations	Kharitonov and Barnes. 2001
	CO	↑	Increased exhaled CO related to impaired gas exchange	Antuni et al. 1999
Obstructive Sleep Apnoea	Pentane	↑	Increased in patients with obstructive sleep apnoea	Olopade et al. 1997
Pulmonary Hypertension	NO	↓	Decreased NO production related to hypertensive state	Sumino et al. 2000
Infections	NO	↓	Production decreased during recurrent bacterial infection	Kharitonov and Barnes. 2001
		↑	Exhaled NO increased during viral infections	
	CO	↑	Exhaled CO increased during lower respiratory tract infections	Biernacki et al. 1998
Veterinary medicine				
Equine RAO	Ethane	↑	Concentrations increased with environmental antigen (dust) exposure	Wyse et al. 2005b
	CO	↑	Concentrations increased with environmental antigen (dust) exposure	Wyse et al. 2005b
	NO	-	BAL concentration of NO not significantly different between horses with RAO and healthy horses	Costa et al. 2001
Bovine respiratory disease	NO	↑	Concentration higher in calves with BRD, compared to healthy calves at a feedlot	Burciaga-Robles et al. 2009
	CO	↑	Concentration higher in calves with BRD, compared to healthy calves at a feedlot	Burciaga-Robles et al. 2009
	NO	↑	Significant relation between increased exhaled NO and severity of bovine respiratory disease	Roller et al. 2007

1.2.1.1 Nitric Oxide

The presence of exhaled NO (eNO) in EB was first reported by Gustafsson et al. (1991) who detected the gas in the breath of rabbits, guinea pigs and humans and determined that NO is produced in the respiratory epithelium by the activity of NO synthase (NOs) on L-arginine (Gustafsson et al. 1991). Constitutive and inducible isoforms of NOs exist in a variety of tissues including vascular endothelial cells and epithelial inflammatory cells (Kharitonov and Barnes, 2000). Bernareggi et al. (1999) demonstrated increased eNO following stimulation with L-arginine in tracheotomised rabbits and guinea pigs, providing evidence of inducible NO production in the lungs. Exhaled NO is a widely investigated biomarker of respiratory inflammation in humans, with increased concentrations documented in asthmatic patients (Kharitonov et al. 1994; Horvath et al. 1998a). Oxidative stress is considered to play a primary role in inducing NO production from inflammatory cells (Horvath et al. 1998a). Nitric oxide has also been detected in EB of seals (Stanek et al. 1995), elephants (Lewandowski et al. 1996) and horses (Mills et al. 1996).

The sources of NO within the respiratory system are unknown; however contributions from the lower and upper airways are likely (Silkoff et al. 2005). Nitric oxide concentration in human EB is dependent on expiratory flow rate (Kharitonov and Barnes, 2000), with lower eNO concentrations recorded at higher expiratory flow rates. This flow rate dependence is characteristic of a diffusion based process for NO transfer from the epithelium of the conducting airways into the airway lumen (Silkoff et al. 2005); at higher expiratory flow rates, less time is available for the transfer of NO into the passing alveolar gas. Therefore within the lower respiratory tract, the conducting airways are considered to be the major source of eNO. The paranasal sinuses contain high NO concentrations (Lundberg et al. 1995), and their contribution to NO in EB was supported by the finding that baboons (the only mammals which lack paranasal sinuses) had reduced NO concentrations compared to several other species (Lewandowski et al. 1998).

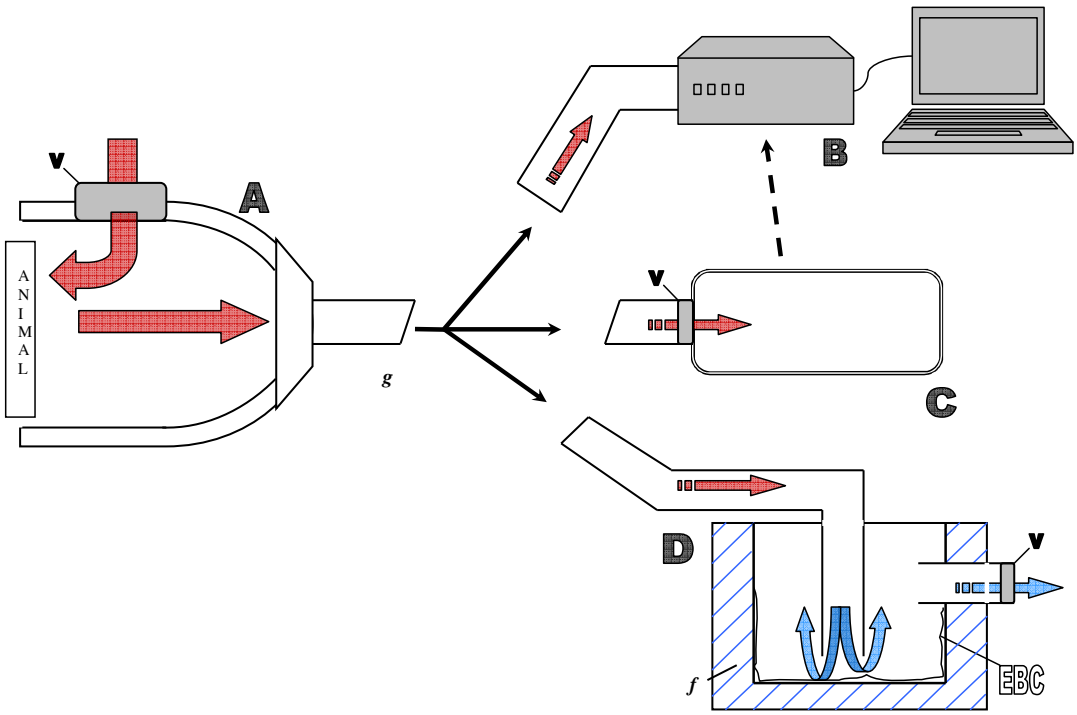


Fig 1.1: Schematic diagram representing some basic methods of exhaled breath (EB) and exhaled breath condensate (EBC) collection in large mammals. The subject breathes through a facemask (**A**), which has one-way valves (**v**) in place to ensure that the full exhalation is directed into the conducting tube (*g)*. The conducting tube diameter should be equivalent to that of the animal's trachea and should be as short as possible to minimise airflow resistance during exhalation. Direct on-line measurement of exhaled gases via chemiluminescence is achieved by directing the exhaled breath (or a sample thereof) into dedicated analysers. Alternatively a sample of exhaled breath can be collected into a non-permeable bag (Tedlar bag, **C**) and stored for off-line analysis. Collection of EBC is achieved by directing the exhaled breath through a condensing chamber (**D**). The condensing surface is creating by placing a removable inert plastic container into a cooling agent (*f)*. One-way valves (**v**) are also incorporated into the sampling devices to ensure no re-breathing of EB and to prevent contamination of the EBC chamber with ambient air.

The presence of NO in the paranasal sinuses has significance in conscious obligate nasal-breathing animals (e.g. horses) where nasal breathing is unavoidable and methodologies employed to prevent nasal contamination of eNO in humans (i.e. use of nose clips, expiring against resistance or gas partitioning) are not transferable.

Veterinary studies of eNO are sparse. Mills et al. (1996) confirmed the flow dependence of expiratory NO in exercising horses, as lower concentrations were recorded at higher flow rates. A linear increase in NO production with exercise intensity and correlation between NO and reduced pulmonary arterial pressure were found, supporting a role of NO in regulation of pulmonary circulatory pressures (Mills et al. 1996). Despite correlations between eNO and LAI in humans (Nickmilder et al. 2007) and the postulated role of NO in equine EIPH (Derksen, 1997), relationships between eNO and equine respiratory diseases are unknown. Exhaled NO has been shown to correlate with severity of bovine respiratory disease in calves (Roller et al. 2007).

1.2.1.2 Carbon Monoxide

CO is produced predominantly as a by-product during the enzymatic conversion of haem to bilirubin, catalyzed by haem-oxygenase (HO) (Choi and Alam, 1996). Haem degradation accounts for 85% of the systemic production of CO, of which 80% is exhaled (Kharitonov and Barnes 2001). At the alveolar level, CO is displaced from haemoglobin by oxygen and is dependent on alveolar gaseous diffusion (Ryter and Sethi, 2007). The majority of endogenous exhaled CO (eCO), therefore, arises from the alveoli, and is less dependent on expiratory flow rates than eNO. Pathological processes that reduce the effective alveolar surface or alveolar diffusive capacity may reduce eCO (Zavorsky, 2004), although CO production can also be increased during periods of airway inflammation.

HO exists in two major isoforms, HO-1 and HO-2. Both are expressed in most tissues, including respiratory epithelial cells, with HO-2 acting as the constitutive enzyme and HO-1 identified as the major heat-shock (stress)

protein (Kharitonov and Barnes 2001). HO-1 can be induced by a variety of stimuli, including pro-inflammatory cytokines, ozone, reactive oxygen and nitrogen species and bacterial toxins (Kharitonov and Barnes 2001). CO has been postulated as a marker of pulmonary oxidative stress. Increased eCO has been documented in untreated asthmatic patients, where the level of eCO was correlated with the expression of HO-1 (Horvath et al. 1998b), cystic fibrosis (Antuni et al. 2000, Yasuda et al. 2011), bronchiectasis (Horvath et al. 1998b) and in lower airway infections (Biernacki et al. 1998). Wyse et al. (2006) demonstrated the expression and activity of HO-1 in neutrophils, macrophages and alveolar type II epithelial cells of healthy equine lung. Despite this evidence for constitutive activity of HO-1, CO could not be detected in EB of horses and greyhounds following exercise (Wyse et al. 2005a). eCO concentrations were found to increase significantly in horses kept in conventionally-managed stables compared to low-dust stables; however results were often below the monitor detection limit (Wyse et al. 2005b). Increased concentrations of eCO have also been documented in calves with bovine respiratory disease compared to healthy age-matched controls (Burciaga-Robles et al. 2009).

1.2.1.3 Ethane and Pentane

The oxidation of cellular lipids results in the production of hydrocarbon gases, including the low molecular weight hydrocarbons pentane and ethane (Riely et al. 1974). These hydrocarbon gases are considered non-specific biomarkers of lipid peroxidation, and concentrations of these gases in EB are representative of the global *in vivo* production of hydrocarbons. Pentane has been detected in EB of humans with a variety of systemic diseases associated with oxidative stress, including inflammatory bowel disease (Wendland et al. 2001); ischaemic heart disease (Mendis et al. 1995), sleep apnoea (Olopade et al. 1997) and cancer (Hietanen et al. 1994).

Investigations into detection of hydrocarbon gases in veterinary species are limited. A gas chromatograph assay has been validated for detection of pentane in equine EB (Wyse et al. 2004b), and exhaled ethane increased significantly in exercising horses and greyhounds (Wyse et al. 2005a), suggestive of exercise-

induced oxidative stress. In addition, exhaled ethane concentrations increased in horses exposed to dusty environments (Wyse et al. 2005b), which may reflect pulmonary oxidative stress as dust exposure in horses with RAO is known to recruit neutrophils into the airways (Pirie et al. 2002). To date, there are no reports of pentane use in the investigation of respiratory disease in animals, and the equipment required for analysis of hydrocarbon gases limits their use in veterinary research.

1.2.2 Exhaled Breath Condensate

The epithelial lining fluid (ELF) of the lungs contains numerous non-volatiles (proteins, lipids, oxidants and nucleotides), which can be transported as aerosols in exhaled breath (Scheideler et al. 1993). These aerosolised particles can be collected by condensing expired air, either as a liquid or in a frozen state (Figure 1.1), thereby forming exhaled breath condensate (EBC).

Table 1.5: Advantages and limitations of collection and analysis of exhaled breath (EB) and exhaled breath condensate (EBC)

Advantages
Non-invasive
Simple to collect samples
Samples can be collected outside of the laboratory, e.g. in patients home
Does not influence the local environment of the respiratory tract
Applicable for longitudinal sampling
Potentially applicable to a wide range of settings
Useful for monitoring disease progression and response to treatment
Limitations
Lack of standardisation of methodology
Sophisticated analytical equipment required for many compounds in EB
Biomarker concentrations are often centred around the lower detection limit of the analytical equipment
Anatomical origin & contribution of biomarkers is unknown
Variable dilution of solutes in EBC
Poorly defined reference ranges for many biomarkers
Commercially available collection devices are not available for veterinary use
Concentrations of some biomarkers are dependent on respiratory flow rates, collection temperature and humidity
Environmental contamination (particularly relating to the hydrocarbon gases) must be taken into account
Many biomarkers in EBC are highly labile, requiring storage at -80ºC

It is assumed that the concentration of the non-volatile molecules in EBC reflects their concentrations in the ELF (Knowles et al 1997), and the detection of these molecules (or changes in the concentration thereof) within EBC forms the basis of the application of this non-invasive modality in the investigation of lower airway inflammation. While there are advantages to EB/EBC collection, including simplicity, non-invasiveness and application to longitudinal sampling, limitations are recognised also and several are applicable to veterinary studies (Table 1.5).

The non-volatile molecules present in EBC include adenosine, arachidonic acid metabolites, cytokines, 8-isoprostane, surfactant, lipids, histamine, serotonin and electrolytes (Horvath et al. 2005). In addition, water-soluble volatiles, including hydrogen peroxide (H_2O_2), ammonia and ethanol are present (Hunt 2002a). Many of these macromolecules exist in EBC as by-products of airway oxidation or inflammatory processes and thus serve as biomarkers of lower airway inflammation (Table 1.6). The concentration of a specific biomarker within EBC is, however, dependent upon a number of different endogenous, environmental and methodological factors that are independent of the presence or degree of airway inflammation (Horvath et al. 2005)

The production of aerosols within the lower airways depends on the velocity of the expired air and the surface tension of the ELF (Mutlu et al. 2001). Higher velocities, which will occur at higher respiratory rates, are associated with increased production of aerosols, and in disease states where bronchoconstriction may increase air flow turbulence and lower ELF surface tension, the production of aerosols will be further accentuated. This balance of airway velocity and surface tension results in marked variation in the delivery of non-volatiles in EBC, irrespective of their concentrations in the respiratory lining fluid (Mutlu et al. 2001).

Table 1.6: Reported values for the pH and concentrations of hydrogen peroxide and leukotreine B_4 in exhaled breath condensate of healthy mammalian species.

BIOMARKER	SPECIES	COLLECTION METHOD condensing medium (temp ºC) and breathing conditions	ANALYTICAL METHOD	REPORTED VALUES	Reference
Hydrogen Peroxide	Horse	Ice Water (0ºC) Conscious breathing	Spectrophotometric assay	0.4 ± 0.2 µM	Deaton et al. 2004
	Horse	Liquid Nitrogen/Ethanol slurry (-80ºC) Conscious breathing	Spectrophotometric assay	2.3 ± 1.8 µM	Duz et al. 2009
	Dog	Condensate collected at -80ºC Conscious breathing	Spectrophotometric assay	1.0 – 3.6 µM	Wyse et al. 2004c
	Cats	Condensate collected at 0ºC Plethysmography	Spectrophotometric assay	0.7 ± 0.1 µM	Kirschvink et al. 2005
	Calves	Condensate collected at 0ºC Spontaneous breathing	Glucose-based Biosensor	0.02-2.2 µM	Knobloch et al. 2008
pH	Horse	Condensate collected at -80ºC Conscious breathing	Model pH21 meter Non-dearated samples	6.0 ± 0.61	Duz et al. 2009a
	Horse	Condensate collected at -75ºC Conscious Breathing	Model pH21 meter Non-dearated samples	4.3 - 4.6 (pasture) 4.61 - 5.02 (stabled)	Whittaker et al. 2009
Leukotreine B_4	Dog	Condensate collected at 0ºC General anaesthesia	LTB_4 Enzyme immunoassay	15-94 pg/ml	Pietra et al. 2003
	Calves	Condensate collected at -15 to -20ºC, conscious breathing	Enzyme linked immunosorbant assay	60-170 pg/ml	Reinhold et al. 2000

The aerosols in EB contain >99% water vapour and variations in EBC solute concentrations vary considerably in healthy human subjects as a direct result of variable dilution of ELF droplets in the aerosolised water vapour (Effros et al. 2002). By comparing plasma and EBC electrolyte concentrations (Effros et al. 2002) or by analysing the total non-volatile cations, conductivity or urea concentration of EBC (Effros et al. 2003), this dilutional factor can be calculated, allowing for more accurate quantification of EBC solute, however, these techniques have not yet been standardised. Further, biological characteristics of EBC (including solute concentrations) are influenced by environmental temperature and humidity (Kullmann et al. 2008) and collection temperature (Goldoni et al. 2005).

Table 1.7: Influences of pulmonary diseases on exhaled breath condensate (EBC) biomarkers reported in human and veterinary medicine. (BAL, bronchoalveolar lavage; CF, cystic fibrosis; CO, carbon monoxide; COPD, chronic obstructive pulmonary disease; H_2O_2, hydrogen peroxide; IAD, inflammatory airway disease; NO, nitric oxide; NH_3, ammonia; NH_4^+, ammonium; RAO, recurrent airway obstruction)

Pulmonary condition	Biomarker	Effect	Comments	References
Human medicine				
Asthma	pH	↓	Consistent finding between studies, correlates with disease severity and treatment response	Hunt et al. 2000; Kostikas et al., 2002; Carraro et al., 2005
	Hydrogen peroxide	↑	H_2O_2 concentrations increased in steroid-naive and unstable steroid treated asthmatics compared to stable steroid treated patients	Horvath et al., 1998b
	Nitrate/nitrite	↑	EBC nitrate and nitrite concentrations increased in clinically stable asthmatics, with significant correlation to EBC H_2O_2	Ganas et al., 2001
	8-isoprostane	↑	Increased concentrations of 8-isoprostane in asthmatics compared to healthy controls, no correlation with exhaled CO or NO	Montuschi et al., 1999
	Leukotriene B_4	↑	Increased in asthmatic patients treated with steroids	Hanazawa et al., 2000
	NH_3/NH_4^+	↓	Decreased EBC ammonia in asthmatics correlates with EBC pH	Hunt et al., 2002b
COPD	Hydrogen Peroxide	↑	H_2O_2 and 8-isopratsane concentrations increased in patients with COPD. H_2O_2 more reliable with higher sensitivity	Kostikas et al., 2003
	8-isoprostane	↑		
	pH	↓	Decreased EBC pH in patients ventilated due to severe COPD, accompanied by a decrease in EBC concentrations of ammonia	Gessner et al., 2003
	Nitrite	↑	Increased nitirites and nitrothiols in patients with COPD compared to healthy controls and healthy smokers	Corradi et al., 2001
Cystic fibrosis	Nitrite	↑		Formanek et al., 2002
	pH	↓	EBC pH reduced in CF patients with further reductions during exacerbations of disease	**Ojoo et al. 2005**
	8-isoprostane	↑	3-fold increase in patients with clinically stable CF compared to healthy controls	Montuschi et al., 2000
	Hydrogen Peroxide	-	No difference detected between clinically stable CF patients and normal controls	Ho et al., 1999
Bronchiectasis	Hydrogen Peroxide	↑	H_2O_2 concentrations increased in patients with bronchiectasis with strong correlation with disease severity	Loukides et al., 1998
Sarcoidosis	8-isoprostane	↑	Increased concentrations present in patients with sarcoidosis, no correlation with BAL cell types	Montuschi et al., 1998
Acute Respiratory Distress Syndrome	Hydrogen Peroxide	↑	Significant increase in EBC H_2O_2 concentrations in ventilated patients which developed ARDS compared to those which did not	Baldwin et al., 1986
Veterinary medicine				
RAO	Hydrogen peroxide	↑	Significant increases in horses with RAO compared to healthy controls	Deaton et al., 2004
		-	No difference after environmental antigen exposure compared to healthy horses	Deaton et al., 2005, Wyse et al., 2005
		-	No difference between horses with RAO or IAD and healthy horses	**Duz et al. 2009a**
	pH	-	Non-significant trend in horses with RAO/IAD compared to healthy horses	
		↑	Stabling of horses causes significant increase in pH of EBC	Whittaker et al., 2009
	Leukotriene B_4	↑	Increased concentrations in horses with RAO. Samples collected in standing, intubated horses	Fey et al., 2001
Bacterial pneumonia in calves	Leukotriene B_4	↑	Increases detected in experimental *Pasteurella multocida* infection	Rheinhold et al., 2000
Viral pneumonia in calves	Leukotriene B_4	↑	Increases detected in experimental bovine respiratory syncytial virus infection	Rheinhold et al., 2000
Feline lower airway inflammation	Hydrogen peroxide	↑	Allergen-challenged *Ascaris suum*-sensitised cats used in experimental study	Kirschvink et al., 2005
Porcine pneumonia	Leukotriene B_4	↑	Experimentally-induced *Chlamydia suis* infection	Reinhold et al., 2008

EBC has been collected successfully from dogs (Wyse et al. 2004c), cats (Sparkes et al. 2004) horses (Deaton et al. 2004; Wyse et al. 2005b; Duz et al. 2009a) and calves (Knobloch et al. 2008). In veterinary species, most research of EBC has focused on H_2O_2, pH and leukotriene B_4 (LTB_4) (Table 1.7).

In line with the methodological differences that confound data interpretation between EBC studies in human medicine (Horvath et al. 2005), the techniques used in veterinary studies have also varied substantially.

1.2.2.1 Hydrogen Peroxide

Reactive oxygen species (ROS) are oxidizing agents produced by phagocytes which play a key role in destroying invading micro-organisms, but which can also inflict damage on host tissues (Babior 2000). Superoxide (O_2^-) is produced during the respiratory burst of neutrophils, eosinophils and macrophages from O_2 and NADPH;

$$2O_2 + NADPH \rightarrow 2O_2^- + NADPH^+ + H^+$$

This reaction is catalysed by the enzyme NADPH oxidase, a membrane-bound enzyme that is activated by a variety of stimuli including bacteria and inflammatory polypeptides. O_2^- is rapidly converted to O_2 and H_2O_2, via the activity of superoxide dismutase;

$$2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$$

The lung is protected from the potential damaging effect of these reactive oxygen species by the presence of antioxidants (ascorbic acid, glutathione and uric acid) in the ELF (Deaton et al. 2004), however; the increased ROS production during states of oxidative stress can overwhelm the anti-oxidative capacity of the ELF resulting in damage to host tissues.

