THE ROLE OF INHALED ENDOTOXIN IN THE AETIOPATHOGENESIS OF EQUINE HEAVES

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DECLARATION

I declare that the contents of this thesis are my own work and that they have not been presented to any University other than the University of Edinburgh.

Robert Scott Pirie,

Edinburgh, August 2001

ABSTRACT

Soluble lipopolysaccharide (LPS) inhalation challenge induced a dose-dependent bronchoalveolar lavage fluid (BALF) neutrophilia in both heaves-susceptible and control horses, and significant lung dysfunction in the heaves group. The response thresholds were lower for the heaves group, yet were markedly greater than airborne endotoxin exposure during the 5h dusty hay/straw challenge. In addition, there was no significant difference in BALF neutrophil numbers between the 2 groups following challenge with the middle and high LPS dose. There was a significant difference in the airway inflammatory response of the heaves group to 2 separate hay/straw exposures. This response was not related to the level of airborne endotoxin exposure. These findings indicated that inhaled endotoxin is not solely responsible for the induction of naturally occurring heaves.

Inhalation challenge of the heaves group with 3 incremental doses of soluble A. fumigatus extract resulted in an increase in a BALF neutrophilia and lung dysfunction, which plateaued following inhalation of the middle dose. Inhalation challenge with LPS-depleted A. fumigatus extract resulted in a significant reduction in airway neutrophil numbers, of a magnitude that was greater than predicted by extrapolation from soluble LPS dose response inhalation experiments. These findings indicated that inhaled endotoxin may act synergistically with mould antigens, and contribute to the pulmonary inflammation observed in heaves.

Inhalation challenge with hay dust suspensions (HDS), prepared from fine hay dust particles, induced an airway neutrophilia, airway dysfunction and mucus hyper-secretion in the heaves group only. Inhalation challenge of the heaves group with the soluble fraction of HDS (SUP) failed to induce the magnitude of response measured following HDS challenge, despite containing almost all of the endotoxin activity of the HDS. These findings supported the involvement of HDS components, other than endotoxin, in the aetiopathogenesis of heaves. Inhalation challenge of the heaves group with the particulate fraction of HDS (WP) induced only a mild BALF

neutrophilia, however a combined challenge with SUP and WP induced a neutrophilic response approaching the magnitude of that following HDS challenge. These findings indicated a synergistic action between the soluble and particulate fractions of HDS.

Inhalation challenge of the heaves group with LPS-depleted HDS resulted in a significant reduction in BALF neutrophil numbers, of a magnitude that was greater than predicted by extrapolation from soluble LPS dose response inhalation experiments. Replacement of the depleted LPS resulted in the re-establishment of the original level of BALF neutrophilia. Inhalation challenge of the heaves group with WP reconstituted in LPS solution (containing an equivalent LPS activity to SUP) resulted in a BALF neutrophilia that was not significantly different from that following challenge with combination of WP and SUP. These findings indicated that the endotoxin content of HDS acts synergistically with other HDS components, most notably the particulate fraction.

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DEDICATION

To the memory of Stewart Pirie (13th February, 1963 – 14th August, 1963)

The brother I never knew, but am certain knows me.

To my parents, Frank and Jen Pirie, and my brother Iain Pirie, for their continuous, uncompromising support, their guidance and their encouragement.

I am eternally grateful.

TABLE OF CONTENTS

ABBREVIATIONS

AFE	*Aspergillus fumigatus* extract
A React	Airway reactivity
Art BG	Arterial blood gas
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
Cdyn	Dynamic compliance
Clin Ex	Clinical examination
cm	centimetres
$dPpl_{max}$	Maximum transpulmonary pressure change
h	hours
Haem	Haematology
H/S	Hay/straw exposure challenge
kg	kilogrammes
l	litres
LF	Lung function
LPS	Lipopolysaccharide
M	molar
m^3	cubic metres
mg	milligrammes
min	minutes
ml	millilitres
mm	millimeters
ng	nanogrammes
nm	nanometres
$PaCO_2$	Arterial carbon dioxide tension
PaO_2	Arterial oxygen tension
PCCdyn70	Concentration of inhaled methacholine chloride solution required to reduce the Cdyn to 70% of the baseline value
PMN	Polymorphonuclear cells
Ptp	Transpulmonary pressure
$RL_{E25\%}$	Lung resistance at 25% expired volume