H_2O_2 has been shown to correlate with the presence and severity of asthma (Horvath et al. 1998b; Antczak et al. 2000), response to treatment in asthmatics (Loukides et al. 2002) and bronchiectasis (Loukides et al. 1998).
Like eNO, the H_2O_2 concentration of EBC is flow-dependant, suggesting that a significant proportion of exhaled H_2O_2 is produced in the conducting airways (Schleiss et al. 2000).

EBC H_2O_2 as a biomarker for LAI has been investigated in horses. Deaton et al. (2004) reported H_2O_2 concentration was correlated with the degree of tracheal inflammation in horses with RAO. However, significant increases in H_2O_2 in horses with RAO exposed to environmental challenge were not found in later studies (Deaton et al. 2005; Wyse et al. 2005b). Further, Duz et al. (2009a) found no increase in H_2O_2 concentration in horses with LAI compared to healthy horses. Conversely, H_2O_2 concentration was correlated with percentages of eosinophils in BALF from allergen-challenged *Ascaris suum*-sensitised cats (Kirschvink et al. 2005), supporting the use of H_2O_2 for monitoring LAI in cats.

A common finding in studies of EBC H_2O_2 is large intra- and inter-subject variability. In healthy humans and patients with COPD, the coefficients of variation (CV) of EBC H_2O_2 concentration were 42% and 45%, respectively, likely dependent on expiratory flow rates and circadian rhythms (Beurden et al. 2002). Despite this variability, concentrations are often higher in patients with asthma (Loukides et al. 2002), viral respiratory disease (Jöbsis et al. 2001), COPD (Nowak et al. 1999) and bronchiectasis (Loukides et al. 1998), compared to healthy humans. Substantial intra-day (60-68%) and inter-day variation (64-103%) in EBC H_2O_2 concentrations in healthy horses and horses with LAI was reported recently (Duz et al. 2009a) possibly explaining the lack of significant difference in H_2O_2 between the two groups. These results suggest H_2O_2 is not a useful biomarker for equine LAI. Wyse et al. (2006) found H_2O_2 concentrations were highly variable between and within individuals in healthy dogs. Similarly, in a study using healthy calves, Knobloch et al. (2008) found marked inter-animal variability in EBC H_2O_2 concentration. However, measurements made from individual animals over a 4-week period were highly repeatable (Knobloch et al. 2008), suggesting a potential monitoring application of this biomarker in cattle. The authors also standardised measurement of H_2O_2 in EBC by use of spirometric determination of EB volume and correction for ambient air H_2O_2 concentration (Knobloch et al. 2008). Similar techniques may be useful in horses to determine whether concentrations of H_2O_2 and other constituents are flow dependent.

Analysis of H_2O_2 has been done mostly using spectrophotometric assays of oxidation of tetramethylbenzidine by H_2O_2 (Gallati and Pracht, 1985), although a fluorometric assay has been developed also (Beurden et al. 2002). H_2O_2 is highly unstable at room temperature, photo- and thermo-sensitive and reactive to many materials. To limit degradation of H_2O_2, EBC must be collected frozen or assayed immediately if collected at room temperature (Horvath et al. 2005). Another consideration is diurnal variation; Nowak et al. (1999) found EBC H_2O_2 in healthy humans peaked at 12:00 h and 24:00 h, while in healthy calves, concentrations increased significantly throughout the day (Knobloch et al. 2008). Other possible influences on H_2O_2 concentration are feed intake (Knobloch et al. 2008), patient age (Nowak et al. 1999), respiratory rate and ambient humidity.

1.2.2.2 Leukotriene B_4

LTB_4, a metabolite of arachidonic acid, acts as non-specific pro-inflammatory mediator by recruiting leukocytes and stimulation of pro-inflammatory cytokines (Crooks et al. 1998). Increased LTB_4 concentrations have been found in the EBC of smokers (Carpagnano et al. 2003), patients with COPD and asthmatics (Kostikas et al. 2005). Information of LTB_4 in EBC from animals is limited. Reinhold et al. (2000) detected LTB_4 in EBC of healthy calves using an ELISA and found good intra-animal repeatability between two consecutive days. In that study, induction of bacterial pneumonia resulted in increased LTB_4 concentrations, however the concentrations remained within the range obtained from healthy calves, suggesting LTB_4 is not a useful indicator of bacterial pneumonia. In contrast, infection with bovine respiratory syncytial virus caused a 300-400% increase in EBC LTB_4 concentration in 2 (of 4) calves (Reinhold et al. 2000). In experimental chlamydial pneumonia in pigs, LTB_4 concentration increased after infection (Reinhold et al. 2008). LTB_4 was found in EBC from horses with RAO (Fey et al. 2001), but healthy horses were not examined. LTB_4 has been measured in EBC samples collected from anaesthetised dogs (Pietra et al. 2003); however no information was available on the disease status of the animals.

1.2.2.3 Exhaled Breath Condensate pH

Several studies have demonstrated a lower EBC pH from humans with inflammatory lung diseases. This association was reported first by Hunt et al. (2000); EBC pH was significantly lower in asthmatics compared to healthy humans and treatment with corticosteroids resulted in increases in pH to values similar to healthy humans. Subsequently, airway acidification has been found in COPD (Kostikas et al. 2002), bronchiectasis (Kostikas et al. 2002), cystic fibrosis (Tate et al. 2002) and acute lung injury (Gessner et al. 2003), suggesting pH may represent a useful biomarker of disruption to ELF acid-base homeostasis by inflammation.

EBC pH in healthy humans has minimal variation (Vaughan et al. 2003) and excellent intra-sample repeatability (Borrill et al. 2005). In healthy humans, inter-day and intra-day CV for pH within and between individuals were found to be small (<5 %) (Vaughan et al. 2003; Bloemen et al. 2007). While there is greater variability in EBC pH from humans with respiratory disease, likely reflecting differing lung pathology (Borrill et al. 2005; Do et al. 2008), repeated measurements from individuals may have disease monitoring applications (Do et al. 2008). In humans, EBC pH is not affected by ventilatory pattern, patient age, collection temperature or duration or storage time (when frozen) (Vaughan et al. 2003; McCafferty et al. 2004), whereas influences of environmental temperature and humidity have been found (Kullmann et al. 2008). EBC pH is considered to reflect the pH of ELF. In humans, EBC pH is similar to that of ELF as measured directly using a pH probe (Jack et al. 1994) and has been correlated with the pH of breath isolated from the lower airways in endotracheally intubated patients (Vaughan et al. 2003) and undiluted tracheal secretions (Hunt et al. 2000).

EBC pH is highly unstable when stored at room temperature (Horvath et al. 2005). The stability of EBC pH can be improved via removal of volatiles, principally CO_2, by de-aeration with an inert gas, which raises pH by approximately 1 unit (Borrill et al. 2006).

The method of EBC collection and measurement of pH in humans has yet to be standardised, making comparisons between studies difficult. Some authors consider gas standardisation of EBC with argon (Vaughan et al. 2003) or CO_2 partial pressure (Kullmann et al. 2007) necessary to obtain reliable pH measurements. However, others argue CO_2 is a relevant EBC component and pH should be measured without gas standardisation (Bloemen et al. 2007; Leung et al. 2006). Further, an unpredictable effect of gas standardisation of EBC with a pH < 6 has been reported (Borrill et al. 2006). Repeatable results have been obtained with analysis immediately after EBC collection without gas standardisation (McCafferty et al. 2004; Ojoo et al. 2005). In a study of equine EBC, intra-sample repeatability of pH was more repeatable using plain samples than after argon deaeration (Duz, 2009b).

Limited information of EBC pH in animals is available. Knobloch et al. (2008) reported the pH of EBC sampled from calves was 5.3 to 6.5 (non-deaerated). The values were not influenced by breathing patterns but were found to increase throughout the day. In horses, pH was significantly higher when EBC was collected as a liquid compared to frozen, suggesting differences in concentrations of volatiles (Whittaker et al. 2012). In another study, the pH of non-deaerated EBC was highly repeatable within and between days in healthy horses and horses with LAI; for both groups, the CVs for intra- and inter-day pH measurements were ≤ 5% (Duz et al. 2009a). While there was no significant difference in pH between groups, a trend for a lower pH in horses with LAI was observed (Duz et al. 2009a). In a study investigating the effect of stabling on healthy ponies, correlations between EBC pH and ambient and exhaled ammonia concentrations were found (Whittaker et al. 2009b). Similarly, an association between EBC pH and ammonia in humans has been demonstrated (Wells et al. 2005). Ammonia is produced from glutamine by bronchial epithelial pH-regulatory glutaminase and may contribute to airway pH homeostasis by acting as a buffer (Hunt et al. 2002b). Further, acidification of ELF may trap ammonium ions, preventing volatile egress of ammonia from the lung in EBC (Hunt et al. 2002a).

The reported values of EBC pH of calves (Knobloch et al. 2008) and horses (Duz et al. 2009a, Whittaker et al. 2009b) are lower than those of human EBC (Hunt et al. 2000; Paget-Brown et al. 2006). These veterinary studies used non-deaerated EBC, which could partially explain the lower pH; however values are still lower than those of non-deaerated human EBC (Kullmann et al. 2007).

The regulation of airway acid-base homeostasis is incompletely understood. Respiratory epithelium may be involved, through proton pump activity (Burvall et al. 2002), secretion of glutaminase (Hunt et al. 2002b), buffering from bicarbonate and proteins (Ricciardolo et al. 2004) and secretion of surfactant (Ricciardolo et al. 2004). There is evidence that airway glands secrete acid and base equivalents (Inglis et al. 2003). Mechanisms for airway acidification in pulmonary disease are not known. Possible contributors include granulocyte production of acids (Kostakis et al. 2002), upregulation of epithelial proton pump activity (Ricciardolo et al. 2004), microaspiration of gastric contents (Ricciardolo et al. 2004) and decreased production of buffers in the ELF (Hunt et al. 2002b). One mechanism postulated for reduced pH in asthma is reduced ammonia production from down regulation of glutaminase activity from exposure to pro-inflammatory cytokines (Hunt et al. 2002b). In cystic fibrosis, mutations in the cystic fibrosis transmembrane conductance regulator may contribute to reduction in pH (Ojoo et al. 2005). Investigations of relationships between granulocytes influx, cytokine expression and EBC pH in animals with lung diseases are necessary to determine if dysregulation of airway acid-base homeostasis occurs.

1.2.2.4 Methodological considerations in collection of EBC

The numerous methodological variances that can impact upon EBC collection and influence the concentrations of exhaled biomarkers have been extensively reviewed (Horvath et al. 2005) and their consideration in relation to EBC collection in veterinary species has received similar attention (Reinhold et al. 2010; Cathcart et al. 2012).

Ventilation parameters are particularly influential during EBC collection with alterations in ventilation flow rates affecting the volume of EBC collected (Gessner et al. 2003), the production of aerosols from the epithelial lining fluid (Mutlu et al. 2001) and the transfer of biomarkers from the conducting airways into the expired breath (Mills et al. 1996; Schleiss et al. 2000). Whilst resistors can be incorporated in to EBC collection devices in attempt to standardise the intra- and inter-subject expiratory flow rates of human subjects, the current recommendation is for EBC to be collected during tidal breathing (Horvath et al. 2005). The use of resistors has not been applied to studies of consciously breathing animals, as their presence would significantly restrict tidal breathing. The reproducibility of biomarker measurements can be improved by incorporating a spirometer into the collection apparatus, allowing specific quantification of respiratory dynamics. Using this methodology Reinhold et al. (2010), obtained good reproducibility of the H_2O_2 concentration of EBC by re-calculating the concentration of H_2O_2 per 100 litres of exhaled breath. Again, the use of spirometers to record the tidal and minute volumes of exhaled breath in equine studies is restricted largely by to the disparities between the working flow-rate range of commercially available spirometers and the higher expiratory flow rates which occur during even passive breathing in Thoroughbred horses.

In general, both inspiratory and expiratory resistance will influence respiratory rate and tidal volume, the significance of which relates not only to the changes in expiratory flow rates, but also in the potential for changes in the exhaled gas concentrations, including carbon dioxide (CO_2). The concentration of CO_2 in exhaled breath, or end-tidal CO_2 ($ETCO_2$), is considered to reflect the alveolar CO_2 concentration, which in itself is closely associated with the partial pressure of CO_2 in the blood (Taylor & Clarke 2007). $ETCO_2$ can be altered by changes in respiratory rate or tidal volume, with increases and decreases in $ETCO_2$ associated with hyperventilation and hypoventilation, respectively. When in solution, CO_2 forms carbonic acid;

$$CO_2 + H_2O \rightarrow HCO_3^- + H^+$$

Carbonic acid is a weak acid, thus higher concentrations of CO_2 will reduce the pH of the solution, i.e. higher concentrations of CO_2 in the airways may artificially lower the pH of the epithelial lining fluid, and thereby lower the resultant EBC pH. Therefore, whilst EBC pH has been shown to be independent of expiratory flow rates (Vaughan et al. 2003; Knobloch et al; 2008), a theoretical influence of altered ventilation on EBC pH may exist via changes in expired CO_2 concentrations.

When EBC is collected from human patients it is done so through open mouth breathing, wherein the breath is exhaled through the oral cavity. This has led to concerns regarding the influence of salivary bases and acids in determining the pH of the EBC collected. Ammonia is the most abundant cationic buffer in EBC and is critical in determining EBC pH (Effros et al. 2006a). Ammonia is also present in saliva (as a product of bacterial degradation of urease) and gaseous ammonia present in the oral cavity is considered to significantly contribute the ammonia concentration in EBC in man (Effros et al. 2006b). Contrary to this, there is a body of evidence that refutes the contribution of oral ammonia in determining the EBC pH. Vaughan et al. (2003) found that EBC pH is correlated with the pH of breath isolated from the lower airways in endotracheal-intubated patients. Further, Wells et al. (2005) found that the pH of EBC was not influenced by the oral ammonia concentration. Other than isolating exhaled breath from the lower airways (via endotracheal intubation), ammonia can be excluded from EBC by rinsing the mouth with lightly acidic solutions or condensing the EB as a solid, below minus 40°C (Wells et al. 2005). Lyophilisation of EBC also removes the entire ammonia content, with varying effects on the final EBC pH reported (Wells et al. 2005; Effros et al. 2006a).

Whilst saliva is not considered a dominant contributor to EBC, current recommendations in medical research are for gross salivary contamination to be rigorously excluded, which can be achieved by use of a saliva trap at the level of the mouth-piece, by elevating the condensing unit above the level of the mouth and by separating the mouth-piece from the condenser by a length of tubing.

(Horvath et al. 2005). Detecting salivary amylase has been adopted in human EBC studies to demonstrate an absence of salivary contamination, documented by a 10,000 fold reduction in EBC amylase relative to the salivary concentration (Effros et al. 2002; Effros et al. 2006b).

Salivary contamination during EBC collection in horses has historically not been considered a significant contributor to EBC constituents, largely based on the fact that the horse is an obligate nasal breather. The arcus palatopharyngeus and the relationship between the epiglottis and soft palate provide an airtight seal in the horse; even during intense exercise, horses do not breathe through the mouth. As a result, exposure of the expired breath to saliva and the influence of salivary acids and bases on EBC pH would be considered negligible. Furthermore, the methodologies employed to collect EBC in horses invariably incorporate mechanisms which exclude the possibility of direct salivary contamination, namely the use of tubes to connect the face-mask to the condensing unit, and the collection of EBC in a solid, frozen state.

1.2.3 Summary

The potential for EB and EBC analysis to provide a non-invasive diagnostic modality in the investigation of IAD in Thoroughbred racehorses holds considerable appeal. The advances in EB and EBC analysis in human medicine and the clinical application that has already been established (i.e. analysis of eNO in paediatric asthmatic patients) provide encouragement for the application of these techniques in veterinary medicine.

It was hypothesised that methodological, subject-related and environmental variables would all have significant influence on the concentrations of exhaled biomarkers, and that standardisation of these methodologies, detailed knowledge of the endogenous and exogenous influences on exhaled biomarkers and validation of biomarker concentrations to standard markers of LAI would be required before any clinical application can be pursued.

1.3 Aims of the Study

The study contained in this thesis had three aims:

1. Develop and modify the methodologies for EB and EBC collection for the specific application for use in Thoroughbred racehorses.

2. Assess the influence of the following methodological and environmental variables on exhaled markers of inflammation;
   I. Use of the condensing chamber
   II. Salivary contamination during EBC collection
   III. Effect of exercise *per se*
   IV. Effect of exercise in extremes of ambient temperature

3. Investigate the association between exhaled markers of inflammation and respiratory secretion cytology in poor performing Thoroughbred racehorses.

Chapter 2: Materials and Methods

2.1 Collection of Exhaled Breath and Exhaled Breath Condensate

The collection of exhaled breath (EB) from Thoroughbred racehorses prior to and following exercise has been previously described for the assessment of exhaled carbon monoxide and ethane (Wyse et al. 2005a), however; there have been no studies that have documented the collection of exhaled breath condensate (EBC) in this population of animals, at rest or in association with strenuous exercise. Previous research at the University of Glasgow's School of Veterinary Medicine led to the development of a variety of EBC collection devices for use in horses (Duz 2009b) and ponies (Whittaker et al. 2012), and provided the frame-work for the development of a system which could be applied to EBC collection from Thoroughbred racehorses.

2.1.1 Development of the EBC collection apparatus

The specific objectives in developing the EBC collection apparatus for use in this study were:

1. To ensure the collecting apparatus would not interfere with normal respiration of the horses, cause undue stress or be of detriment to the normal training regimen.
2. To develop a portable system that could be carried by the operator at all times, and would present minimal risk to the horse or the operator.
3. To ensure that the system could be rapidly cleaned after each collection, thereby minimising any risk of disease transmission.
4. To isolate the condensing agent in a secure fashion.

2.1.1.1 Optimising the collecting apparatus

Regardless of the methodology employed to collect EB or EBC, the expired air must be collected in its entirety without any contamination of ambient air and without causing the animal to 're-breath' any exhaled breath. This is achieved in horses by isolating the muzzle via a rubber facemask, thereby encompassing both nares in an air-tight seal, and directing the inhaled and exhaled breath

accordingly using one-way, non-rebreathing valves. The exhaled air can then be directed into a reservoir bag for collection of EB or into a condensation chamber for the collection of EBC (Figure 1.1).

The face-mask used in this project was adapted from the design utilised by Duz (2009b) and Whittaker et al (2012); a fibreglass horse muzzle with a latex rubber shroud enclosing the open end, which conforms to the horse's nose forming an air-tight seal. Two holes on the dorsal aspect of the fibreglass muzzle act as bilateral common inlet and outlet conduits to which one-way inhalational and exhalational valves are attached (Figure 2.1). The one-way valves (Salford non-rebreathing valve 35mm diameter, Cranlea & Company, Birmingham, UK) are placed at either end of a T-piece connection tube that facilitates the unidirectional flow of ambient air into the facemask during inhalation and ensures all exhaled air is directed into the collecting apparatus during exhalation. Both exhalation outlets are then connected to the condensing chamber using 35mm corrugated polypropylene tubing which, in the studies by Duz et al. (2009b), was arranged in Y-shaped configuration such that exhaled breath was ultimately directed through a single piece of 35mm tubing into the condensing chamber.

Figure 2.1: Photograph depicting the fibreglass facemask (A) used to direct the exhaled breath of the horse into the collecting apparatus. The latex shroud (B) ensures an air-tight seal around the muzzle; the horse respires through one-way valves arranged such that there is unidirectional flow of inspired (blue arrow) and expired (red arrow) air.

During early experimentation with the system used by Duz et al. (2009b) in Thoroughbred horses, it was found to create undesirable resistance to both inhalation and exhalation, evident by increased excursions of the respiratory musculature and excessive movement of the facemask to and fro as negative and positive pressures were created within the mask during respiration. The airway impedance in any breathing system is largely dependent on resistance (R), which is principally determined by the radius of the airway (Robinson 2007). Resistance during laminar airflow (as occurs in the upper respiratory tract) can be calculated using a rearrangement of Poiseuille's Law;

$$R = 8h\mathit{l} \div \pi r^4$$

Where *l* and *r* are the length and radius of the airway, respectively, and h is the viscosity of the air.

By virtue of its elevation to the fourth power, radius is the most important variable in determining resistance; a reduction of the radius of the airway by half results in a 16-fold increase in resistance. The narrowest points of the equine respiratory tract during passive respiration are the nasal valve (located immediately caudal to each nostril), the larynx and the trachea (Robinson 2007). Of these, the rima glottidis of the larynx represents the point at which greatest resistance is encountered within the equine respiratory system. The cross-sectional area of the equine rima glottidis has been recorded, at rest, as 1130 ± 117 mm^2 during inhalation and 640 ± 224 mm^2 during exhalation (Lafortuna et al. 1999). The T-piece connector, non-rebreathing valves and conducting tubing used for the collection of EBC all have a standard radius of 17.5mm, therefore; when the apparatus is arranged in the configuration used by Duz et al. (2009b), the resultant surface area for exhalation is 962.13 mm^2 is for and a 1,924mm^2 for inhalation (2 inhalation valves), based on the formulas;

$$\text{Surface Area of one circle} = \pi r^2$$

$$\text{Combined surface area of two circles} = \pi(r1^2 + r2^2)$$

Whilst these values exceed that recorded for the rima glottidis in resting horses (Lafortuna et al. 1999), these values are likely to be over-estimates as the true

surface area for each non-rebreathing valve will be reduced by the diaphragm and the central bar which anchors the diaphragm within each valve. Based on these calculations and the observations made whilst using the apparatus, it could be concluded that the use of two inhalation valves and one common connecting tube for expired air was resulting in greater or equivocal resistance to airflow than the horse's own respiratory system, thereby creating undesirable interference with normal respiration.

To reduce the resistance to inspiration, two further one-way valves were incorporated into the front face of the fibreglass facemask, resulting in four valves being available for inspired air to be drawn into the mask. Using the above calculations, this would result in a total surface area of 3,848.35 mm^2 available for inspiration, far exceeding that of the rima glottidis, and significantly reducing the resistance to inspiration. Reducing the resistance to expiration could simply be achieved by maintaining the two expiratory 'arms' of the collection apparatus in parallel, rather than employing the Y-shaped configuration (Figure 2.2). This would double the surface area available for expiration, reducing the resistance by a factor of 16.

Figure 2.2: Photograph depicting expiratory 'arms' of the collecting apparatus, which were maintained in parallel and kept to a maximum length of 1 metre, to reduce resistance to expiration.

The length of a tube is also a significant contributing factor in determining the resistance to laminar airflow; therefore, reducing the length of the conducting tubes would further reduce the resistance to expiration. A one metre length of tubing was selected for each expiratory 'arm' of the collecting device, which is similar to the length of the Thoroughbred's trachea (70-80cm; de Freitas et al. 2001), but which ensured that the condensing chamber was kept a safe distance away from the horse, and that the system remained portable (Figure 2.2). By employing the use of two 1-metre, 35mm diameter corrugated polypropylene tubes (Cranlea & Company, Birmingham, UK) and incorporating the four inspiratory valves into the facemask, the resistance to inspiration and expiration created by the placement of the collection apparatus became negligible (based on observations of respiratory patterns during EBC collection).

2.1.1.2 Developing the condensing chamber

A number of different commercially developed EBC collection devices are available for EBC collection in humans, including the R-Tube™ (Respiratory Research Inc, Austin, Texas) and the EcoScreen II (FILT GmbH, Germany). Using these devices, researchers can obtain standardised and repeatable results that greatly facilitate comparisons between studies. Indeed both devices have been shown to produce similar results for EBC pH in normal and asthmatic human patients (Koczulla et al. 2009). The R-Tube™ has the advantage of being portable and can be used by patients in their own home without professional assistance, making it very useful for large-scale studies. The EcoScreen II allows for fractioning of the exhaled breath, and provides data relating to respiratory mechanics via an in-built spirometer. Whilst the EcoScreen II has been used to successfully collect EBC from calves (chosen due to similar body weight and ventilatory volumes to that of human subjects (Knobloch et al. 2008), the majority of veterinary studies into EBC rely on the development of custom-made collection devices and condensing chambers.

A variety of condensing agents have been used in the collection of EBC in horses, the most common of which are iced water (Deaton et al. 2004), dry ice (frozen

carbon dioxide; Whittaker et al 2012) and liquid nitrogen/ethanol mixtures (Duz et al. 2009b). Using iced water, EBC is collected as a liquid at 0°C and this method is only applicable to studies where the condensate can be immediately analysed (Horvath et al. 2005). Dry ice and liquid nitrogen will condense EB at approximate temperatures of -78°C and -100°C, respectively, and maintaining the EBC in a frozen state facilitates any delay encountered between sample collection and analysis. The pH of EBC has been found to be stable in a frozen state for up to one month in equine samples (Whittaker et al. 2012) and up to two years in human samples (Vaughan et al. 2003). Hydrogen Peroxide (H_2O_2) has been found to be stable in human EBC for up to one month (Jöbsis et al. 1998), however; snap freezing and subsequent thawing of equine EBC was found to reduce the H_2O_2 concentration by 57% compared to analysis within 5 minutes of collection (Deaton et al. 2004).

The use of dry ice as a condensing agent comes with the potential complication of EBC contamination with CO_2. When in solution, CO_2 forms carbonic acid, which, acting as a weak acid, will lower the pH of the solution (see section 1.2.2.4; Methodological considerations in collection of EBC), which has obvious implications in the measurement of EBC pH. Using ethanol solution, cooled with liquid nitrogen provides a very efficient method of condensing EB (Duz et al. 2009b), but represents a logistical obstacle in relation to the transporting and storage of liquid nitrogen, and could be considered more hazardous to use in a field setting.

In a methodological study comparing different EBC collection devices and different condensing agents (Whittaker et al. 2012), the pH of EBC (de-aerated and non-de-aerated) was not significantly different when either dry ice or liquid nitrogen/ethanol were used in the collection device described by Duz et al. (2009b). Based on these results it was decided that dry ice, which can be transported and handled safely and which has no hazardous welfare or environmental considerations, would be used as the condensing agent for the studies described in this thesis.

The condensing chamber used in the following studies was adapted from that used by Duz et al. (2009b). The conducting tubes directed the expired air into two 400ml polypropylene beakers via a modified polypropylene FloPlast AntiVac Bottle Trap (B&Q plc, UK) that maintained the internal diameter of each expiratory arm at 35mm and permitted flow of the expired air around the internal surface of the beaker before exiting via the outlet of the trap. Further one-way valves were placed at each outlet to ensure one-way flow of expired air through the condensing chamber and to prevent any contamination with ambient air during sample collection.

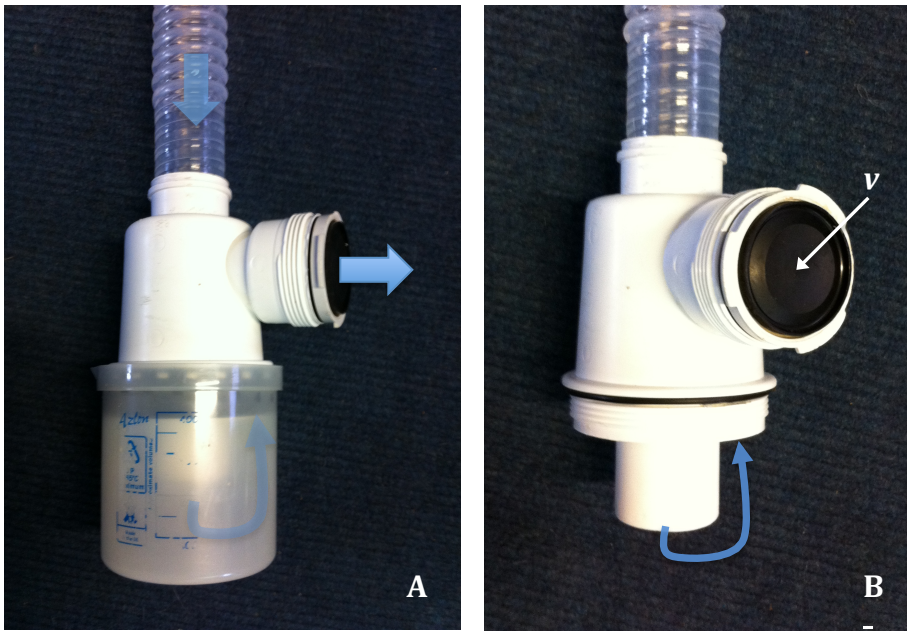


Figure 2.3: Photographs of modified polypropylene FloPlast AntiVac bottle trap, attached to end of 35mm corrugated polypropylene tubing, with (A) and without (B) the 400ml condensing beaker attached. Blue arrows indicate direction of airflow, which is maintained unidirectional by placement of a one-way valve at the outlet (*v*).

The polypropylene beakers were chilled to -70°C with dry ice such that the condensate of the exhaled breath was collected in a frozen state on the inside of the beakers. During the initial development of the apparatus, the dry ice was housed within a polystyrene lined cool bag and the beakers were placed through the top of the cool bag directly into the dry ice. This system resulted in rapid evaporation of the dry ice, and further concern regarding the sublimation of CO_2 in the immediate vicinity of the condensing unit. To overcome these issues a side-opening plastic container was used to contain the dry ice, into which two

conforming cylindrical 'sleeves' had been placed, attached to the lid of the container. The sleeves were made of wide-meshed casting material molded to the beakers, which kept the beakers separate from the dry ice but in sufficiently close apposition as to chill the condensing surface (Figure 2.4). The entire condensing chamber was housed within the polystyrene lined cool bag that could be carried by the operator during sample collection.

Figure 2.4: Photograph depicting the condensing box used to house the dry ice; the design allowed for close apposition between the condensing beakers and the dry ice, whilst fully enclosing the dry ice, thereby preventing sublimation of evaporating CO_2 into the condensing surface. The box could be opened from one side.

2.1.2 Sample Collection

Prior to sample collection, all horses were examined for any overt signs of respiratory disease (cough, nasal discharge, increased respiratory effort at rest) or for any signs of cutaneous infectious disease, i.e. dermatophytosis, which could be spread from horse to horse via use of the facemask. All horses were restrained with a head-collar and lead rope and samples were collected without sedation. Initially the facemask was placed over the muzzle, without the condensing chamber attached, to allow the horses to become accustomed to breathing through the facemask. Horses that reacted adversely to the placement of the facemask on more than two attempts were excluded from the studies.

Placement of the facemask often induced an initial transient period of hyperventilation. This was considered to be representative of a sympathetic response to the novel stimulus of the facemask, rather than any compromise to respiratory function, as the horse's respiratory rates would quickly normalise whilst the mask remained in place. Furthermore, this response was less likely to occur in horses that had previously been sampled. Once the facemask was in place and the horse's respiratory rate had returned to normal, a single expiration of EB was collected. This was achieved by placing a stop-cock over one of the expiratory outlet valves during inspiration and placing a reservoir bag attached to a large bore 3-way tap over the other valve during one single expiration. The EB was then transferred in to a gas-impermeable Tedlar® bag for storage prior to sample analysis. For each EB sample obtained, a corresponding sample of ambient air was collected in the immediate vicinity of the horse's breathing zone, directly into a separate Tedlar® bag.

Following collection of the EB sample, the condensing chamber was attached to the facemask by attaching the connecting tubes to the expiratory valves, through which the horse would breathe for a period of 5-10 minutes. Each horse was attended at all times with two people present throughout the collection period. The EBC that formed in each of the condensing beakers was subsequently transferred in its frozen state into a 50ml sterile polypropylene universal tube (Griener Bio-One Ltd, Leighton Buzzard, UK) and stored on dry ice until analysed. All precautions were taken to ensure contamination of the EBC sample did not occur. In between different horses, the facemask and latex shroud were cleaned with biguanyde hydrochloride, alkyldymethyl benzyl ammonium chloride and dodecyl dymethil ammonium chloride (Trigene Wipes), rinsed and dried. Different connecting tubes and condensing beakers were used for each sample collection, thereby avoiding excessive washing and drying of equipment and facilitating a rapid turn-around between sample collection times. The connecting tubes and beakers were subsequently all washed in de-ionised water, rinsed and dried prior to further use.

2.2 Optimisation of exhaled breath condensate collection techniques

The numerous methodological variances that can impact upon EBC collection and influence the concentrations of exhaled biomarkers have been discussed in Chapter 1.2.2.4. Based on the current literature, and the observations made during the development of the EBC collection apparatus (see section 2.1.1), two specific methodological considerations were deemed pertinent enough to necessitate further investigation and validation prior to conducting in-field studies on Thoroughbred horses. The two considerations were 1) the influence of patient ventilation on the pH of the EBC, and 2) the assessment as to whether salivary contamination occurred during EBC collection

2.2.1 Influence of ventilation on EBC pH

The influence of EBC collection on ventilation in horses has not been previously investigated with investigators reporting only subjective observations relating to the changes (or lack thereof) in respiratory patterns during EBC collection (Duz et al. 2009b; Whittaker et al. 2009b). As the collection of EBC has the inherent potential to influence ventilation through the creation of resistance to inspiration and/or expiration (section 2.1.1.1), a short study was devised in attempt to try and assess the influence of ventilation on EBC pH.

In the absence of any spirometric equipment suitable to use in Thoroughbred horses, it was proposed that measuring $ETCO_2$ would provide a means by which respiratory dynamics could be assessed during EBC sample collection. $ETCO_2$ can be easily measured using a capnograph, a non-invasive, side-stream sampling device that measures respiration rate and the concentration of inspired and expired CO_2 via infra-red absorption. By incorporating a portable capnograph (N85-1 capnograph, Woodley equipment company Ltd. UK) into the EBC collection device (Fig 2.5), it was possible to record the respiratory rate and $ETCO_2$ of horses during sample collection.

Figure 2.5: Schematic photograph representing the use of capnography during EBC collection. The microstream sampling tube (A) of the capnograph (B) was placed at one of the outlet tubes, between the level of the inspiratory (C) and the expiratory (D) valve.

The aims of the study were to investigate the impact of EBC collection on ventilation by identifying any associations between $ETCO_2$, RR and EBC pH. The details of the study, including the material and methods, results and a short discussion can be found in appendix 1.

The results of the study demonstrated that respiratory rates during EBC sample collection did not vary from normal (pre-collection) resting respiratory rates. A significant, indirect association between RR and $ETCO_2$ was identified in the univariate analysis indicating that whilst no changes in RR occurred during sample collection, a change in respiratory dynamic was likely to be occurring. This was also reflected, again in the univariate analysis, with a direct association between RR and EBC pH noted. No significant association between $ETCO_2$ and EBC pH was found, and all associations between RR, $ETCO_2$ and EBC pH lost significance in the final multivariant model. Therefore, in summary, whilst subtle changes in respiratory dynamics were occurring during EBC sample collection, these did not seem to be influencing the EBC pH.

2.2.2 Assessment of salivary contamination during EBC collection

Whilst the anatomical and methodological factors relating to EBC collection in horses (see section 1.2.2.4), in theory, preclude the contamination of EBC with saliva, there have been no published studies to date that can either endorse or refute the assumption that salivary contamination does not occur when collecting EBC from this species.

In an attempt to address this issue, a pilot study was conducted to document whether salivary contamination occurs during EBC collection in Thoroughbred racehorses. Saliva was collected from Thoroughbred horses during the study outlined in Chapter 4, and various methodologies were employed to analyse the EBC for salivary contamination. The details of the study, including the materials and methods, the results and a brief discussion can be found in Appendix 2.

Via the use of the 2D-gel electrophoresis it was possible to demonstrate that paired salivary and EBC samples had distinctly different protein patterns; all samples of saliva produced similar gel patterns, whereas the EBC samples consistently failed to return any discernable protein bands. The results of the study and the observations made during the investigation support the supposition that salivary contamination does not occur when EBC is collected from horses in a frozen solid state, using collection devices that separate the facemask from the condensing chamber.

2.3 Analysis of Exhaled Breath and Exhaled Breath Condensate

2.3.1 Analysis of eCO and eNO in Exhaled Breath

Samples of EB, and the corresponding ambient samples, were analysed for eCO and eNO using a chemiluminescence gas analyser (LR1800, Logan Research) with detection ranges of 1-50 parts per billion (ppb) for NO and 0.25-50 parts per million (ppm) for CO. The gas analyser was calibrated prior to use with gas standards (105ppb NO and 30ppm CO; BOC industrial gases, UK) as per the manufacturer's instructions. The Tedlar® bags were individually attached to the inlet port of the gas analyser and each sample was analysed for 60 seconds during which a real-time trace of NO, CO and CO_2 was recorded and stored electronically. After 60 seconds, the analysis was stopped and the average NO and CO concentrations detected over the 60-second period were recorded. The final eNO and eCO concentrations for each EB sample were calculated as the net difference between the EB and the paired ambient air concentrations, with a null result recorded if the ambient concentrations exceeded those in EB. Each Tedlar® bag was then evacuated by applying negative pressure to the valve and sealed prior to further use.

2.3.2 Analysis of Exhaled Breath Condensate pH

EBC samples were kept frozen until time of analysis. After thawing, each EBC sample was weighed in order to estimate the volume of EBC collected. The universal containers, which the EBC had been stored in, were gently centrifuged (1,000rpm for 1 minute) to maximise the volume of condensate retrieved prior to transfer into a sterile eppendorf tube. The pH of each EBC sample was measured using a bench top pH meter (pH21, Hanna Instruments Ltd, UK; sensitive to 0.01pH) and a 6mm glass body pH electrode (HI-2031B, Hanna Instrument Ltd, UK). The pH meter was calibrated prior each set of measurements using pH standards (pH 4.01 & pH 7.01, Hanna Instruments Ltd, UK), as per the manufacturers instructions.

The pH of each EBC sample was measured at room temperature by immersing the pH electrode into each eppendorf of EBC until a stable pH reading was obtained, as indicated by the pH meter.

2.3.2.1 Gas standardisation of EBC pH

The EBC samples were subsequently de-aerated by bubbling argon (BOC industrial gases, UK) through the sample with the pH probe in place, as described by Hunt et al. (2000). Argon de-aeration was continued until a stable pH reading was obtained (maximum 10 minutes).

2.4 Ethics and Welfare

All studies described in this thesis were approved by the Ethics and Welfare committee, School of Veterinary Medicine, University of Glasgow.

Chapter 3: Influence of high intensity exercise and ambient temperature on concentration of exhaled biomarkers

3.1 Introduction

The aims of this study were to;

(1) Determine whether EB and EBC could be obtained from Thoroughbred racehorses in a field setting.

(2) Investigate the influence of exercise *per se* and exercise in different ambient temperatures, humidity and management regimens on the exhaled concentrations of nitric oxide (eNO) and carbon monoxide (eCO), and EBC pH.

Exhaled CO has been investigated in relation to maximal dynamic exercise in Thoroughbred racehorses, where eCO was detectable in 8/11 resting EB samples, but was undetectable in all of the post-exercise samples (Wyse et al. 2005a). Mills et al. (1996) measured eNO using direct online analysis of EB in Thoroughbred racehorses during high-speed treadmill exercise in laboratory setting, where a linear increase in eNO was found in association with increasing exercise intensity (Mills et al. 1996). There have been no studies utilising 'off-line' measurement of eNO in exercising horses, and no published investigations into the effects of exercise on the pH of EBC in any animal.

No previous studies have collected EB and EBC simultaneously from equine athletes, and given the aforementioned developmental considerations and validation techniques that relate to the collection of EBC in horses (Chapter 2), this study was primarily intended to serve as a pilot investigation into the feasibility of collecting multiple EB and EBC samples from Thoroughbred horses, prior to and following strenuous exercise. The ability to obtain EB and EBC samples in a field setting would provide significant advantages in the investigation of LAI in horses, including the ability to sample a greater number of horses than would be achievable using conventional methods, and sampling of horses within their normal environment, which would ultimately confer a real-time clinical application to the results of the study.

3.2 Materials and Methods

3.2.1 Horses

Exhaled breath and EBC were obtained from Thoroughbred horses at a single racing yard in southwest Scotland during two 5-day periods; in July 2011 (sampled during warm ambient conditions [A_W group]) and February 2012 (sampled during cold ambient conditions [A_C group]). The study was approved by the Ethics and Welfare committee, School of veterinary medicine, University of Glasgow.

A convenience sample of horses undergoing high intensity exercise was selected on each day, which ensured that sample collection did not interfere with the normal daily schedule of the training yard.

Based on the training schedule, alternating training groups of horses were identified and one horse from each group was randomly selected for EB and EBC sample collection. A maximum of four horses were sampled in one day. Horses could be sampled more than once during the study period. All horses were sampled in their stable, located in one of five barns (1-5), and were considered free of respiratory disease based on history and clinical examination. Exhaled breath and EBC samples were obtained immediately pre-exercise, and post-exercise EB and EBC samples were taken when respiration approximated the pre-exercise respiratory rate (RR), 20 to 30 minutes after exercise. The horses were exercised under saddle on an all-weather one-mile track at half-pace canter, full-pace canter or gallop according to the current training regimen for each horse. For each sample point, the following data was recorded; ambient temperature and humidity; respiratory rate prior to, during and after sample collection; subjective assessment of respiratory effort and depth during sample collection; level of exercise; barn number; bedding type and forage provision. For each horse and sampling period, the ambient temperature and humidity were recorded with a hand-held thermohydrometer (Model 800016, Sper Scientific).

3.2.2 Sample Collection

Exhaled breath and EBC samples were collected as previously described in Chapter 2. For each EB sample, a sample of ambient air was simultaneously collected at the level of the horse's nostrils. Different polypropylene tubes and beakers were used for each EBC collection on any given day to avoid cross contamination of breath condensate samples. After use, the polypropylene tubes and beakers were rinsed with deionised water, wiped with absorbent paper towel and left at room temperature to dry completely. At each sampling point, EBC was collected over a 5-minute period. The dry ice was housed within a self-retaining container to ensure the condensing unit was separated completely from the dry ice. One-way valves at the outflow of the condensing chamber ensured no contamination with environmental air.

The concentrations of NO and CO in exhaled breath samples and ambient air samples were analysed as previously described in Chapter 2. Samples of EBC were kept frozen until time of analysis. After thawing, the pH of neat EBC was measured as previously described, with and without de-aeration using argon.

3.2.3 Statistical Analysis

All numerical data were recorded and stored using Microsoft Excel®, with categorical data (gender, exercise level and bedding type) converted to numerical data as appropriate. Data analysis was completed using Minitab 16® statistical software. All data were tested for normality using the Anderson-Darling normality test. Paired pre- and post-exercise data were compared using a paired *t* test or Wilcoxon signed-rank test where appropriate. Non-paired data (A_W and A_C groups) were compared with two sample *t* tests or Mann-Whitney analysis.

With outcome set as the difference between the post-exercise and pre-exercise value for each exhaled biomarker (NOdiff, COdiff and EBC pHdiff), univariate linear regression models were used to investigate associations between

outcomes and recorded environmental and individual variables. Multivariable models were built for each outcome by inclusion of explanatory variables found to have a *P* value of < 0.2 during univariate analysis. Significance was set at *P* < 0.05.

3.3 Results

Forty-two paired EB and EBC samples were collected from 28 horses (median age 4 years, range 1-10; 32 geldings, 10 mares). Twenty-three (23) paired samples were collected in the A_W group, with nineteen (19) collected in the A_C group. Horses were bedded on either shavings or paper and all received the same forage.

Median ambient temperature was significantly higher for the A_W group (15.8 ºC, range 12.9-20.9) compared to the A_C group (6.0 ºC, range -0.8-7.5 ºC, *P* < 0.001; Fig 3.1). Humidity differed significantly between the groups (A_W 67%, range 54-82; A_C 75%, range 57-99, *P* = 0.009), but this difference was influenced by a single day for the A_C group where humidity was 99%. With this outlier removed, the humidity between the two groups was not significantly different (*P* = 0.06; Fig 3.2).