$RL_{E50\%}$	Lung resistance at 50% expired volume
$RL_{E75\%}$	Lung resistance at 75% expired volume
$RL_{I25\%}$	Lung resistance at 25% inspired volume
$RL_{I50\%}$	Lung resistance at 50% inspired volume
$RL_{I75\%}$	Lung resistance at 75% inspired volume
RL_{iso}	Isovolumetric lung resistance
RL_E	Expiratory lung resistance
rpm	Revolutions per minute
RR	Respiratory rate
S	seconds
t	Time
T_E	Time for expiration (seconds)
T_I	Time for inspiration (seconds)
$T_I:T_E$	Ratio of T_I to T_E
V'_{Emax}	Maximum expiratory flow
V'_{Imax}	Maximum inspiratory flow
V_T	Tidal volume
WBC	White blood cells
Wb	Work of breathing
Wb_{el}	Elastic work of breathing
Wb_{Eres}	Expiratory resistive work of breathing
Wb_{Ires}	Inspiratory resistive work of breathing
Wb_{Itot}	Inspiratory total work of breathing
Wb_{res}	Resistive work of breathing
Wb_t	Total work of breathing
<	Less than
>	Greater than
µg	microgrammes
µl	microlitres
µm	micrometres
%	percent
@	at
°C	degrees centigrade

CHAPTER 1: INTRODUCTION

The domestication of the horse has resulted in the exposure of many horses to environments that have proved to be detrimental to their health. Frequently horses are housed in poorly ventilated environments (Clarke *et al.,* 1987; Jones *et al.,* 1987), fed on poorly saved hay and bedded on poorly saved straw (Clarke, 1987b). As a result the housed horse is frequently exposed to high levels of airborne pollutants including inorganic dusts, ammonia (Clarke, 1987a), endotoxins (Dutkiewicz *et al.,* 1994; McGorum *et al.,* 1998), bacteria (Clarke, 1987a), viruses (Clarke, 1987a), fungi and actinomycetes (Clarke, 1987a; Clarke and Madelin, 1987; Clarke, 1993) and forage mites. Consequently large numbers of housed horses develop environmental pulmonary diseases, the most commonly recognised of which is heaves (previously termed Chronic Obstructive Pulmonary Disease). While an association between heaves and mouldy hay was documented in the veterinary literature over 200 years ago (Clarke, 1788), there is increasing awareness that other forms of stable dust-induced respiratory disease may also affect horses.

Over the past decade it has become increasingly evident that the clinical features of equine heaves are consequences of pulmonary inflammation (Derksen, 1993). Effective management of heaves therefore relies upon the reduction of the inflammatory response, ideally by removing the inciting inhaled agents or by the use of anti-inflammatory therapy. Improved understanding of the inhaled agents responsible for inducing pulmonary inflammation is a prerequisite to the

establishment of airborne dust safety threshold levels and the development of new strategies to control this disease.

1.1 Equine Heaves

1.1.1 Aetiological agents

Heaves results from housing horses in certain environments. When horses are stabled and fed hay, they are exposed to and inhale airborne dusts rich in organic material (Crichlow *et al.,* 1980; Clarke and Madelin, 1987; Clarke *et al.,* 1987; Webster *et al.,* 1987; Woods *et al.,* 1993), which induce pulmonary inflammation and airway obstruction (Robinson *et al.,* 1996). Clinical remission can be achieved by moving the horses to pasture (Thomson and McPherson, 1984; Derksen *et al.,* 1985a; Derksen *et al.,* 1985b). The relative importance of each organic dust component in the aetiopathogenesis of heaves is unknown, and it is probable that more than one component of organic dust has the potential to cause pulmonary disease when inhaled in sufficient quantities. Indeed the inhalation of a combination of these agents may result in complicated additive and synergistic activities.

1.1.1.1 Moulds (fungi and thermophilic actinomycetes)

A wide variety of airborne moulds have been identified in equine stables which contain hay and straw (Clarke and Madelin, 1987), since, if baled with a moisture content exceeding 20%, this herbage is rich in spores of fungi and actinomycetes such as *Aspergillus fumigatus, Faenia rectivirgula* and *Thermoactinomyces vulgaris*