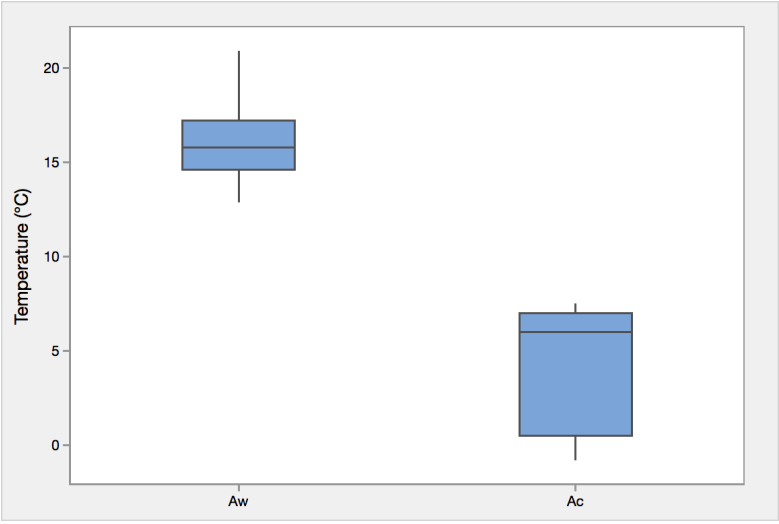


Figure 3.1: Box and whisker plots documenting significant difference (*P* <0.001) in ambient temperature (ºC) between the warm (A_W) and cold (A_C) sample groups

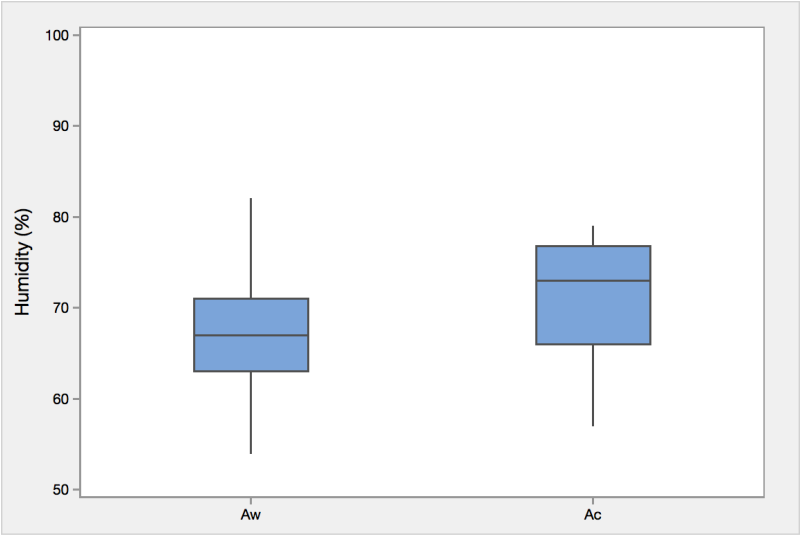


Figure 3.2: Box and whisker plots of humidity in both the warm (A_W) and cold (A_C) sample groups (outlier removed). There was no significant difference between the groups (*P* = 0.06)

Exhaled breath and EBC were collected successfully from all horses pre- and post-exercise and all horses tolerated the collection process. During sample collection the median RR pre- and post-exercise did not differ (*P* = 0.185; Fig 3.3). The median RR during sample collection was significantly lower than that prior to sampling, both pre- and post-exercise (*P* < 0.0001) and the median resting RR was significantly higher post-exercise (14 breaths/min) than pre-exercise (12 breaths/min) (*P* = 0.03).

The concentration of eNO was above the limit of detection for the chemiluminescence gas analyser (1 part per billion) in only 8/42 (19%) and 11/42 (26%) of pre- and post-exercise EB samples, respectively, only 4 of which were paired EB samples. Due to this poor return of interpretable eNO values, and the inability to conduct valid statistical analysis of these data, eNO was excluded from further analysis and interpretation.

The volume of EBC collected was 1.1 ± 0.4 mL and 1.4 ± 0.6 mL pre- and post-exercise, respectively.

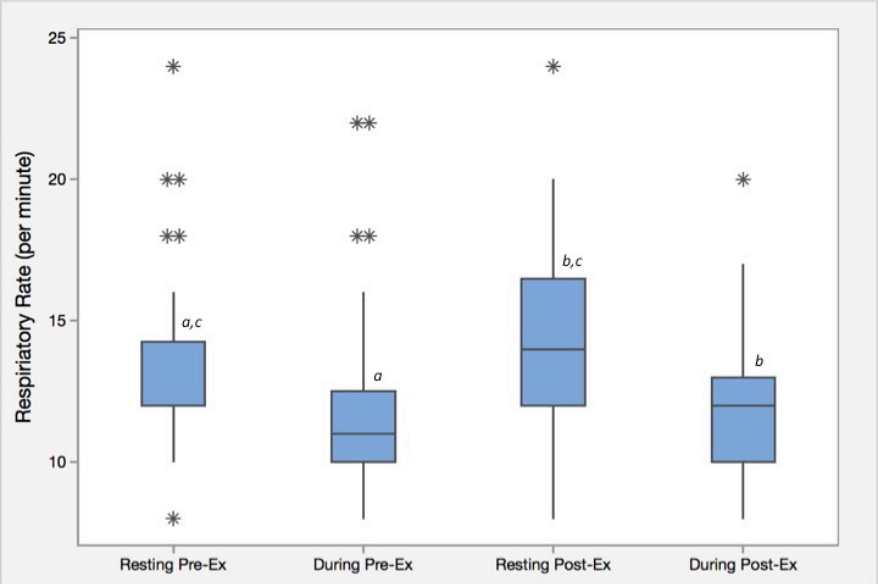


Figure 3.3: Box and whisker plots of respiratory rates (RR) prior to (resting) and during sample collection; pre- and post-exercise. The median RR during sample collection was significantly lower than the median resting RR, both pre- (*a*; *P* <0.0001) and post- (*b*; *P* <0.0001) exercise, and the median resting RR was significantly lower post-exercise than pre-exercise (*c*; *P* = 0.03)

Exhaled CO was detectable in 39/42 pre-exercise EB samples (median 1.3 ppm, inter-quartile range 0.9-1.7) and in 27/39 corresponding post-exercise samples (median 0.8 ppm, inter-quartile range 0.6-1.2). Using these paired data (n = 27), eCO concentration was significantly lower post-exercise (*P* = 0.013; Fig. 3.4). The decrease in eCO (fig 3.5) was significant in the A_W group (*P* = 0.003) and not in the A_C group (*P* = 0.69).

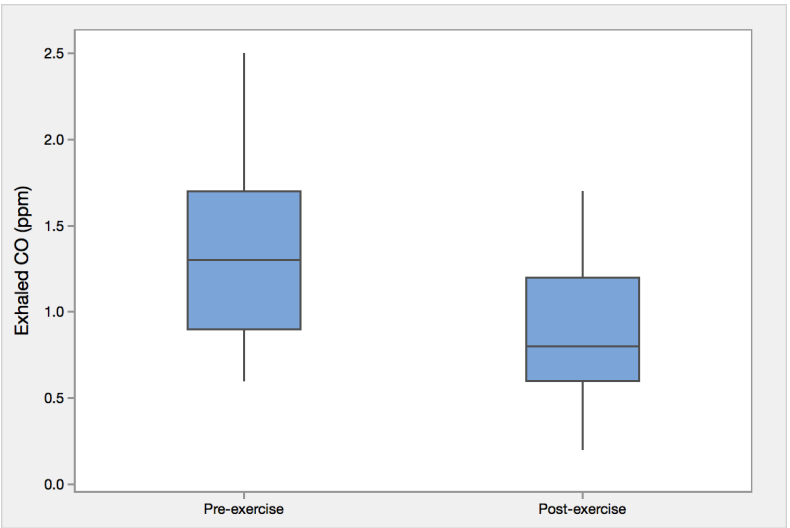


Figure 3.4: Box and whisker plots of exhaled carbon monoxide concentrations (parts per million) in 27 paired pre- and post-exercise exhaled breath samples. Exhaled CO concentrations were significantly lower post-exercise than pre-exercise (*P* = 0.013)

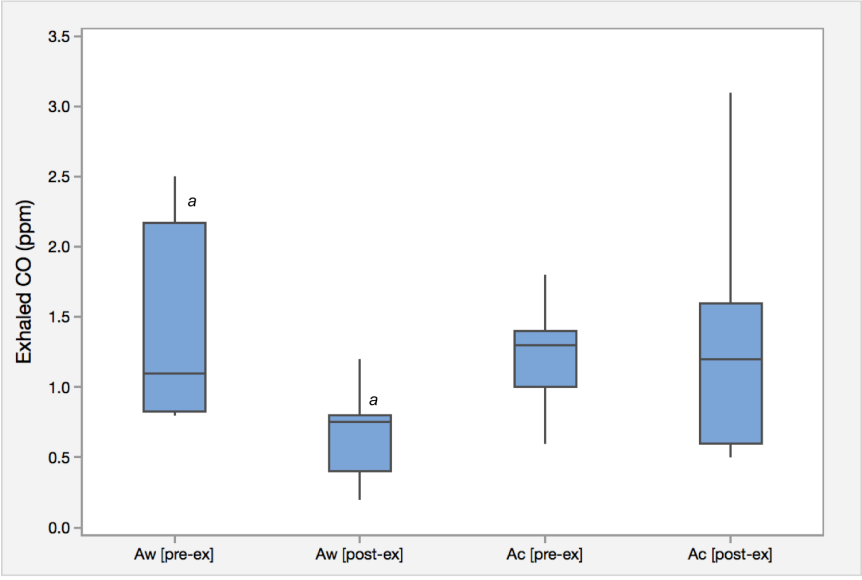


Figure 3.5: Box and whisker plots of exhaled carbon monoxide concentrations (parts per million) pre-exercise and post-exercise in warm (A_W) and cold (A_C) groups. The post-exercise reduction in eCO was only significant in the A_W group (*a*; *P* = 0.003)

Post-exercise, the mean pH of the neat EBC (4.79 ± 0.59) was significantly higher than pre-exercise (4.51 ± 0.23, *P* = 0.003; Fig 3.6). Following de-aeration with argon, mean pH pre-exercise (7.30 ± 0.36) and post-exercise (7.39 ± 0.47) were not significantly different (*P* = 0.24).

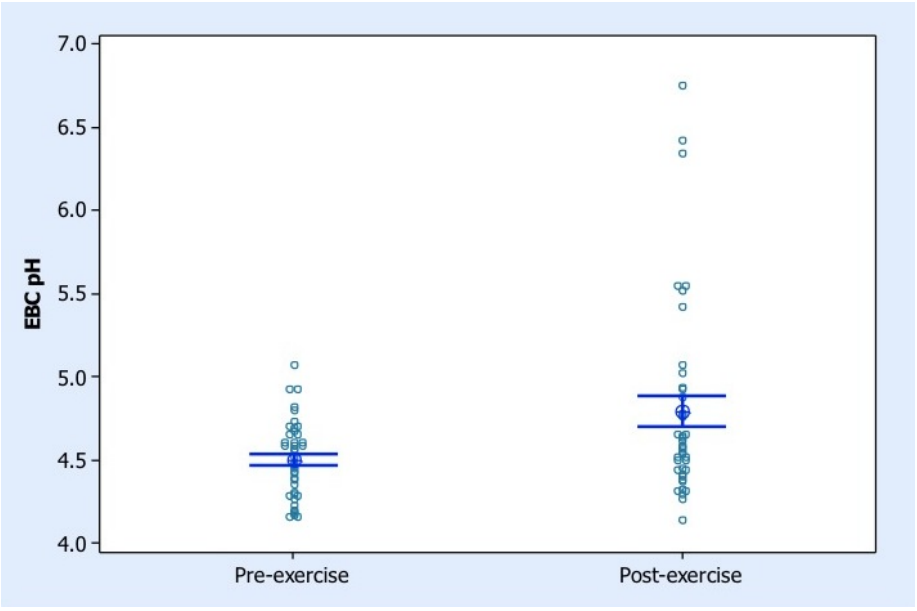


Figure 3.6: Interval plot of exhaled breath condensate pH (EBC pH) pre- and post-exercise. Individual measurements are represented by open circles (dark cirlce at the mean) with standard error interval bars. A significant increase in EBC pH post-exercise was observed (*P* = 0.003).

In relation to the difference in eCO pre- and post-exercise (COdiff; Table 3.1), univariate linear regression analysis identified inverse associations with both ambient temperature (P = 0.001, r^2 = 32.53%) and difference in resting RR (post-exercise value minus pre-exercise value; P = 0.003, r^2= 27.90%). When both these variables were considered together in the multivariable model, only the association between ambient temperature and decrease in eCO post-exercise remained significant (P = 0.023).

In relation to the difference in EBC pH pre- and post-exercise (EBC pHdiff; Table 3.2) an association with type of bedding was found. In the univariate analysis EBC pHdiff was significantly lower in horses housed in barn 2 compared to those in the reference barn (barn 1) (P = 0.016, r^2= 9.40%) and in horses bedded on paper compared to shavings (P = 0.041, r^2= 7.75%). Only horses in barn 2 were bedded on paper, therefore comparisons between barn 2 and the cumulative observations in all other barns equated to the comparison between bedding types. The mean EBC pHdiff in horses bedded on shavings was 0.40 ± 0.60 compared to -0.02 ± 0.44 in horses bedded on paper.

Outcome	Model	Variable	Regression coefficient	95% CI	*P* value
COdiff (ppm)	Univariate				
		Temp^{A}	-0.073	-0.114, -0.032	0.001
		rRRdiff	-0.155	-0.251, -0.059	0.003
	Multivariable				
		Temp^{A}	-0.080	-0.148, -0.012	0.023
		rRRdiff	-0.231	-0.545, 0.084	0.139

Table 3.1: General linear regression analysis with outcome set as difference in exhaled CO pre- and post-exercise (COdiff). Only ambient temperature (Temp^{A}) and the difference in resting respiratory rate post- and pre-exercise (rRRdiff) were significantly associated with the outcome in the univariate analysis with the latter no longer being significant in the multivariable model. Using this model, for each incremental increase in ambient temperature by 1 degree Celsius, the eCO decreased by 0.08 ppm post-exercise.

Outcome	Model	Variable	Category	Regression coefficient	95% CI	*P* value
EBC pHdiff	Univariate					
		Barn	1	REF		
			2	-0.562	-1.013, -0.112	0.016
			3	-0.413	-1.267, 0.441	0.333
			4	-0.138	-0.602, 0.325	0.549
			5	-0.566	-1.204, 0.073	0.081
		Bedding	Shavings	REF		
			Paper	-0.418	-0.819, -0.017	0.041

Table 3.2: Univariate linear regression analysis with outcome set as the difference in EBC pH post- and pre-exercise (EBC pHdiff). EBC pHdiff was significantly lower in horses in barn 2 and those bedded on shavings rather than paper. All horses that were bedded on paper were housed in barn 2 which negated the application of a multivariable model including both barn number and bedding type.

3.4 Discussion

In this study it was successfully demonstrated that both EB and EBC could be collected from Thoroughbred racehorses in a field setting, prior to and following strenuous exercise. No adverse responses or untoward events were recorded, with the process of collecting EB and EBC considered to be both non-invasive and non-influential on the horse's training regimen. In addition, this is the first study to demonstrate that CO can be measured in the EB of horses and significant changes in the concentration of eCO and the pH of EBC occur in response to exercise.

Carbon monoxide can be consistently measured in the EB of humans using electrochemical sensors, laser spectrophotometry or near-infrared analysers (Ryter and Sethi, 2007). Previous studies in horses failed to consistently detect eCO (Wyse et al. 2005a,b); however, an electrochemical analyser with a minimum detection limit of 1 ppm was used. The chemiluminescence analyser used in the current study had a detection limit of 0.25 ppm, and CO was detectable in 80% of the EB samples collected. The mean resting eCO concentration recorded in horses (1.33 ± 0.53 ppm) was comparable to that of healthy children (1.93 ± 0.27 ppm; Horvath et al. 1999) but lower than in healthy adults (3.61 ± 2.15 ppm; Deveci et al. 2004).

In response to high intensity exercise, eCO decreased significantly. Similarly, decreased concentrations of eCO have been identified post-exercise in healthy human athletes (Wyse et al. 2005a). CO is produced in tissues predominantly from the oxidation of haem by the constitutively expressed haem oxygenase-2 enzyme, and is bound largely with haemoglobin in blood (Kharitonov and Barnes, 2001). In health, the presence of CO in EB occurs due to the displacement of CO, by oxygen, from haemoglobin at the alveolar blood-gas interface, a process that is dependent on alveolar gaseous diffusion (Ryter and Sethi, 2007). Whilst any pathological process that reduces the effective alveolar surface or alveolar diffusive capacity may reduce eCO concentrations (Zavorsky, 2004), CO is also

produced locally by inducible haem oxygenase-1 enzyme during states of airway inflammation. As a result of this inducible production of CO, The concentration of eCO has been documented to increase in a number of pulmonary diseases in humans, including asthma (Horvath et al. 1998b), cystic fibrosis (Yasuda et al. 2011) and in smokers (Deveci et al. 2004).

The dynamics of eCO before, during and after exercise in smokers and non-smokers (Yasuda et al. 2011) and in children with cystic fibrosis versus normal controls (Horvath et al. 1999) have been investigated. Both studies found that the fraction of CO (F_ECO) in the EB decreased during exercise, but the total amount of exhaled CO per minute (V_{CO}) increased linearly during exercise, with greater increases in cystic fibrosis (Horvath et al. 1999) and smokers (Yasuda et al. 2011). Therefore, without adjustment for ventilation, the decrease in eCO measured in a single breath sample obtained post-exercise from horses in the current study may reflect dilution due to higher respiratory flow rates and increased airway volumes. Accordingly, the inverse association identified between the difference in resting RRs prior to sample collection (post-exercise value minus pre-exercise value) and change in eCO concentration could also indicate an effect of RR on eCO. Horses with a higher resting RR post-exercise, relative to the resting RR pre-exercise, were more likely to have a decrease in eCO concentration post-exercise.

In healthy humans, the concentration of eCO measured after hyperventilation decreased relative to that recorded during normoventilation (Cavaliere et al. 2009). However, in the current study RRs immediately prior to sampling post-exercise were within the normal reference range for resting RR in horses and were only mildly increased relative to the pre-exercise resting RR. Furthermore, in the studies by Horvath et al. (1999) and Yasuda et al. (2011), F_ECO and adjusted V_{CO} returned to base-line values within 5 min post-exercise, whereas samples in the current study were obtained 20 to 30 min post-exercise, reducing the likelihood that decreases in eCO were due solely to dilution from increased airflow.

In the multivariable regression analysis, only ambient temperature remained significant in relation to the decrease in eCO post-exercise; horses exercised in warmer weather were significantly more likely to have a decrease in eCO post-exercise (and larger eCOdiff), regardless of changes in resting RR. This finding is likely a direct effect of exercising in different conditions as no association was found between ambient temperature and individual pre-exercise eCO concentrations. This effect of ambient temperature on the eCO concentration in single breath samples has not been reported previously, and reasons for this finding are unknown. Horses breathing cold air during exercise have increased pro-inflammatory cytokine expression in BAL fluid 5 hours after exercise (Davis et al. 2005) and pro-inflammatory cytokines induce HO-1 expression (Kharitonov and Barnes, 2001). Therefore it is plausible that eCO production during exercise was greater in the A_C group compared to the A_W group, thereby absolving the theoretical physiological reduction observed in the A_W group.

The repeatability of eCO measurements using the sample collection technique and analytical methodology was not determined in the current study and has not been published previously. The within and between animal repeatability of methodologies used for determination of eCO are important considerations for the analytical and diagnostic performance of the technique. Further work is required to assess the repeatability and reproducibility of the methods and equipment available for quantification of eCO before this biomarker can be used in clinical settings.

Gas standardisation of EBC with argon is recommended by some authors to ensure reliable pH measurement (Vaughan et al. 2003), while others consider CO_2 as a relevant component of EBC and suggest the pH should be measured without gas standardisation (Leung et al. 2006). In the current study, the standard deviation of the de-aerated EBC pH was greater than that of the neat samples, indicating increased variance following gas-standardisation. These results are similar to the findings of Duz et al. (2009b), who documented excellent intra- and inter-animal repeatability of non-deaerated EBC pH in

horses. Borrill et al. (2006) demonstrated an unpredictable effect of gas standardisation of EBC with a pH < 6, which may explain this increased variance and lack of significant difference between pre- and post-exercise de-aerated EBC pH.

The pH of non-deaerated EBC samples increased significantly following exercise; this finding was unaffected by ambient temperature, humidity or respiratory rate. EBC pH has been found to increase after exercise in humans and is associated with alterations in exhaled volatiles, including increased ammonia and decreased propionic acid concentrations (Riediker and Danuser, 2007; Greenwald et al. 2009). With increased airflow velocities and pulmonary blood flow during exercise, production and delivery of solutes from the pulmonary lining fluid to EB may increase, altering EBC pH.

This increase in EBC pH post-exercise may represent a physiological response to exercise through increased systemic, and local pulmonary epithelial ammonia production. Ammonia, produced in the bronchial epithelium by glutaminase, is an important buffer of the airways (Hunt et al. 2002b) and expression of glutaminase, EB ammonia concentration and EBC pH are all decreased in asthmatic humans (Hunt et al. 2002b). The dynamics of airway ammonia production in horses are unknown, however, increased ambient ammonia concentrations were significantly associated with increases in exhaled ammonia concentration and EBC pH (Whittaker et al. 2009b).

In the current study, the univariate linear regression model indicated an influence of bedding type on the increase of EBC pH post-exercise.
The effects of stable and bedding type on changes in EBC pH with exercise are likely to be modest as r^2 values calculated in the current study were very low (< 10%). The impact of increased ambient ammonia concentrations on the equine airway epithelium, including glutaminase activity, and its association with lower airway inflammation remains to be established.

Nitric oxide was not consistently measured in EB samples, when using the methodologies described in this study. Studies that have documented eNO in the horse (Mills et al. 1996) and other mammalian species (Gustafsson et al. 1991; Lewandowski et al. 1998) used direct 'on-line' measurement of eNO in controlled ambient settings.

The results of this study suggest that 'off-line' analysis of NO from single EB samples obtained in a field setting does not have adequate sensitivity for detection of eNO.