(Clarke, 1987b). Although there is little or no difference between healthy and heaves horses with respect to levels of serum antibodies to a number of different moulds (Lawson *et al.,* 1979; Madelin *et al.,* 1991), heaves horses do have exaggerated local pulmonary antibody (both IgG and IgE) responses to certain moulds including *A.fumigatus* and *F.rectivirgula* (Halliwell *et al.,* 1993; Crameri, 2001). McGorum *et al.* (1993c) demonstrated that inhalation challenges with aqueous extracts of either *A. fumigatus* or *F. rectivirgula* induced a neutrophilic pulmonary inflammatory response and associated pulmonary dysfunction in asymptomatic heaves-affected horses, but not in controls, thus indicating that these agents may contribute to the development of naturally occurring heaves. However in this study, the severity of the pulmonary inflammation induced by these challenges was significantly less marked than that induced by long-term mouldy hay/straw exposure. Many differences exist between the two types of challenge systems that may account for this difference. These include a difference in the total quantity and duration of antigen challenge, a possible difference in the proportion and location of antigen deposition within the respiratory tract and the fact that the natural hay/straw challenge results in exposure to multiple mould antigens. Additionally, the potential involvement of other agents present within organic dust should not be overlooked, since these may act in an additive or synergistic fashion with mould antigens, thus amplifying the inflammatory and lung function changes. In contrast to the findings of McGorum et al (1993c), Derksen et al (1988) demonstrated that inhalation challenge with *F. rectivirgula* induced a bronchoalveolar lavage fluid (BALF) neutrophilia in both control horses and asymptomatic heaves horses. The BALF neutrophilia reported in the control ponies may indicate that *F. rectivirgula* inhalation challenges are not a good model of the

disease, as control horses do not develop an airway neutrophilia following natural exposure to a hay/straw environment. Alternatively, the concentration of extract used by Derksen *et al.* (1988) may have been excessively high, thus inducing a non-specific inflammatory response. A similar induction of false positive results has been reported in humans given inhalation challenges with excessively high concentrations of antigen (Townley *et al.,* 1965; Cavanaugh *et al.,* 1977). An alternative explanation for the finding of Derksen *et al.* (1988) may have been contamination of the inhaled extract with other pro-inflammatory agents such as endotoxin.

1.1.1.2 Forage mites

Large numbers of forage mites may be present in poorly stored forage (Halls and Gudmundsson, 1985). Horses with heaves do not have elevated BALF levels of forage mite specific IgE and IgG, suggesting that pulmonary hypersensitivity responses to forage mites are unlikely to be involved in the pathogenesis of the disease (BC McGorum, *personal communication*).

1.1.1.3 Endotoxins

Further investigation is warranted with respect to the potential role of inhaled endotoxin in the aetiopathogenesis of equine heaves, since there are many similarities between equine heaves and endotoxin mediated lung diseases in other species (McGorum *et al.,* 1998). Both are characterised by reversible airway obstruction, airway neutrophilia, bronchial hyper-responsiveness and increased mucus production,

have similar time courses, and result in minimal permanent structural lung changes such as emphysema and fibrosis (Rylander, 1990; Gordon and Harkema, 1995; Robinson *et al.,* 1996). In addition several studies have reported high concentrations of airborne endotoxin in horse stables, often exceeding the recommended safety levels for human exposure (Dutkiewicz *et al.,* 1994; Rylander, 1997b; McGorum *et al.,* 1998; Tanner *et al.,* 1998). The latter section of this review will give a more detailed literature review on the potential involvement of inhaled endotoxin in the aetiopathogenesis of heaves.

1.1.1.4 Other stimuli contributing to airway inflammation and dysfunction

Horses with symptomatic heaves have increased airway reactivity (Derksen *et al.,* 1985a), therefore other inhalants, including particulate matter, cold air, dry air and noxious gases may exacerbate airway inflammation and obstruction in symptomatic horses.

1.1.2 Pathogenesis of heaves

The exact mechanisms that result in pulmonary inflammation and dysfunction in heaves are not fully understood, however both environmental observations and the findings of several studies strongly support the involvement of a hypersensitivity component (Robinson *et al.,* 1996). Some studies have demonstrated an increased frequency and magnitude of skin responses in heaves-susceptible horses following intradermal injections of both stable dust and mould extracts (Halliwell *et al.,* 1979;

McPherson *et al.,* 1979), with a significant difference from the response of control horses at 30min and 4h (Halliwell *et al.,* 1979). As well as supporting the involvement of allergy in heaves, the study by Halliwell *et al.* (1979) also indicated that both a type I (IgE-mediated) and type III (IgG-mediated) hypersensitivity were involved, at least with respect to the dermal response. However the limited value of intradermal mould antigen testing was highlighted by McGorum *et al.* (1993a), who demonstrated that there was no difference between controls and heaves horses with respect to the intradermal end-point titres of various aqueous mould extracts. This study also failed to demonstrate a significant correlation between the pulmonary and dermal response to mould extracts (McGorum *et al.,* 1993a), a finding which has also been reported in human allergen inhalation and intradermal studies using recombinant house dust mite allergens (van der Veen *et al.,* 1998). Further support for IgE mediation (type I hypersensitivity) in heaves has been advocated recently, where bronchoalveolar lavage cells harvested from hay/straw challenged heaves horses, when compared to those from control animals, had increased expression of interleukin-4 (IL-4) and IL-5, and decreased expression of interferon-γ (INF-γ) (Lavoie, 2001), a cytokine profile consistent with a local pulmonary T-helper cell 2 response. In addition, a recent study reported that compared with controls, heaves-susceptible horses had a higher number of IgE positive cells in bronchioles and pulmonary blood vessels following mouldy hay straw challenge (van der Haegen *et al.,* 2001). However, the significant difference between the 2 groups was largely due to 2 individual heaves horses with excessively high IgE positive cells. The study suggested that various immunological reactions are probably involved in heaves, and

that IgE-mediated reactions are possibly only involved in some cases or in some stages of the disease.