A limitation of this study is the absence of objective data of the respiratory health of the horses used. Although no horse had clinical signs of respiratory disease at the time of sample collection, IAD does not always result in overt clinical signs at rest (Couëtil et al. 2007). Comparing exhaled biomarker concentrations, including changes in response to exercise, to cellular and molecular components of corresponding BAL samples would allow investigation of correlations between EB parameters and markers of LAI. Furthermore, whilst standardisation of the intensity of exercise undertaken by each horse would be desirable, this was not feasible in the current field study design. In future studies, quantification of the intensity of exercise could be attained by recording velocity and heart rate during exercise, which would facilitate investigations of associations between the degree of exercise and the magnitude of changes in the exhaled biomarkers.

Finally, ventilation characteristics (other than respiratory rates) were not recorded in this study. Recording inspiratory and expiratory flow rates, minute volume and total expired volume (via spirometry) would confer significant advantages in the analysis of the exhaled biomarkers by adjusting for changes in ventilation.

3.5 Conclusions

Exhaled breath and EBC can be collected from Thoroughbred racehorses in a field setting. The collection process was simple and well tolerated producing reliable samples of EB and EBC in each horse. Carbon monoxide was measurable in EB and concentrations decreased significantly following exercise, possibly due, in part, to dilution effects of increased respiration. The pH of EBC increased following high intensity exercise. Precise mechanisms for these changes cannot be determined from this study. Irrespective of whether physiological or pathological mechanisms are responsible, the findings of this study provide useful information that should be considered in future studies on EB/EBC in athletic animals.

Chapter 4: Association of exhaled biomarkers with respiratory secretion cytology

4.1 Introduction

The cytological analysis of respiratory secretions sampled from the lower airways is frequently employed in the diagnosis of equine pulmonary disease, and is considered essential in the diagnosis of IAD (see section 1.1.4). Evidence of inflammatory changes within BALF, in conjunction with documented pulmonary dysfunction, is considered the gold standard in the diagnosis of IAD in horses (Couëtil et al. 2007), with the former being more commonly adopted in the clinical setting. For the biochemical analysis of EB and EBC to have relevant clinical application in the investigation of IAD, it must first be tested against this current gold standard. When developing a new diagnostic test, three factors are of pivotal importance; first, an appropriate study population must be selected in which the test can be analysed. Second, the relation of the new test to current diagnostic tools should be investigated, before, finally, determining the diagnostic power of the test (including sensitivity, specificity, positive and negative predictive values), relative to the current gold standard (van der Schouw et al. 1995).

To date, there have been no veterinary studies investigating the relationships between exhaled biomarkers and the cytology of respiratory secretions. Human studies have identified an association between eNO and sputum eosinophilia in patients with asthma (Schleich et al. 2010; Hastie et al. 2013) and chronic obstructive pulmonary disease (Chou et al. 2014), with eNO found to be predictive of the degree of sputum eosinophilia in patients with COPD (Soter et al. 2013; Chou et al. 2014), but not in those with asthma (Hastie et al. 2013). EBC pH was inversely associated with both sputum eosinophils and neutrophils in asthmatic smokers, with EBC pH being a better predictor of sputum neutrophilia in comparison to eNO (Hillas et al. 2011). The aims of this study were to investigate whether any associations exist between eNO, eCO and EBC pH, and recognised markers of airway inflammation, namely; (1) endoscopic tracheal mucus scoring, (2) cytology of tracheal mucus, and (3) cytology of BALF, as they occur in poorly performing Thoroughbred racehorses.

4.2 Materials and Methods

4.2.1 Horses

A convenience sample of Thoroughbred racehorses, presenting with a history of poor performance, was obtained between February and July 2012. Cases were selected based on historical factors indicative of respiratory disease, i.e. cases presenting with poor performance and one or more of the following: coughing, nasal discharge, recent EIPH, previously documented respiratory infection or prolonged respiratory recovery from exercise, and where respiratory investigations (endoscopic examination and respiratory secretion sampling) were indicated for clinical diagnostic purposes. Horses were sampled either at the training facility, or at the Weipers Centre for Equine Welfare, dependent upon where the examination and consultation were scheduled.

Signalment, anamnesis (including time since last race and current level of exercise) and the results of a full clinical and respiratory examination were recorded for each case. Horses were classified with EIPH if the owner or trainer had noted epistaxis following exercise, within the last 30 days; no additional information regarding performance or previous treatment was recorded.

Consent was obtained from owners or their representatives prior to collection of EB and EBC from each case. The study was approved by the Ethics and Welfare committee, School of veterinary medicine, University of Glasgow.

4.2.2 Sample Collection

Exhaled breath and EBC samples were collected, as previously described, following initial clinical evaluation, and prior to any diagnostic investigations being conducted. Exhaled breath samples, and the corresponding samples of ambient air were stored in gas impermeable bags (Tedlar®) during transport to the laboratory. Exhaled breath condensate was collected over a 5-minute period, with the resultant condensate being stored in a frozen state within

airtight polypropylene containers during transportation. Respiratory rates prior to and during EBC collection were recorded using visual observation.

4.2.2.1 Endoscopy, tracheal aspirate and bronchoalveolar lavage

Following collection of EB and EBC, all horses were sedated with detomidine hydrochloride (Domidine®, 10mg/ml; Dechra Veterinary Products, Shrewsbury, UK) at 10-20 μg/kg, and butorphanol tartrate (Dolorex, 10mg/ml; MSD animal health, Milton Keynes, UK) at 10μg/kg, intravenously.
Endoscopic examination of the upper and lower respiratory tract was conducted with specific examination of the guttural pouches completed only in those cases with a history of epistaxis. Tracheal mucus scores (TMS) were recorded, as described by Dixon et al. (1995) (table 1.1), by the same observer in all horses.

Where indicated, trans-endoscopic tracheal aspirates (TA), collected for bacteriological analysis, were obtained via instillation and retrieval of 20ml of sterile saline at the level of the thoracic inlet. Where cytological analysis alone was required, disposable single lumen endoscopic catheters were used, whereas if a clinical indication for bacteriological culture of the tracheal aspirate was present, a triple lumen endoscopic catheter (Mila International Inc, Kentucky, USA) was used. At the end of the endoscopic examination, 20mls of local anaesthetic (lidocainehydrochloride; Lignol® 2.0% solution, Dechra Veterinary Products, Shrewsbury, UK) was instilled into the distal trachea and tracheal bifurcation prior to conducting the BAL procedure.

Bronchoalveolar lavage fluid was obtained from each horse via blind passage of a Large Animal Broncho-Alveolar Lavage Catheter (BAL)® (Mila International Inc, Kentucky, USA) to the level of the small bronchi, with subsequent lavage and retrieval of 300mls of sterile saline solution. Gross observations of the BALF sample, including percentage return, quality and colour of the sample, were recorded. All TA and BALF samples from each case were stored in sterile

ethylenediaminetetraacetic acid (EDTA) blood tubes for cellular preservation prior to submission to the laboratory for cytological analysis.

4.2.3 Sample Analysis

Exhaled breath samples and ambient air samples were analysed for nitric oxide and carbon monoxide as previously described (Chapter 2, Section 2.3.1). After thawing, the pH of neat EBC was measured as previously described (Chapter 2, Section 2.3.2), without de-aeration

4.2.3.1 Tracheal aspirate and BALF cytology

All TA and BALF samples obtained during the study were independently analysed by experienced clinical pathologists at the Veterinary Diagnostic Services, School of Veterinary Medicine, University of Glasgow. The clinical pathologists were blinded to the clinical information, the results of the endoscopic examinations, and the recorded values of exhaled biomarkers measured for each case. All samples were analysed within 24 hours of collection.

Fluid cell counts (red blood cell [RBC] and white blood cell [WBC] counts) and differential white cell counts (percentage of macrophages, lymphocytes, neutrophils, eosinophils, mast cells and haemosiderophages) were obtained manually via direct smear examination and cytological analysis of cytospin preparations, respectively. Estimated cell counts for each white cell population were calculated by applying the percentage differential count to the total WBC count for that sample.

4.2.4 Statistical Analysis

All numerical data were recorded and stored using Microsoft Excel®, with categorical data (gender [female or male] and bleeder [yes or no]) converted to binomial numerical data as appropriate. Data analysis was completed using

Minitab 16® statistical software. All data were tested for normality using the Anderson-Darling normality test.

With eNO, eCO and EBC pH set as the individual outcome variables, linear univariate regression analysis was used to identify any associations with explanatory variables, pertaining to both the patient (age, gender, time since last exercise, previous EIPH and respiratory rates prior to and during sampling), and to the respiratory secretion parameters (including TMS and the total RBC count, total WBC count and differential WBC counts of both TA and BALF). Significant univariate associations with *P*-value of < 0.2 were carried into a multivariable general regression model for each individual outcome.

Using set cut-off values for the differential white cell counts in the BALF, horses were further categorised based on the presence or absence of neutrophilic (≥5% neutrophils in BALF) or mast cell associated (≥2% mast cells in BALF) LAI. One-way ANOVA modelling with Fisher's exact test was used to investigate any associations between the outcome variables (eNO, eCO and EBC pH) and the presence of neutrophilic or mast cell-associated LAI.

4.3 Results

Twenty five Thoroughbred horses were sampled between February and July 2012. Twenty-one horses were examined at their home facility, with four travelling to the clinic for assessment. Geldings were over-represented (n = 20) with only 5 females sampled. The median age was 4-years-old (range 2-9). Eight of the horses (32%) presented with a history of recent EIPH, whilst nine (36%) presented with coughing as the primary problem associated with poor performance. Previous infection (n = 2), prolonged respiratory recovery following exercise (n = 2) and nasal discharge (n = 2) accounted for the remainder of the recorded respiratory problems associated with poor performance, whilst 3 cases returned non-specific histories (Fig 4.1).

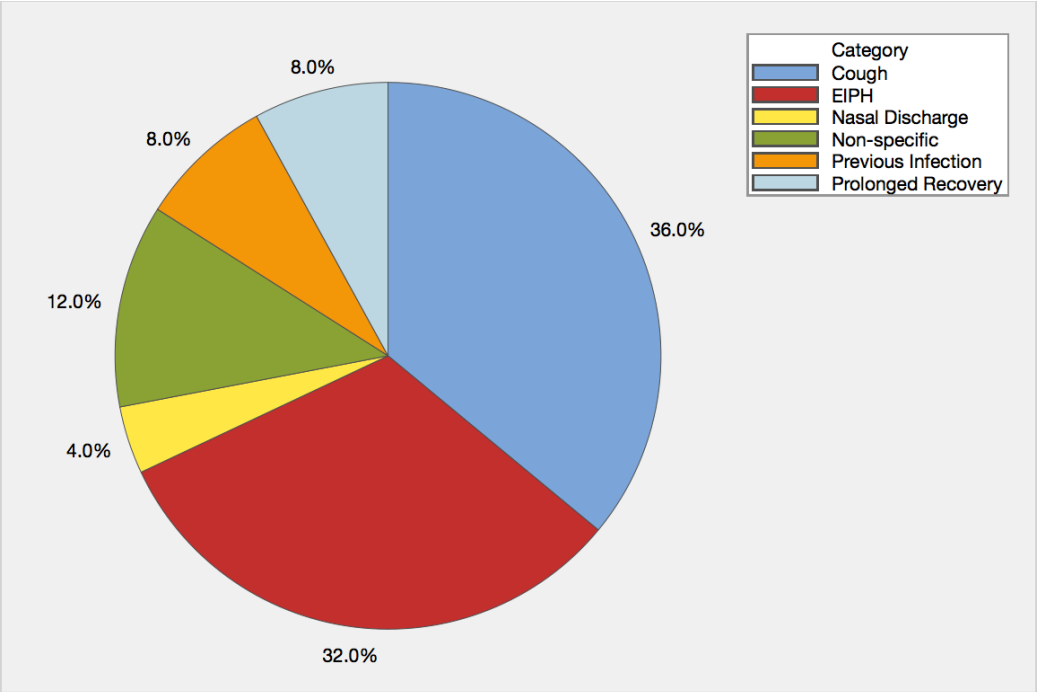


Figure 4.1: Percentage representation of different primary presenting complaints associated with poor performance in 25 Thoroughbred racehorses

Exhaled breath and EBC samples were successfully collected from all horses. The median respiratory rates prior to, and during sample collection were 14 breaths/min (IQR 12 - 16) and 12 breaths/min (IQR 10-14), respectively.

Trans-endoscopic tracheal mucus scoring was recorded in 24 horses, with a normal distribution of scores returned (Fig 4.2). No horses had a TMS of 4 or above. Tracheal aspirates were obtained in 8 cases (all with coughing as the primary presenting problem). Bronchoalveolar lavage fluid was successfully obtained in all horses, with a mean percentage return of 60.2% ± 11.8.

Exhaled NO was detectable in only 4/25 (16%) of samples, and was excluded from further analysis due to the consequent lack of statistical power.

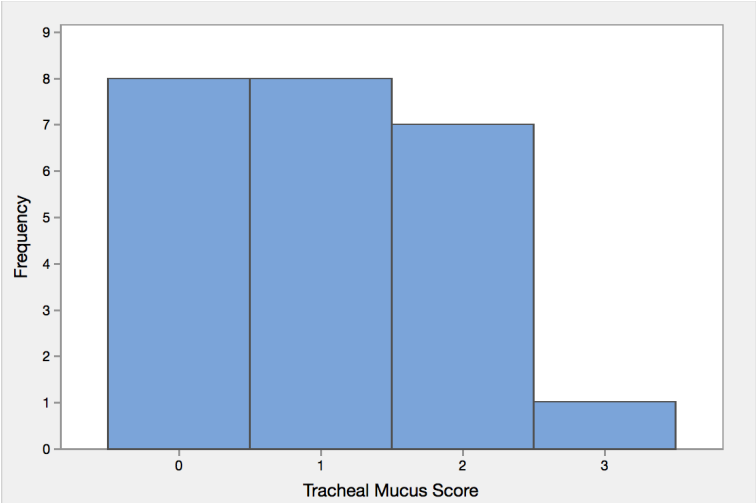


Figure 4.2: Distribution of tracheal mucus scores in 24 Thoroughbred horses presented for assessment of poor performance

Exhaled CO was detected in 16/25 samples with a median of 1.0ppm (IQR 0.5 – 2.9). One eCO reading returned a value of 11.3ppm, which was treated as an outlier and removed from further analysis. This sample point corresponded to the only horse with a TMS grade of 3. Significant associations between eCO and tracheal mucus score (P = 0.031), and eCO and TA WBC count (P = 0.03; Fig 4.4a) were identified in the univariate general regression analysis, with TA neutrophil count (TA #neutro; Fig 4.4b) and respiratory rate prior to sampling (RR pre) returning weaker associations with eCO, but with P-values of < 0.2 (Table 4.1). The association with eCO and RR pre was an inverse linear relationship (Fig 4.4c)

Using one-way ANOVA, the association between eCO and TMS (grades 0,1 and 2) was not significant, however; when grades 0 and 1 were combined, they were significantly different to grade 2 (P = 0.0476, Fig 4.3). Using this binary modelling of TMS in the univariate regression analysis, the same linear association was identified, which was subsequently carried forward into the multivariable regression model.

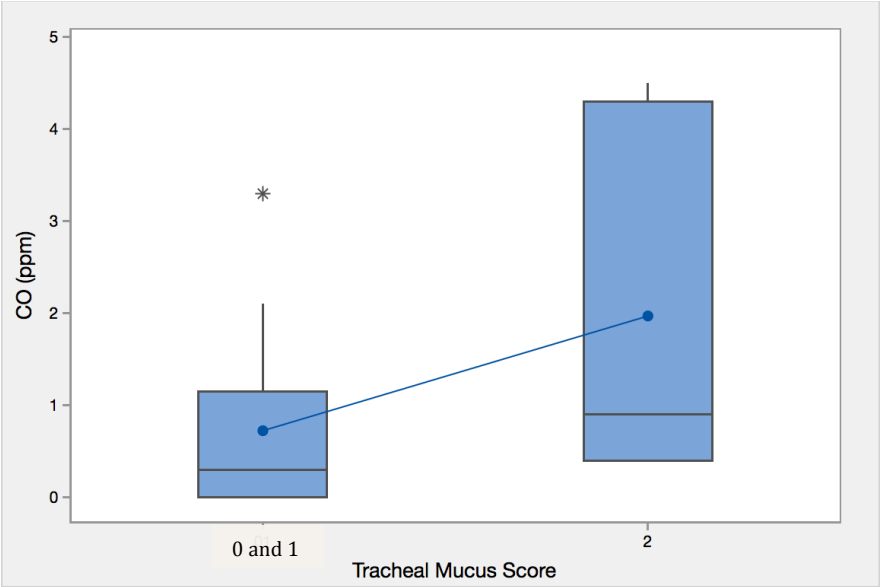


Figure 4.3: Box and whisker plot of exhaled CO (ppm) against tracheal mucus score (TMS). Horses with TMS of 2 combined had significantly higher exhaled CO compared to those with TMS of 0 and 1 combined (p = 0.0476). Boxes represent median and inter-quartile range, dots represent mean

The associations between eCO and both the TA WBC count and TA #neutro were based on 8 sample points for TA collection (Fig 4.4 a&b), with one outlier of TA #neutro visibly skewing the results. Due to this small sample size, the TA parameters were not carried forward into the multivariable regression model.

In the final multivariable regression model for eCO, following removal of unusual observations, the association with TMS remained significant (P = 0.019, Table 4.1) with the association between eCO and respiratory rate prior to sample collection loosing significance (P = 0.117).

No significant differences in eCO were identified when horses were categorised as having neutrophilic or mast cell-associated LAI.

The EBC pH (non de-aerated) was measured in all samples with a median of 5.12 (IQR 4.65 – 5.82) and a mean of 5.29 ± 0.69. In the univariate general regression model (Table 4.1 and Figure 4.5), significant direct associations were identified between EBC pH and both the respiratory rate prior to sample collection (P < 0.0001) and the respiratory rate during sample collection (P < 0.0001; Fig 4.5a).

RR pre and RR during were found to be strongly associated with each other (r= 1.03 ± 0.09; R-Sq(adj) 82.63%, P <0.0001), therefore, respiratory rate during sample collection was carried forward into the multivariable general regression model. The percentage of neutrophils (% neutro) in the BALF was directly associated with EBC pH (P = 0.034), a relationship which strengthened when a BALF neutrophil outlier value (50% neutrophils in BALF) was removed (P = 0.0032; Fig 4.5b). An inverse association between EBC pH and the percentage of mast cells (% mast cell) in the BALF was also identified (P = 0.031; Fig 4.5c). In the final multivariable regression model for EBC pH (Table 4.1), following removal of unusual observations, only the association with respiratory rate during sample collection remained significant (P < 0.0001) whilst the association between EBC pH and both the percentage of neutrophils (P < 0.190) and percentage of mast cells (P = 0.245) in the BALF lost significance.

Outcome	Model	Variable	Regression Coefficient (r)	Standard error coefficient	P value
eCO (ppm)	Univariate				
		RR pre	-0.190	0.098	0.066
		TA WBC	0.757	0.253	0.03
		TA #neutro	1.457	0.428	0.055
		TMS	0.769	0.332	0.031
		TMS 01 v 2	1.246	0.592	0.0476
	Multivariable				
		TMS 01 v 2	0.533	0.196	0.019
		RR pre	0.059	0.034	0.117
EBC pH	Univariate				
		RR pre	0.188	0.033	<0.0001
		RR during	0.204	0.039	<0.0001
		BALF %neutro	0.026	0.011	0.034
		BALF %mast	-0.189	0.082	0.031
	Multivariable				
		RR during	0.121	0.021	<0.0001
		BALF %neutro	0.044	0.005	0.190
		BALF %mast	-0.037	0.031	0.245

Table 4.1: General linear regression analysis with outcome set as exhaled CO (ppm) and EBC pH.

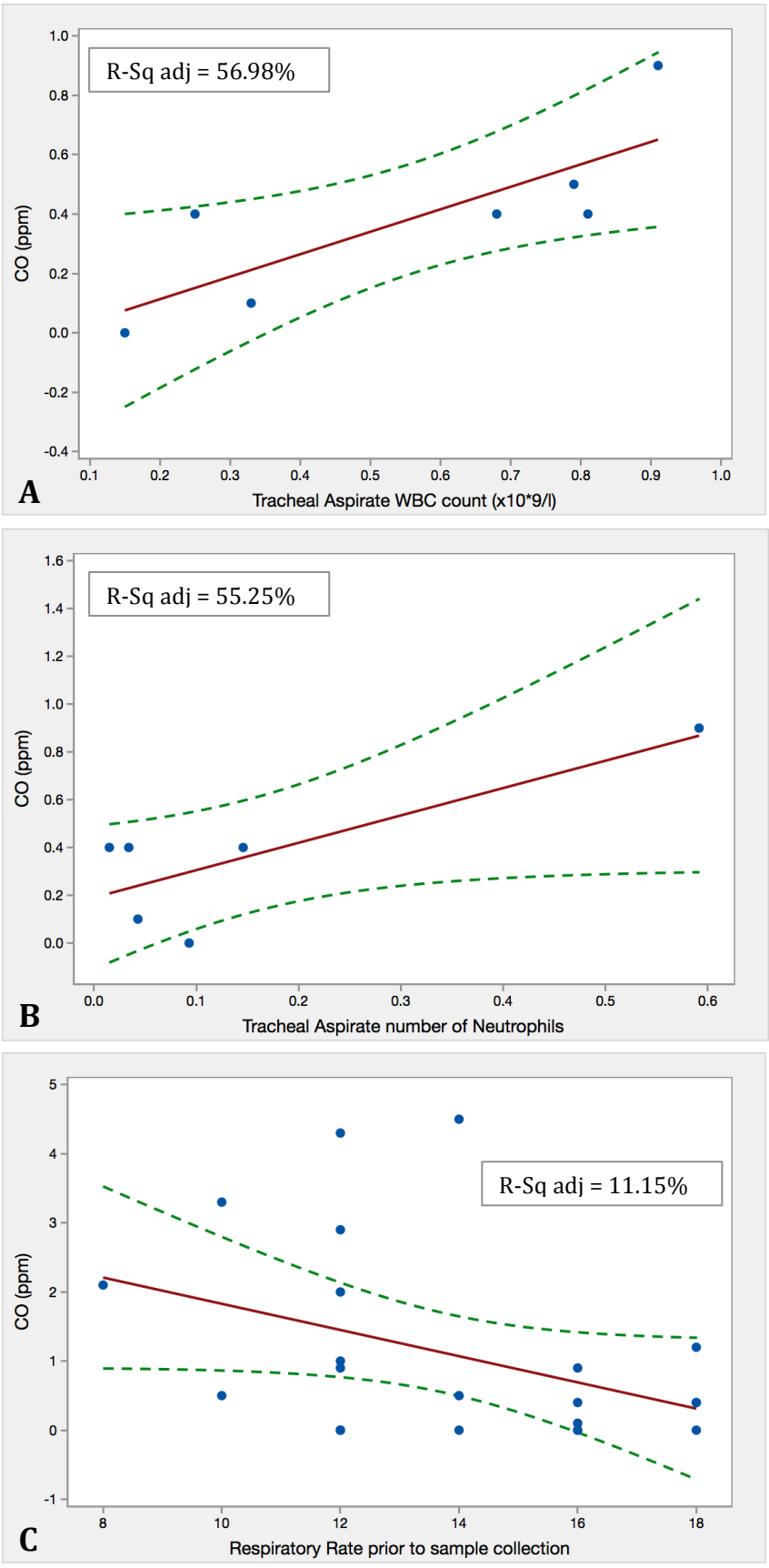
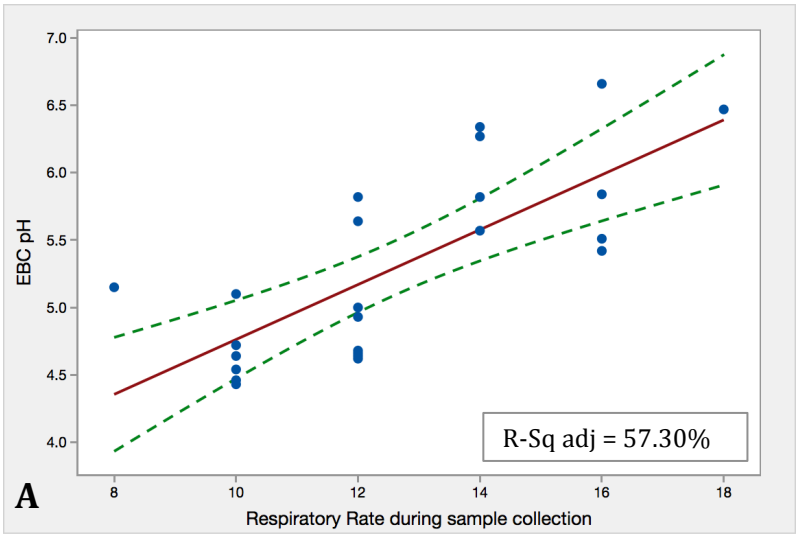
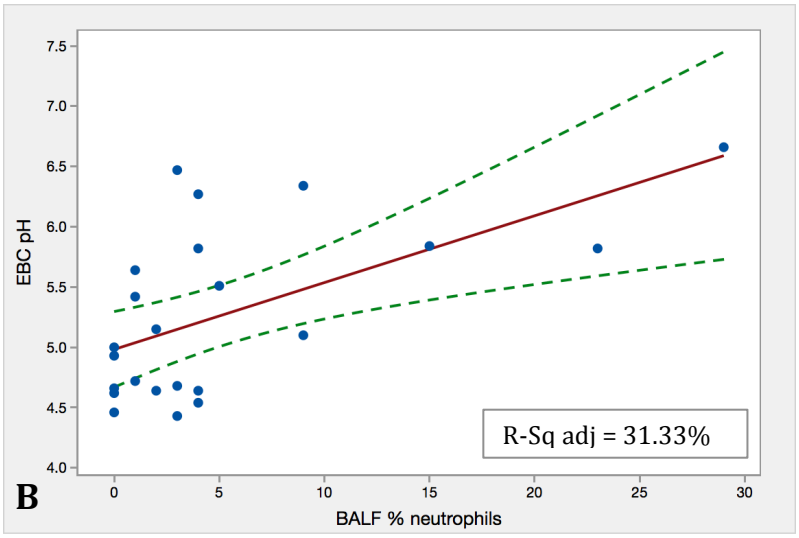


Figure 4.4: General linear regression models of exhaled CO versus tracheal aspirate WBC count (A), tracheal aspirate neutrophil count (B) and respiratory rate prior to sample collection (C). Blue dots = data points; red line = fitted regression line; green dashed line = 95% confidence intervals





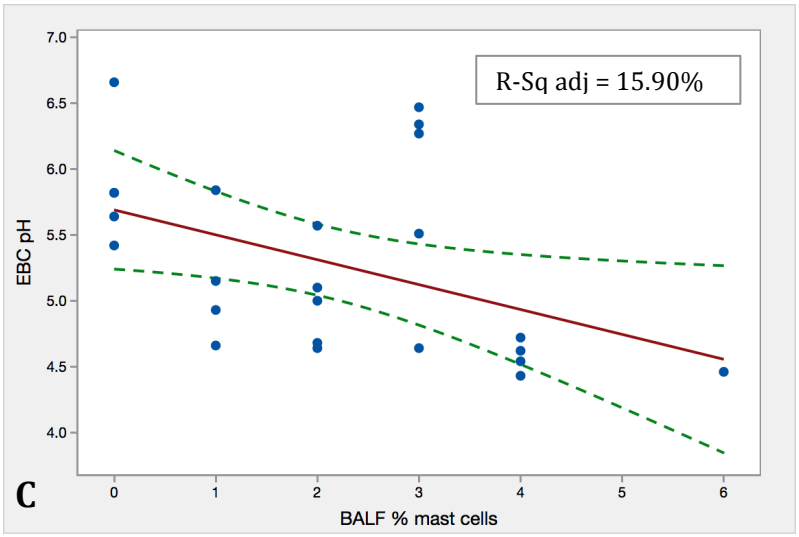


Figure 4.5: General linear regression models of EBC pH versus respiratory rate prior to sample collection (A), BALF neutrophil percentage (B, outlier removed) and BALF mast cell percentage (C). Blue dots = data points; red line = fitted regression line; green dashed line = 95% confidence intervals

A significant increase in EBC pH was identified in horses with neutrophilic LAI (5.83 ± 0.52) compared to those without a neutrophilic response in the BALF (5.06 ± 0.63, *P* = 0.01; Figure 4.6). No difference in EBC pH was found when the presence or absence of mast cell-associated LAI was compared.

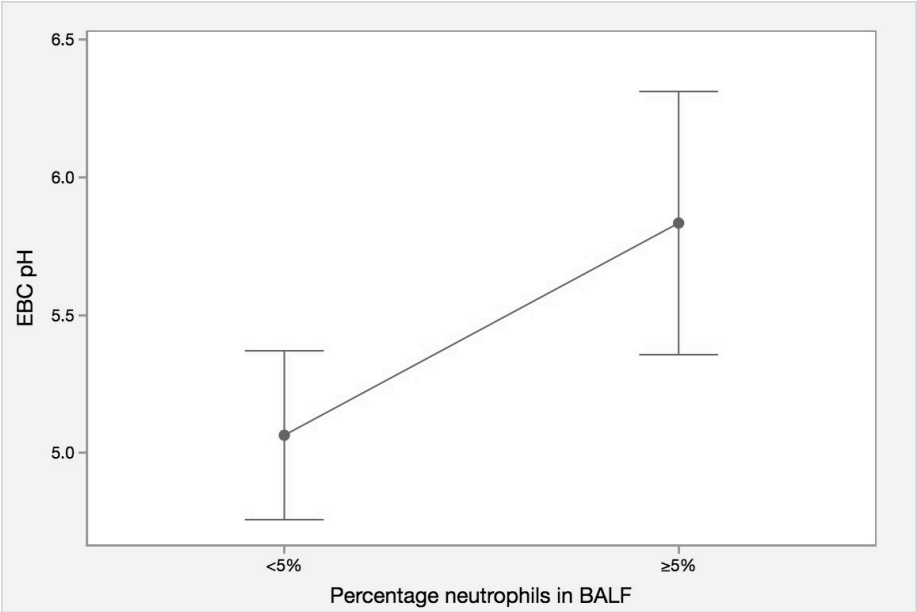


Figure 4.6: Interval plot (mean and standard error of the mean) of EBC pH in horses with (≥5%) and without (<5%) neutrophilic LAI. A significant increase in EBC pH in horses with neutrophilic LAI was identified (*p* = 0.01)

4.4 Discussion

This is the first study in veterinary medicine to investigate the relationship between exhaled biomarkers and established indices of LAI in horses. Thoroughbred racehorses were selected due to the high prevalence or IAD identified in these horses (Wood et al. 2005b), with a relevant study population created by specifically recruiting poor performance cases that had a reported history of respiratory disease.

Whilst this selection process inherently created bias, the study was not designed to investigate the diagnostic accuracy of measuring exhaled biomarkers to diagnose, or rule out, lower airway inflammation, nor was it intended to specifically investigate and compare affected versus unaffected animals.

Eighty percent (20/25) of the sampled horses were diagnosed with IAD based on the presence of one or more abnormality in the BALF differential nucleated cell counts (>5% neutrophils; ≥ 2% mast cells; >0.1% eosinophils). This represents a high prevalence of IAD in this very specific population of horses, which is undoubtedly an over-representation of this condition in the general population of Thoroughbred racehorses, associated with the selection bias. It does, however, highlight the importance of this condition in those racehorses that are performing poorly with associated signs of respiratory disease. The age demographic of the study population also highlights that IAD is not restricted to young (2 or 3-year-old) racehorses.

Of the 5 horses that did not have IAD based on BALF cytology, all had a tracheal mucus score of 0 or 1; therefore, it is likely that these horses were under performing due to conditions unrelated to the lower respiratory tract. It should be noted, however, that no tracheal aspirate cytology was recorded in any of these 5 horses, and consequently, exclusion of lower airway disease is not possible.

The poor return of EB samples with eNO concentrations above the detection limit of the analyser in this study (16%) is consistent with the results obtained in the study of the effects of exercise on EB biomarkers (chapter 3), where eNO was detected in only 19% of the resting EB samples. This result further supports that, using the specific collection and analytical methodology employed in these studies, eNO is not of value in the investigation of LAI in horses.

Exhaled breath condensate pH readings were obtained from all horses, with a mean value of 5.29 ± 0.69, which was higher than that previously recorded in Thoroughbreds prior to exercise (4.51 ± 0.23; Chapter 3). The strongest association of a recorded independent variable to EBC pH was the respiratory rate of the horse during sample collection. A strong positive linear relationship was identified whereby with each single unit increase in respiratory rate during sample collection, the EBC pH increased by 0.2 ± 0.04. This association was also the only one to remain significant in the multivariable model, indicating that

patient respiratory variables have a greater influence on the EBC pH than the cytological profile of the BALF.

Taken individually, the univariate linear associations between EBC pH and the percentage of neutrophils and eosinophils would suggest that varying states of LAI might influence the acid-base balance of the epithelial lining fluid. This is further supported by the significant increase in EBC pH in those horses classed as having neutrophilic LAI in comparison to those without.

Changes in EBC pH in relation to specific cytological changes consistent with LAI in any veterinary species have not been documented previously. In human medicine, changes in EBC pH have been identified in association with neutrophilic (and eosinophilic) cytological profiles of induced sputum taken from asthmatic patients (Hillas et al. 2011), however; direct comparison between the two studies cannot be drawn due to significant differences in EBC collection technique, and in the source of the respiratory secretions analysed. It was unfortunate that a greater number of tracheal aspirate samples were not obtained in the current study, as it is probable that the low sample number reduced the significance of the associations between EBC pH (or eCO) and the different cytological parameters of the TA samples.

The results of this study contradict the vast body of evidence in human literature that documents a decrease in EBC pH (airway acidification) in the face of pulmonary inflammation (Hunt et al. 2000; Kostikas et al. 2002; Tate et al. 2002; Gessner et al. 2003). If the positive relationship between BALF neutrophilia and EBC pH were to be an accurate association, it would reflect a significant difference in the manner in which the acid-base homeostasis of the lower airways became disrupted in horses with airway inflammation, in comparison to people. Differences in cytokine expression at the alveolar level, granulocyte acid production or an enhanced buffering capacity within the inflamed airways of horses may, in theory, explain this discrepancy, but this cannot be supported by the results of this study.

The significant association between respiratory rate and EBC pH confounds the interpretation of the association between EBC pH and BALF neutrophil percentage. Until the respiratory dynamics of the horse during sample collection are determined, or the results are standardised against known ventilatory parameters, no accurate conclusions in relation to EBC pH and respiratory secretion cytology can be drawn.

Exhaled CO was more consistently measured in the EB of the horses in comparison to NO. The median value of eCO recorded in this study (1.0ppm; IQR 0.5 – 2.9) was similar to that obtained in Thoroughbred racehorses prior to exercise (1.3 ppm, IQR 0.9-1.7; Chapter 3). The identification of an association between eCO and the tracheal mucus score represents the first documented association between eCO and a known marker of airway inflammation in any species.

Tracheal mucus scoring has been widely studied in relation to inflammatory conditions of the equine respiratory tract, including RAO (Robinson 2003) and IAD (Couëtil and DeNicola 1999), and the presence of a TMS of greater than 1 has been associated with pulmonary disease (Dixon et al. 1995). Increased accumulations of tracheal mucus have also been associated with increased concentrations of local airborne particulate matter in Thoroughbred racehorses (Millerick-May et al. 2013).

In the current study, horses with a TMS of 0 and 1 were grouped together and compared to those with a TMS of 2. This form of categorisation is not without precedent; a TMS of 0 represents no mucus accumulation in the tracheobronchial tree, but a TMS of 1, representing a mild accumulation of individual mucus flecks, is also considered a normal variant in horses (Dixon et al. 1995). Furthermore, in a wide-scale study of Thoroughbred racehorses in the USA, where horses with a TMS of 0 or 1 were grouped together as normal, a TMS of greater than or equal to 2 was associated with poor racing performance (Holcombe at al. 2006).

Accumulation of mucus in the trachea has been identified in horses immediately post exercise (Burrell et al. 1985) and is recognised in association with recent long distant transport due to persistent elevation of the head restricting normal mucociliary clearance (Raidal et al. 1995). In the current study, all endoscopic examinations and sampling were undertaken at rest. Following transportation, the horses that travelled to the place of examination were given sufficient rest time in a loose box prior to endoscopic examination.

Although no significant associations were identified between the TMS and the different percentage or relative numbers of inflammatory cells in the TA or BALF in this current study, TMS has previously been correlated with the percentage of neutrophils in both the TA and BALF samples of horses (Gerber et al. 2004). Holcombe et al. (2006) also identified significant associations between TMS and absolute numbers of neutrophils, macrophages and lymphocytes in the tracheal mucus of horses.

This published evidence of TMS being correlated with neutrophilic inflammation of the equine respiratory tract allows for some inferences to be drawn as to the potential influence that increased numbers of inflammatory cells in the airways may have on the concentration of CO in EB. That is, if the higher tracheal mucus scores recorded in this study were, in accordance with previously published data, representative of increased numbers of neutrophils in the airways, then an association may exist between neutrophil activity and increased eCO.

Exhaled CO has been postulated as a marker of pulmonary oxidative stress (Kharitonov and Barnes 2001) through the activity of the inducible form of the haem-oxygenase enzyme (HO-1) in pulmonary disease states. This association between inflammation, increased HO-1 activity and increased eCO has been documented in human asthmatic patients (Horvath et al. 1998a). The activity and expression of HO-1 has been documented in neutrophils, macrophages and alveolar type II epithelial cells of healthy equine lung (Wyse et al. 2006;

Rendle et al. 2010), however no equine studies have investigated the expression of HO-1 in states of airway inflammation, nor have any associations between HO-1 and eCO been documented in equidae.

Indirectly, by demonstrating an association between airway inflammation (as evidenced by TMS) and eCO in horses with respiratory disease, this study has gone some way to further establish a link between airway inflammation and increased CO in exhaled breath. Whether this is due to increased expression of HO-1 remains to be established, however, the results indicate that eCO may be a valid and useful biomarker as a non-specific marker of airway inflammation in horses.

4.5 Conclusions

In conclusion, the results of this study indicate that eCO may serve as a non-invasive biomarker for the detection of LAI in Thoroughbred horses. Exhaled CO was directly associated with tracheal mucus accumulation and did not seem to be affected by individual horse or specific respiratory variables.

EBC pH was primarily influenced by the respiratory rate of the horse during sample collection and therefore cannot, at this stage, be recommended for use as a biomarker for airway inflammation. Further work is required where the respiratory dynamics of the horse during sample collection are measured such that the EBC pH results can be standardised against known ventilator parameters, before a true association between EBC pH and cytological markers of airway inflammation can be established. Finally, this study confirms that using off-line measurement of eNO is not a valid means by which to assay eNO in horses.

Chapter 5: General Discussion and Conclusions

5.1 Collection of EB and EBC in Thoroughbred racehorses

The initial aim of this study was to develop and modify the methodologies employed for the collection of EB and EBC, for the specific application in Thoroughbred racehorses, thereby facilitating the completion of the subsequent study objectives. A precedent existed in relation to the collection of EB from Thoroughbred horses, before and after exercise (Wyse et al. 2005a), but this was to be the first documented use of EBC collection, in a field setting, in this population of horses.

Through considerable modification of pre-existing collection devices used at the University of Glasgow, the author was able to demonstrate that EB and EBC can be collected easily and safely from Thoroughbred racehorses, and without any adverse effects on the daily training regimens. The collection of single EB samples is simple and, other than the necessity for impervious tedlar bags, requires minimal equipment.

In relation to the collection of EBC, modifications were required to reduce the resistance to airflow initially encountered when using pre-existing systems, and to make the collection systems safer and more portable for use in a field setting. Modification of the device employed by Duz et al. (2009b), through the use of two inert polypropylene 35mm tubing conduits and 400ml polypropylene beakers in parallel in the condensing chamber, resulted in negligable resistance to airflow, as documented by the lack of significant differences between respiratory rates prior to and during EBC sample collection. The use of dry ice as the condensing medium negated any of the health and safety concerns that are inherent in the sourcing, handling, transport and disposal of liquid nitrogen. Whilst the sublimation of frozen CO_2 could have an influence on EBC pH, the design of the condensing unit, supported by the documentation that dry ice does not influence the EBC pH relative to that obtained when using liquid nitrogen/ethanol slurry (Whittaker et al. (2012), negated this concern. Dry ice is readily accessible to veterinary health care professionals, and should be considered an appropriate alternative to liquid nitrogen.

Some limitations relating to the EBC collection system were encountered during the studies, which are relevant to its application to clinical veterinary medicine. Primarily, whilst the system was portable (it could be carried on the shoulder of the investigator during EBC collection), the total amount of equipment required to obtain the EBC samples was, at times, cumbersome and increased relative to the number of horses being sampled on a single day. To ensure that there was no cross-contamination between samples, it was necessary to use two clean, dried polypropylene conduit tubes and two 400ml beakers for each sample. Refining this system to allow easy and quick cleaning of the contact surfaces between samples would facilitate ease of use in a field environment. Also, the transferal of the frozen EBC collected within both 400ml beakers into the 50ml universal tube, as required for transportation to the laboratory, was a limiting factor in maximizing the amount of EBC obtained. Retaining the EBC within the primary condensing chamber would confer obvious advantages.

Across all of the studies described within this thesis, 109 EB samples and 139 EBC samples were obtained from 70 horses, with no adverse effects documented (or subsequently reported) in any of the horses sampled. Only one horse (located at the racing yard visited in the study described within chapter 2) resented the placement of the facemask to a degree that resulted in that horse being excluded from the study. Therefore, irrespective of nuances pertaining to the refinement of the EBC chamber, it can be concluded, that the collection of EB and EBC in Thoroughbred racehorses is a safe, non-invasive procedure, which is amenable to repeat sample collection, within single, or on consecutive days.

These findings support the findings of previous investigators (Wyse et al. 2004a; Duz et al. 2009b; Whittaker et al. 2009b) in highlighting the potential appeal of EB and EBC analysis in the investigation of IAD in horses, where the current gold standard for diagnosis is the collection of BAL fluid; an invasive procedure that is not only associated with more distress on the animal (to the point where sedation is a prerequisite for sample collection) but which also is inappropriate for short-term serial monitoring as the procedure itself induces (focal) lower airway inflammation (Sweeney et al. 1994).

5.2 Expired NO and CO as biomarkers for lower airway inflammation

A common finding across all the studies described within this thesis was the inconsistent measurement of nitric oxide in the exhaled breath of the horses. Of the 109 EB samples obtained, eNO was above the detection limit (1 part per billion) for the chemiluminescence gas analyser in only 23 samples (21.1%). This poor return of eNO readings consistently negated any meaningful statistical interpretation of the presence of NO in the expired breath and, consequently, no inferences can be drawn as to the suitably of eNO as a biomarker for IAD in Thoroughbred racehorses.

The only other study to investigate the presence of NO in the exhaled breath of horses sampled the EB at set flow-rates, collected larger volumes of EB (50 litres) over a set period of time and used a chemiluminescence analyser with a detection limit of 0.25ppb (Mills et al. 1996). Using this methodology, the investigators recorded eNO concentrations of 0.89 ± 0.3 ppb in horses at rest, which would have been below the detection limit of the analyser used in the current studies, and significantly higher concentrations of eNO during exercise (7.0 ± 0.4 ppb). Studies of eNO in other mammalian species have employed similar 'on-line' collection systems and used analysers with lower detection limits (Gustafsson et al. 1991), some being sensitive enough to detect eNO at concentrations of <50 parts per trillion (Lewandowski et al. 1998).

Roller et al. (2007) utilised off-line measurement of eNO in calves and, using laser absorption spectroscopy, documented concentrations of eNO in the magnitude of 0.3ppb. Importantly, these investigators employed two methodologies outlined within the American Thoracic Society's recommendations for the off-line measurement of eNO (Silkoff et al. 2005); the fractionation of the exhaled breath to exclude environmental 'dead-space' air, thereby sampling only the EB from the lower airways, and the use of a NO-scrubbing filter in the inspiratory arm of the collection device to remove contamination of EB with inspired NO. This later consideration is particularly

worthy of note, given that the eNO results returned in the current study were frequently below the detection limit of the analyser due to the NO concentration of the ambient air being greater than that of the corresponding EB sample. Roller et al. (2007) suggest that mechanical equipment that burn fossil fuels (e.g. tractors) would contribute significantly to the ambient NO concentration, which may have been a particular influence in the study described in Chapter 3 of the thesis, where tractors were used regularly within the barns housing the horses as part of the daily cleaning regime.

Ultimately, the results of the current study lead unequivocally to the conclusion that the off-line collection of EB and the methodology of eNO analysis employed were not appropriate for the detection of eNO in Thoroughbred racehorses. Until suitable devices are developed where the airflow of the EB can be quantified (or controlled), and the EB arising from the lower airways can be isolated and fractioned off from the dead-space air, the analysis of eNO will be restricted to on-line measurement using chemiluminescent analysers with a detection limit of less than 1 ppb.

The results of the study in relation to eCO are, however, more encouraging. Indeed this is the first study to successfully document that, not only can eCO be consistently detected in the EB of horses, but that significant changes in eCO occur in response to exercise and in relation to recognised markers inflammation in horses with lower airway disease.

Previous studies into the dynamics of eCO in relation to exercise and LAI in veterinary species are sparse. Using a portable electrochemical CO sensor attached to the outlet port of a non-rebreathing facemask, Wyse et al. (2005a) could only identify eCO (at concentrations of 1.0 ppm) in 3 of 11 horses at rest, and failed to identify CO in the EB of the same population of horses following exercise. The authors cited insufficient sensitivity of the electrochemical sensor and inability to employ specific respiratory manoeuvres (i.e. breath holding) in horses as explanations for the poor return of eCO (Wyse et al. 2005a). This is in spite of consistent evidence that HO-1 expression occurs in alveolar epithelial

cells and inflammatory cells found in the equine respiratory tract (Wyse et al. 2006; Rendle et al. 2010), documented evidence of increased eCO being associated with HO-1 expression in human asthmatic patients (Horvath et al. 1998a) and the demonstration of increased eCO in humans in response to exercise (Yasuda et al. 2001).

Via the collection of a single sample of exhaled breath, off-line, and measuring CO via a chemiluminescence analyser with a lower detection limit of 0.25ppm the author was able to measure eCO more consistently than previous studies, and has documented an association between eCO and tracheal mucus score; a well-established marker of LAI. This provides support for the use of eCO as a biomarker for airway inflammation, as it is in human medicine (Ryter and Sethi, 2007). The direct association between eCO and the TMS, and the significant association between eCO and the WBC count, and neutrophil percentage, of the tracheal aspirate identified in the univariate analysis provide evidence that eCO increases in horses with LAI. As previously mentioned, conducting a study where larger numbers of tracheal aspirates were obtained may reveal associations between eCO and individual cytological parameters. The postulate that this increase in eCO is due to increased expression of HO-1 from the inflammatory cells present is yet to be fully elucidated in horses, but extrapolation of data from human medicine would support this causality.

Whilst eCO was identified in 78% (82/106) of all the EB samples collected throughout the studies, supporting the use of the chemiluminescence analyser in the off-line measurement of eCO relative to other analytical methodologies, 22% of the samples returned null eCO concentrations, limiting the applicability of this method as a diagnostic tool in clinical settings. This suboptimal return of eCO measurements may relate directly to the EB collection method, in particular, the reliance upon a single breath sample for measuring eCO, and the imprecise method utilised to account for inhaled, ambient CO. As such, developing a collection system where exhaled breath could be partitioned, where a series of breaths obtained at one sample time point, and which has filters in place to absorb or remove inhaled, ambient CO, would undoubtedly

improve the sensitivity for detecting the low concentrations of CO that exist in the EB of horses.

Importantly, both studies showed statistical evidence of decreasing eCO in association with increased RR prior to sample collection, not only following exercise, but also at rest. Whilst this inverse association was not identified as the primary explanatory variable for eCO in either study (with ambient temperature during exercise and tracheal mucus score each maintaining significance over RR in the multivariable analysis), the influence of respiratory rate cannot be overlooked. As discussed in chapter 3, studies in which an increase in eCO in association with exercise were identified in human subjects, employed continuous collection of EB during exercise and the measurement of respiratory dynamics of the subject during sample collection (Horvath et al. 1999; Yasuda et al. 2011). These studies clearly demonstrated that the *fraction* of CO exhaled per individual breath decreased at higher respiratory rates and tidal volumes, which was attributed to these changes in respiratory dynamics having a dilutional effect on eCO within a single expired breath. This reflects the findings of the current studies, and provides support to the requirement for continuous EB samples and recording of ventilation parameters during sample collection.

On the proviso that such a system could be developed for use in horses, the current studies provide good evidence supporting the utilisation of eCO as a biomarker for LAI.

5.3 EBC pH as a biomarker for lower airway inflammation

With features including small intra- and inter-subject coefficients of variation, good repeatability and minimal influence attributed to physiological variables, EBC pH is presented in the literature as a robust biomarker for LAI (Vaughan et al. 2003; McCafferty et al. 2004; Borrill et al. 2005; Duz et al. 2009a). However, in the current study, the most significance influences on EBC pH were those

attributable to physiological (recent exercise and respiratory rate) and management (type of bedding) variables.

The finding that EBC pH increased post-exercise in horses concurs with investigations in human medicine, which also documented an increase in EBC pH in response to exercise (Riediker and Danuser, 2007). The mechanisms by which the pH of the ELF, and therefore the EBC pH, increase in this manner are not clear and cannot be elucidated from this study, but this finding represents an important methodological consideration for future studies into equine EBC pH.

The associations between EBC pH and both the percentage of neutrophils and eosinophils in the BALF, identified in chapter 4, provide fair indication that EBC pH may be a valuable biomarker of LAI in horses. Furthermore, the significant increase in EBC pH in horses with neutrophilic airway inflammation (>5% neutrophils in BALF) provides support for the ability of this biomarker to differentiate between groups of affected versus unaffected horses in relation to IAD or RAO. Further studies with larger subject numbers would be necessary to identify cut-off values of EBC pH as a predictor for LAI, with appropriate positive and negative predictive values established to validate the clinical relevance of this biomarker. One must be mindful, however, of the fact that the documented increase in EBC pH in relation to markers of LAI in horses vastly contradicts the body of evidence in human literature, which consistently identifies a decrease in EBC pH in the presence of LAI (Hunt et al. 2000; Kostikas et al. 2002; Tate et al. 2002; Gessner et al. 2003). Whether this increase in EBC pH in horses with LAI is a true finding, or merely represents an anomaly, manifest of unaccounted physiological variables, will only be established by employing more robust studies where the respiratory dynamics during EBC collection are taken into consideration.

This study is the first published investigation of equine EBC pH where any form of respiratory dynamic recording has been utilized. By employing the most simplistic form of such recording, namely a visual respiratory rate, a positive linear relationship between RR during collection and EBC pH was identified in

both the study outlined in chapter 4, and the methodological pilot study documented in appendix 1. Whilst these findings contrast results from human studies (Kullman et al. 2007) and those in calves (Knobloch et al. 2008) where respiratory rate was not considered to be an influential factor in EBC pH, it further emphasized the need for more accurate and objective recording of respiratory variables during EBC collection.

The mean EBC pH recordings (non de-aerated) measured in this study of 4.51 (range 4.19 – 5.07) prior to exercise and 5.29 (range 4.43 – 6.66) in horses at rest), were comparable to values of 5.65 (range 5.08-6.02) documented by Duz et al. (2009a) and the mean value of 4.5 recorded in adult horses by Whittaker et al. (2012). This represents a consistent finding across all equine studies, where the EBC pH is lower than that documented in humans (mean pH 6.89, range 6.17 - 7.19; Kullmann et al. 2007). Reasons for the disparity between EBC pH in horses and humans are not clear. Whilst methodological variances may play a significant role (Whittaker et al. 2012), it is equally plausible that the consistency of this low EBC pH in horses is representative of differences in the acid-base homeostatic balances of ELF which should be taken into consideration when interpreting equine EBC pH in future studies.

This study also supports findings from previous investigations of equine EBC pH in providing further evidence that the de-aeration of equine EBC with argon produces EBC pH values with greater variance than non de-aerated EBC samples. As previously mentioned, Borrill et al. (2006) demonstrated an unpredictable effect of gas standardisation of EBC with a pH < 6, which provides reason for the fact that no significant findings were identified in any statistical analysis using de-aerated EBC pH throughout this study. Overall, these findings provide support to the argument that the removal of volatiles, including CO_2, from EBC artificially interferes with the true acid-base balance within EBC (Bloemen et al. 2006; Leung et al. 2006) and gives just reason for the future use of non de-aerated EBC pH in further equine studies.

5.4 Limitations of the study and directions for future research

The limitations of the individual studies described within this thesis are addressed in detail within the relevant chapters. The most notable, and arguably most pertinent overall limitation is the absence of any objective, quantifiable data pertaining to the respiratory dynamics of the horses being sampled.

Obtaining such data on respiratory dynamics, including basic parameters such as tidal volumes, minute volumes and expiratory flow rates, is achievable in horses within laboratory settings (exercising on a treadmill under controlled environments), but has not been described adequately for in-field analysis. The use of portable spirometers would confer an effective manner in which these base respiratory parameters could be measured in a field setting, however, as discussed in the material and methods section of this thesis, no spirometers are commercially available that are suitable for recording the flow rates and air-flow volumes that occur during tidal breathing in horses. Ultrasonographic pneumotachographs provide a plausible method by which expiratory flow rates can be measured without affecting resistance, and respiratory inductance plethysmography can provide estimates for tidal and minute volumes. The simple observation that EBC pH is associated with the horse's respiratory rate during sample collection indicates that, even for this biomarker (which is considered to be unaffected by respiratory dynamics in other species), definitive associations between markers of inflammation and states of pulmonary disease cannot be made in equine subjects without first accounting for respiratory rates and flow dynamics.

Further studies into the concentrations of exhaled gases should pay particular attention to the stringent exclusion of inspired, ambient air from the EB sample. By comparing the current studies of eCO and eNO in horses to other published investigations in veterinary species, it is clear that the simultaneous collection of ambient air in a separate impervious bag is an imprecise methodology with

which one cannot accurately account for the contamination of EB with inspired gas. Work completed by Roller et al. (2007) outlines a more robust collection methodology, which adheres to basic requirements of filtering ambient air during inspiration and using CO_2 sensors to partition the brochoalveolar component of EB from the dead-space.

If, by employing these methods, the sensitivity of detecting eCO in EB for horses is improved, then further studies should focus on further establishing the association between eCO and current markers of LAI in tracheal wash or BAL fluid. The results of the current studies provide some early evidence for the suitability of eCO as a biomarker for LAI, and by establishing studies with larger subject numbers, the association between eCO and the tracheal mucus score (and the percentage neutrophilic infiltrate of the tracheal mucus) can be further interrogated with a view to establishing reference ranges of eCO for horse with and without LAI. The characterisation of a such a biomarker profile in relation to specific clinical diseases would lend itself favourably to the non-invasive monitoring of treatment efficacy for pulmonary disorders, as has been described for corticosteroid treatment of human asthma (Hunt et al. 2000).

Before direct associations between LAI and the concentration of gases, such as NO and CO, in EB can be made, further work is required to investigate the precise source of these gases from within the respiratory system of horses. Isolating airflow from different anatomical locations of the respiratory tract and associating the presence of the gases with the known enzymatic processes that underlie their production is an essential next step in this field of research. By reproducing the studies outlined by Rendle et al (2010), where the HO-1 expression was identified in neutrophils and macrophages within BAL fluid, and combining this with the eCO the more sensitive detection methods employed in the current studies, associations between HO-1 expression and eCO could be investigated. Quantifying the level of HO-1 expression via eCO would confer even greater advantage in the investigation of LAI in comparison to relating eCO to absolute neutrophil numbers or relative percentages in BAL fluid, as it would

represent the inflammatory-induced activity of the neutrophils rather than their number alone.

The large intra- and inter-subject variability for many EB/EBC biomarkers investigated to date (excluding pH), may ultimately limit their use as sensitive diagnostic markers for individual respiratory diseases of animals. Improving the sensitivity of the assays, as discussed above, may help to reduce the coefficient of variance within these tests, and is an inevitable logical progression in the field of exhaled breath analysis. This is demonstrated by the results of the current studies where eNO was not reliably detectable using a chemiluminescence analyser with a lower detection limit of 1ppm, and supported by frequent documentation in the literature of eNO existing at concentration below 1ppm in EB. Using laser absorption spectrometry, mass spectrometry or gas chromatography will significantly improve the sensitivity of EB and EBC analysis (Wyse et al. 2004a), although will currently make it less applicable to the in-field, clinical setting.

Recently, Muscatello et al. (2009) reported the use of bacteriological culture and colony blotting to detect *Rhodococcus equi* in the EB of foals with *R. equi* pneumonia. The authors were able to identify the virulent strain of *R. equi*, and demonstrate that the concentration of this virulent strain was higher in the EB of the foals in comparison to the environmental air, however; no significant differences were noted between diseased and non-diseased foals (Muscatello et al. 2009). The concurrent use of measuring exhaled biomarkers of inflammation may play a role in this situation to further differentiate between affected and non-affected individuals. The concurrent detection of pathogens and inflammatory biomarkers in EB/EBC may facilitate development of non-invasive screening tests for infectious diseases. Whether infectious lung diseases may be detected or predicted by analysis of EB/EBC using conventional microbiology, antigen detection or molecular techniques requires further elucidation.

5.5 Conclusions

Exhaled breath and exhaled breath condensate samples can be safely collected from Thoroughbred racehorses, in their own environment, without adverse effect or interference with the horse's training regimen.

Exhaled breath condensate pH can be consistently measured in Thoroughbred horses. The use of off-line collection and analysis of exhaled gases via chemiluminescence is suitable for the measurement of eCO, but is not appropriate for analyzing eNO.

Significant changes in the concentration of eCO and the pH of EBC occurred in response to strenuous exercise with physiological and environmental variables identified as confounding factors in the assessment of EBC pH. Exhaled CO was found to be associated with the tracheal mucus score (and the number of neutrophils in the mucus) of Thoroughbred horses, and EBC pH was significantly different in horses with evidence of neutrophilic lower airway inflammation in comparison to those without.

Individual respiratory rate prior to EB collection, and during EBC collection, was a consistently significant explanatory variable influencing the concentration of exhaled biomarkers, and further studies in EB and EBC analysis in horses should only be completed where investigators can objectively account for key respiratory dynamics during sample collection.

With developments in methodological validation and standardisation and understanding of physiological and disease effects on biomarkers, safe, reliable and clinically relevant breath tests may be achievable in the field of equine veterinary medicine.

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Appendix 1: Pilot study into the influence of ventilation on EBC pH

Objectives

This pilot study had the following objectives;

- To investigate the impact of EBC collection on ventilation by recording end tidal carbon dioxide ($ETCO_2$) and respiratory rate (RR) prior to and during sample collection.
- To investigate any associations between $ETCO_2$, RR and EBC pH.

Materials and Methods

Animals: Three resident teaching horses owned by the School of Veterinary Medicine, University of Glasgow (2 mares and 1 gelding, all of cross-breeding) were used for the study. The horses were considered free of respiratory disease based on history and clinical examination. All examinations and sampling was conducted in the horse's own stable.

Study Design: Each horse was sampled twice daily for five days, once in the morning and once in the afternoon. Prior to EBC sample collection, a portable capnograph (N85-1 capnograph, Woodley equipment company Ltd., UK) was used to record the RR and $ETCO_2$ during normal, unrestricted breathing. To achieve this, the microstream sampling tube port was held under the alar cartilage at the level of the external nares. This technique was found to be the most compliant method of sampling that did not cause irritation to the horse (in comparison to placing the sample tubing further into the nasal cavity). Readings of $ETCO_2$ and RR were recorded every 30 seconds for five minutes during conscious, unrestricted breathing. A sample of EBC was then collected as described (section 2.1.2) over a period of 10 minutes. During EBC collection, the microstream sample tube was incorporated into the facemask, between the inspiratory and expiratory valves, and $ETCO_2$ and RR were recorded every 30 seconds for the 10 minute sampling period. Following collection, EBC was stored in dry ice until EBC pH was measured, as previously described (section 2.2.2). All EBC samples were analysed within 2 hours of collection.

Data analysis: The values recorded for RR and $ETCO_2$ were averaged for each sample collection period data and the resultant data was tested for normality using the Anderson-Darling normality test. Paired data for RR and $ETCO_2$ pre- and during sample collection were compared using paired *t* test or Wilcoxon signed-rank test where appropriate. With EBC pH set as the outcome, univariate linear regression models were use to investigate associations between EBC pH and RR or $ETCO_2$ during sample collection. Multivariable models were built by inclusion of explanatory variables found to have a *P* value of < 0.2 during univariate analysis. Significance was set at $P < 0.05$.

Results

End tidal CO_2 and RR were successfully recorded using capnography prior to and during EBC collection in all horses at each sample time point. This resulted in a total of 30 paired sample points for the mean $ETCO_2$ and the mean RR prior to and during EBC sample collection, and 30 corresponding EBC pH readings (Table 1). The overall mean RR during EBC collection was 6.7 ± 1.1 breaths/min, which was not significantly different from the overall mean RR prior to sample collection (6.6 ± 1.2; $P = 0.6$). A significant reduction in $ETCO_2$ during sample collection was noted ($P < 0.005$); decreasing from an overall median of 50.8 ± 7.1 mmHg before sampling to an overall median of 43.4 ± 1.0 mmHg during EBC collection (Fig 1).

In the univariate general regression analysis, a significant inverse association between mean RR during sample collection and mean $ETCO_2$ ($p = <0.005$; figure 2) and a direct association between mean RR and EBC pH ($p = 0.007$; figure 3) were identified. An association between mean $ETCO_2$ and EBC pH was found which approached, but did not achieve significance ($p = 0.066$). Both the association between RR and $ETCO_2$ and between RR and EBC pH lost significance when horse, day and sample time explanatory variables were included in the multivariable regression model.

Sample Number	Horse A			Horse B			Horse C		
	Mean RR	Mean $ETCO_2$	EBC pH	Mean RR	Mean $ETCO_2$	EBC pH	Mean RR	Mean $ETCO_2$	EBC pH
1. Pre	5.8	63.5		8.1	54.3		6.1	46.9	
During	6.1	55.1	5.20	6.6	47.6	6.96	6.7	33.7	5.99
2. Pre	6.1	61.4		6.3	53.0		8.4	44.9	
During	5.9	53.3	5.22	6.6	42.8	4.68	9.1	36.6	5.11
3. Pre	6.5	57.6		6.6	55.8		7	33.2	
During	6.6	50.9	4.90	5.7	47.1	4.96	9	32.5	6.45
4. Pre	5.4	31.7		7.9	52.2		6.8	45.8	
During	7.3	38.4	5.29	5.6	44.4	4.89	7.3	38.4	5.10
5. Pre	6.2	57.1		6.9	56.6		6.2	46.9	
During	6.8	46.3	4.66	5.7	47.7	4.60	8.1	38.0	5.51
6. Pre	5.3	52.1		6.3	53.6		8.5	44.4	
During	6.5	46.1	5.00	6.1	44.6	4.76	8.6	35.9	5.57
7. Pre	6.4	52.6		5.3	56.8		5.5	51.2	
During	5.8	49.8	4.35	5.7	49.1	5.02	5.9	41.3	4.91
8. Pre	4.9	49.4		6.5	54.8		9.2	44.5	
During	6.3	46.3	4.59	5.8	45.3	4.78	8.5	37.0	5.09
9. Pre	5.6	58.3		7.2	52.6		8.6	49.0	
During	5.9	48.1	4.65	6.1	47.7	4.55	6.9	40.5	5.25
10. Pre	7.9	49.4		8.0	48.8		6.3	46.4	
During	6.9	42.7	4.86	6.1	42.9	4.58	9.0	35.6	4.46

Table 1: Averaged respiratory rates (RR) and end-tidal CO_2 ($ETCO_2$) prior to and during EBC sample collection, with corresponding EBC pH readings, in 3 adult horses.

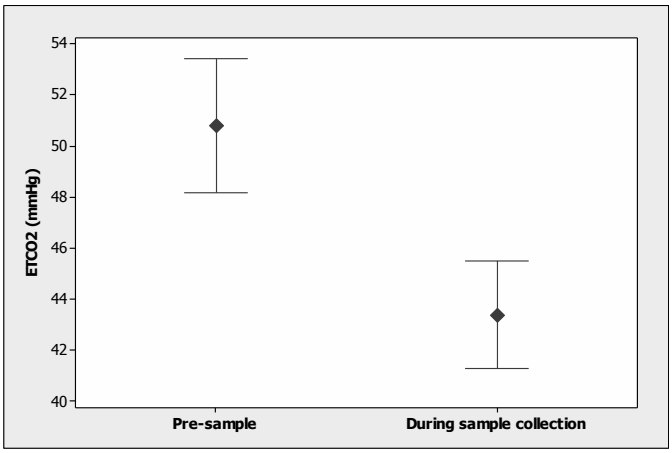

Figure 1: Interval plot (mean plus standard error of the mean) of End-tidal carbon dioxide ($ETCO_2$) prior to and during EBC sample collection

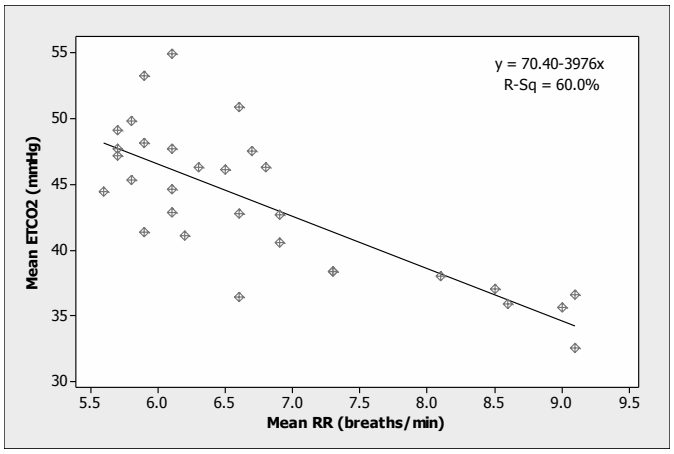

Figure 2: Scatter plot of mean respiratory rate (RR) during sample collection against mean $ETCO_2$ during collection. Linear regression demonstrates the inverse association between RR and $ETCO_2$ ($P < 0.005$).

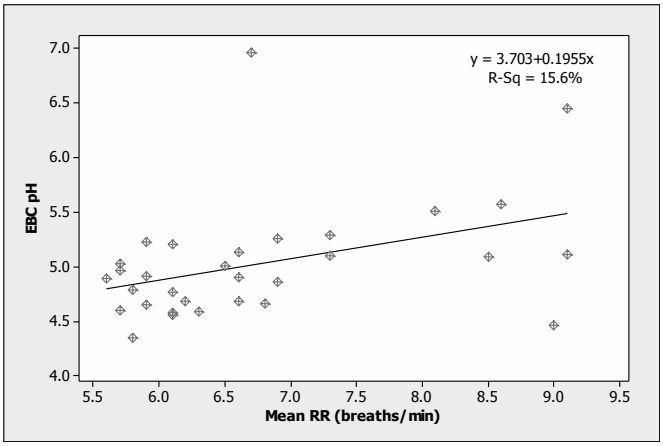

Figure 3: Scatter plot of mean respiratory rate (RR) during sample collection against exhaled breath condensate (EBC) pH. Linear regression demonstrates the direct association between RR and EBC pH ($P = 0.007$).

Based on the documented difference in mean $ETCO_2$ prior to and during sample collection and with an *n* of 30, the power of the pilot study was calculated at 0.998.

Discussion

The utilisation of the portable capnography allowed for the successful monitoring of the horse's RR and $ETCO_2$ during EBC collection. The $ETCO_2$ values obtained in consciously breathing horses ranged from 31.7-63.5mmHg. No reference ranges for $ETCO_2$ in conscious horses have been published with the only published reference ranges relating to horses under general anaesthesia (35-45mmHg; Reed at al. 2006). Whilst the mean $ETCO_2$ recorded in the consciously breathing horses (50.8mmHg) does fall outside this range, direct comparisons cannot be made.

The method used to obtain the pre-sampling readings of $ETCO_2$ in this study was developed for the purpose of this investigation and has not been validated. Nasal cannulas placed at the external nares have been used to monitor $ETCO_2$ in conscious adults (Roy et al., 1991) and children (Tobias et al., 1994), with an $ETCO_2$ of 40.5 ± 5.5 recorded in healthy adults (Roy et al., 1991). The technique employed in the current study is similar in principal to the use of nasal cannulas in people, albeit in a less controlled environment. By placing the sampling port of the capnograph at the level of the horses nostril it cannot be assumed that only inhaled and exhaled breath is being sampled, and some influence of ambient air could be expected. However, atmospheric air contains very low concentrations of CO_2 (0.035%[1]) and thus the contribution of ambient air to the $ETCO_2$ can be considered negligible.

The overall mean $ETCO_2$ during EBC collection (43.4 mmHg) was significantly lower than that recorded prior to sample collection. Whilst no significant difference in the mean respiratory rates prior to and during sample collection were present, a subjective increase in tidal volume was occasionally observed during sample collection. Without changes in respiratory rate, an increase

in tidal volume would increase ventilation of the small airways and improve gas exchange at this level, which would, in theory, cause a decrease in the $ETCO_2$. Also, a significant association between median RR and median $ETCO_2$ during sample collection was identified in the univariate general regression analysis, supporting an individual association between RR and $ETCO_2$.

In this inverse association, as the mean RR increased during sample collection, the mean $ETCO_2$ decreased (Fig 3.3). This counterintuitive relationship ($ETCO_2$ would be expected to increase at higher respiratory rates) could be explained by the changes in tidal volume discussed above or by longer periods of 'breath-holding' present at lower respiratory rates, which would increase the $ETCO_2$ at the alveolar level. Therefore, whilst the inherent expiratory resistance of the collection apparatus was not sufficient enough to alter respiration rate, the RR during sample collection did influence the $ETCO_2$, an effect that may have been accentuated by unrecorded changes in respiratory dynamics (e.g. tidal volume).

No significant association between median $ETCO_2$ and EBC pH was found in the univariate regression analysis, indicating that the concentration of CO_2 in the expired breath does not have any influence on the pH of the EBC. However, a direct association was found between median RR during sample collection and the EBC pH, where increased RR was associated with higher EBC pH. Given the previously discussed association between median RR and $ETCO_2$, it is hard to ignore the possibility that the three factors are inter-related and that by increasing $ETCO_2$, lower respiratory rates were associated with a reduction in EBC pH. This is supported by the fact that the association between median $ETCO_2$ and EBC pH was approaching significance ($p = 0.066$), and this association may have achieved significance with a higher sample number.

All associations identified in the univariate analysis lost significance when horse, day and sample time explanatory variables were included in the multivariable regression model. Thus when accounting for daily (intra-subject) variability and horse (inter-subject) variability, the influence of RR and $ETCO_2$ on EBC pH was no longer significant.

Based on the results of this pilot study, it can be concluded that respiratory rates during EBC sample collection do not vary from normal resting rates, and whilst respiratory rate does seem to influence the $ETCO_2$, this does not significantly affect EBC pH when intra- and inter-subject variability is accounted for.

Appendix 2: Assessment of salivary contamination during EBC collection

Objectives

This study had the following objective;

- To investigate whether salivary contamination occurred during EBC sample collection in Thoroughbred racehorses.

Materials and Methods

Animals: This pilot study was conducted in conjunction with the sampling of EBC from horses in group A_W of the study; *Influence of high intensity exercise and ambient temperature on concentrations of exhaled biomarkers of airway inflammation*, described in chapter 4 of this thesis. A convenience sample of Thoroughbred racehorses in active training (signalment presented in chapter 4), were selected for sample collection over a 5-day period.

Collection of saliva from horses

Saliva has previously been collected from horses for biochemical analysis (Eckersall 1984), cortisol assays (Reijerkerk et al., 2009; Peeters et al., 2010) and measurement of lactate concentration (Lindner et al., 2000). The methodologies employed to obtain saliva from horses have varied, ranging from use of a custom-made fenestrated 'bit' through which saliva could be aspirated (Eckersall et al., 1984), to placement 10-30 cotton balls sequentially into the horses' mouth with subsequent extraction of the saliva by expressing the cotton balls using a 10ml syringe (Reijerkerk et al., 2009). More recent studies have utilised commercially available cotton swabs designed for saliva collection in humans, which were found to absorb sufficient volumes of saliva within 30-40s (Peeters et al., 2010).

The oral swabs chosen for this study (Salimetrics Oral Swab, Salimetrics Europe Ltd., UK) were absorbent inert polymer cylindrical swabs commercially available for use in people, which had been approved for the analysis of cortisol,

testosterone and α-amylase. Optimal placement of oral swabs for collection of saliva in horses is considered to be at the opening of the major salivary ducts; adjacent to the frenulum of the tongue (mandibular and sublingual gland) and at the level of the 3rd maxillary premolar (parotid duct), with placement at these sites achieved by holding the oral swabs with metal forceps (Peeters et al., 2010). Initial attempts at recreating this methodology were met with difficulty (poor patient compliance and incessant mastication) and were not without risk of causing damage to the horse.

Placement of the salimetrics oral swab (SOS) in the ventral buccal cavity at the level of the mandibular cheek teeth with a gloved finger was well tolerated by the horses and thus was employed for the purpose of this study. Due to the indirect contact with any salivary duct opening, the sample time for saliva collection was doubled to 90s.

Prior to EBC sample collection the right buccal cavity of each horse was rinsed with tap water to remove any organic matter. Following EBC collection (5 minutes), a sample of saliva sample was obtained as described above. The SOS was then placed in a custom-designed filter 'swab storage tube' (Salimetrics Europe Ltd., UK), snap-frozen in dry ice and stored at -80°C. Prior to analysis, the samples were brought to room temperature and the oral swabs, within the filter swab storage tube, were centrifuged for 15 minutes at 3,000rpm to extract the saliva.

Establishing a methodology for comparison of saliva and EBC

Alpha Amylase assay: Based on the precedent set in medical research for the use of α-amylase assays to document the absence of saliva in EBC samples (Effros et al., 2006b), extrapolation of this methodology to the equine samples was attempted. A pilot subset of paired saliva and EBC samples (n = 10) were analysed for salivary α-amylase using a commercially available salivary α-amylase liquid phase enzymatic assay (IBL International GmbH, Hamburg, Germany) that incorporated two control reference solutions of human α-amylase

and a blank negative control. Every sample of saliva and EBC returned results below the limit of detection for the assay, with none registering any α-amylase activity above the level of the negative control.

Protein assay: The protein patterns of EBC and corresponding saliva samples in people have been analysed by means of 2-dimensional protein gel electrophoresis (Griese et al., 2002). This study consistently documented the presence of 3 distinct proteins in all EBC samples analysed (*n* = 10).

The proteins were also present in 60-70% of the matched saliva samples, which the authors, having stringently excluded salivary contamination, concluded was representative of the independent presence of these proteins in both saliva and secretions of the lower airway (Griese et al., 2002).

In the absence of any assay specific for equine salivary α-amylase, the relative total protein concentrations and protein patterns of the paired equine EBC and saliva samples were measured and compared using spectrophotometric protein quantitation and 2-dimensional protein gel electrophoresis.

For each EBC and saliva sample, 500µl was transferred into a quartz crystal cuvette and analysed by ultraviolet absorption spectroscopy at 280nm as described by Noble and Bailey (2009). Where <500µl of saliva was retrieved from the oral swab, appropriate dilutions of the samples were made with distilled water and the relevant dilutional factor was accounted for in the final protein concentration calculation.

Two-dimensional polyacrylamide gel electrophoresis was conducted using 18 well gel plates, with one well containing the marker, ten wells containing each EBC sample in duplicate, and five wells containing samples of saliva. 10µl of sample, 18µl of H_2O, 10µl of sample buffer and 2µl of reducing agent were all added to an eppendorf tube. The samples were then heated at 95ºC for 4 minutes. Next 10µl of the sample/marker was placed into each of the wells in the gels plates and the electrophoresis run at 200V for 60 minutes.

The gels were then removed from the plate and stained with Coomassie Brilliant Blue dye for 1 hour, prior to destaining with acetic acid, methanol and water for 24-hours. The gels were scanned using Magiscan™ and digital images were created using Adobe Photoshop™ software, allowing for digital enhancement of the gels to discern the protein bands present on the gel.

In attempt to improve the sensitivity of the protein gel electrophoresis for the EBC samples, the EBC samples from each day were pooled and concentrated prior to repeat gel electrophoresis. Exhaled breath condensate samples were concentrated using Microcon® centrifugal filter devices (Millipore Ltd., Watford, UK). One hundred and fifty microliters of each EBC sample from a single day (6-8 samples) were sequentially placed in the same filter cap and centrifuged at 5500rpm for 90 minutes, resulting in a cumulatively formed, concentrated retentate. The 5 resultant concentrated retentates were then re-suspended in 20µl of sample buffer and analysed by repeat gel electrophoresis, in conjunction with the corresponding saliva samples, which had been pooled relative to their day of collection, but not concentrated.

Results

A total of 38 paired saliva and EBC samples were obtained from 19 Thoroughbred horses. Sample collection was generally well tolerated in all horses with an average of 2.5ml saliva retrieved from each sample.

The mean and median estimated protein concentration for the EBC and saliva samples are presented in Table 1. Non-parametric analysis between the two groups (Mann-Whitney *U* test) found the difference to be statistically significant ($P < 0.0001$, Fig 1), However, large coefficients of variance were identified for both EBC and saliva protein concentrations, resulting in a large range in the relative proportions of protein content between the paired EBC and saliva samples. Salivary protein concentration was between 5.4 and 540 times higher than the corresponding EBC samples.

Sample	Mean	SE mean	StDev	Median	Min	Max	CoV
EBC	0.183	0.031	0.189	0.116	0.038	1.050	103.4
Saliva	8.035	0.838	4.587	6.447	3.073	20.240	57.1

Table 1: Descriptive statistics of the estimated total protein concentration of EBC and saliva samples, as determined by ultraviolet absorption spectroscopy

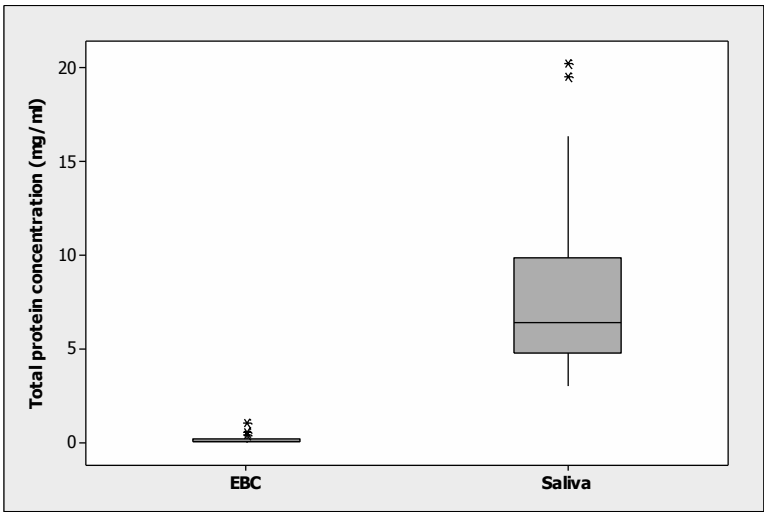

Figure 1: Estimated total protein concentrations of EBC and corresponding saliva samples. The protein content of the saliva samples was significantly higher than the EBC samples (*P* < 0.0001)

Distinct protein bands were identified in the saliva samples following 2-dimensional protein gel electrophoresis, which were not present in the paired EBC samples (Fig 2a). No trace of protein could be detected in any of the EBC sample wells analysed. These results were consistent across all of the gels analysed.

Given that the EBC protein concentrations were consistently low, many being below the limit of detection for protein electrophoresis, the EBC samples were pooled and concentrated as described. A similar gel pattern was found when the pooled and concentrated EBC samples were run against the matched pooled saliva samples, with no bands detectable in the concentrated EBC samples (Fig 2b). By pooling the saliva samples alone, without concentrating the protein content, distinct and repeatable protein patterns became apparent on the gels.

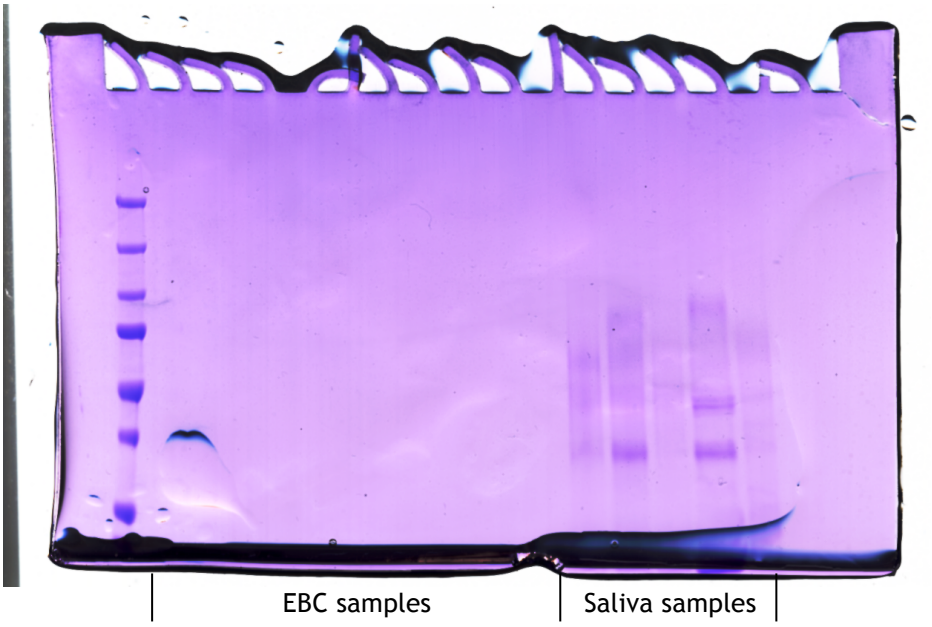

Figure 2a: Protein gel electrophoresis of paired EBC (duplicated) and saliva samples. Protein patterns can be identified in each of the saliva wells, with distinct protein bands evident in two of the samples.
No trace is evident in the EBC samples.

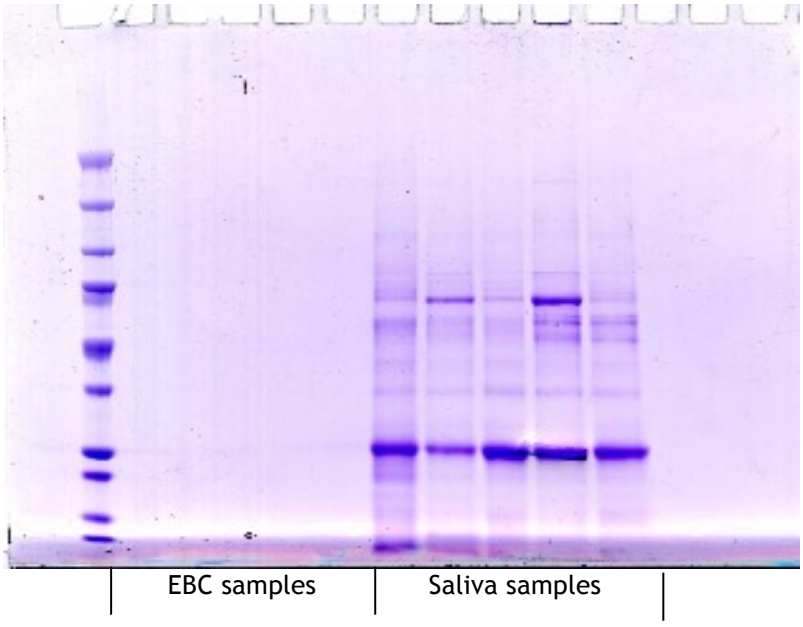

Figure 2b: Protein gel electrophoresis of pooled and concentrated EBC samples together with pooled saliva samples. The protein bands present in saliva are more distinct and repeatable across all well. The EBC samples still show no band pattern relative to the saliva samples and the marker.

Discussion

This technique of saliva collection employed in this study was successful in obtaining saliva from all the horses sampled, and was simple to conduct with most horses being compliant. There were, however, some limitations to this technique: although several dental syringes full of water were used to clean out the mouth prior to sample collection some organic matter was occasionally found on the oral swab after sample collection. This was invariably fibre based feed, which would be of low protein content, but could potentially influence the results of the protein analysis. Not accounting for sample contamination with organic matter is a limitation of this technique and should be addressed in future studies.

The ultraviolet absorption spectroscopic protein assay employed in this study is particularly sensitive for the detection of aromatic amino acids (Noble and Bailey, 2009), and thus was considered a suitable assay for protein analysis of saliva, which contains high levels of aromatic amino acids. The protein concentrations obtained from the saliva samples demonstrated high inter-subject variability. No reference ranges for protein content of equine saliva have been published, and the expected variance between biological samples with a consistent composition such as saliva in this species is not known. Whilst intra-subject variability (variance in autonomic tone, proximity to feeding, hydration status) may influence the protein content of the saliva, the high coefficients of variance in the protein concentrations of the saliva samples collected in this study may also indicate that pure saliva retrieval was variable using the technique employed. This is also reflected in the variable intensity of the protein patterns identified on 2D-gel electrophoresis of the individual saliva samples.

As expected, the estimated protein concentration of the EBC samples was significantly lower than that found in saliva. However, given the large inter-

subject variation for the protein content of both EBC and saliva samples, and without knowing the different protein composition of the solutions, this method of analysis alone would be insufficient to ascertain whether salivary contamination has occurred during EBC collection. A 10,000 fold lower concentration of α-amylase needs to be documented in EBC relative to matched saliva samples to document the absence of saliva in human EBC (Horvath et al., 2005); whether the same fold difference is relevant to animals which are obligate nasal breathers has not been investigated. The greatest differential protein content identified between matched EBC and saliva samples in this study was 540-fold, indicating that relative to the α-amylase assay, the quantitation of protein by ultraviolet absorbance spectroscopy lacks the sensitivity to adequately exclude the presence of saliva in EBC.

The lack of a suitable α-amylase assay for use in equine studies relates to the absence of any specific assay for equine salivary α-amylase. Amylase exists in two main isoforms in mammals, salivary and pancreatic and each isoenzyme is species specific. Therefore assays specific for human salivary α-amylase or canine pancreatic α-amylase lack sensitivity for α-amylase in equine saliva, as documented by the failure of the assays in the methodological development of this study.

Protein gel electrophoresis was consistently more sensitive for detecting distinct proteins bands in the saliva samples, and when the inter-subject variation of sample quality (and protein concentration) was accounted for via the pooling of the saliva samples, repeatable protein bands were identified across all of the samples. The individual EBC samples failed to produce any discernable protein pattern, with no uptake of the Coomassie Brilliant Blue dye in any of the wells. This in itself was considered insufficient evidence that no salivary proteins were present in the EBC samples, as it may have been a manifestation of the low protein concentrations, some of which were below the minimal level for detection by electrophoresis. In an attempt to improve the sensitivity of the gel electrophoresis, the EBC samples for each day were pooled and concentrated. Again, no band patterns were apparent on the gels from the wells containing the

EBC samples. This finding provided further support to the observation that the minute concentrations of protein detected in the EBC samples did not correspond to the proteins present in the saliva samples.

Characterisation of the protein bands identified in the saliva samples would have been of interest, and may have strengthened the observations made relating to the protein content of saliva and EVC. This was unfortunately out with the remit of this pilot study.

In conclusion, this study has gone some way toward addressing the question as to whether salivary contamination occurs during EBC collection in horses, and the observations made during the investigation support the assumption that salivary contamination does not occur. Two-dimensional gel electrophoresis lacks the sensitivity to detect the very low concentrations of protein present in individual EBC samples, which, in combination with the time and cost required to run each gel, would prevent the utilisation of this technique in routine monitoring of equine EBC for salivary contamination. However, the results of this strongly indicate that such assessment is not a stringent requirement when EBC is collected from horses in a frozen solid state, using collection devices that separate the face-mask from the condensing chamber.