Certain studies have suggested that the mast cell may play a key role in the pathogenesis of heaves (Mair *et al.,* 1988), and mast cells have been identified in greater numbers in lungs from horses with heaves (Winder and Vonfellenberg, 1990). In addition, the pulmonary epithelial lining fluid (PELF) concentration of histamine was found to be elevated in heaves horses following hay/straw challenge, and the histamine concentration was found to correlate with the numbers of metachromatically staining cells, presumed to be mast cells and/or basophils (McGorum *et al.,* 1993b). However, although the potential involvement of mast cells and mast cell-derived mediators is also consistent with an IgE mediated hypersensitivity response, it is possible that other factors such as bacteria, fungal spores and endotoxin result directly in mast cell degranulation (Clementsen *et al.,* 1991; Norn *et al.,* 1994; Larsen *et al.,* 1996; Brown *et al.,* 1998; Iuvone *et al.,* 1999) causing a non-IgE mediated mast cell-induced airway response (Mehlhop *et al.,* 1997). In addition, the *sole* involvement of a type I hypersensitivity in heaves is unlikely given the usual clinical presentation of heaves susceptible horses following exposure to mouldy hay/straw environments where an early (<1h) onset dyspnoea is absent.

Therefore despite the evidence for a hypersensitivity response in heaves, it would appear that this does not exclusively involve an IgE mediated type I response,

although the balance between the involvement of a type I and type III (and possibly type IV) response may vary among individual subjects (Halliwell *et al.,* 1979). The involvement of a hypersensitivity response is further supported by the fact that only certain susceptible individuals develop all the characteristic features of heaves, namely airway neutrophilia, obstructive pulmonary disease and bronchial hyperreactivity, when exposed to dusty environments, which have little or no effect on the majority of the horse population. Of the above characteristics, airway obstruction is the most likely to result in overt clinical signs and thus initiate further diagnostic intervention. Therefore it is probable that less severely affected horses remain undiagnosed, and that a larger spectrum of disease severity than is currently appreciated likely exists. Upon consideration of the variety of components present in stable dust, it is possible that the distinction between clinical and sub-clinical disease may not only reflect variation in the degree of sensitivity to inhaled allergens, but also variation in the response to other inhalants through non-allergenic mechanisms.

1.1.3 Pathology of heaves

The principal lesion in heaves-affected horses is bronchiolitis (Nicholls, 1978). Airway wall thickness increases are due to increased thickness of the epithelium, submucosa and smooth muscle. Mucus accumulates within the lumen of airways and adjacent alveoli. Peribronchial accumulations of inflammatory cells, principally lymphocytes, are accompanied by intraluminal accumulations of neutrophils, and very rarely eosinophils (Derksen *et al.,* 1985b; Yamashiro *et al.,* 1985; McGorum *et al.,* 1993d). Alveolar epithelial changes include necrosis of type 1 alveolar epithelial

cells with replacement by type II alveolar epithelium. Mucus cell metaplasia and hyperplasia is seen, in addition to Clara cell degranulation and loss (Kaup *et al.* 1990a). Focal loss of ciliated cells occurs in the larger airways followed by replacement by undifferentiated cells in a hyperplastic epithelium (Kaup *et al.* 1990b).

1.1.4 Pathophysiology of heaves

The airway dysfunction and resultant clinical abnormalities can largely be related to the airway obstruction. This obstruction occurs both as a result of airway narrowing associated with inflammation and to contraction of bronchial and bronchiolar smooth muscle. Airway smooth muscle contraction is caused by the complex actions of inflammatory mediators on smooth muscle and on the afferent and efferent neural arcs which control airway calibre (Robinson *et al.,* 1996).

Some of the clinicopathological changes associated with heaves are fully reversible following a relatively short period (4-24 days) of exclusion from the causal agent(s) (Thomson and McPherson, 1984). Consequently, animals in disease remission have no clinically detectable pulmonary dysfunction, airway neutrophilia nor increased airway reactivity, however some structural airway changes are longer lasting and include epithelial metaplasia and hyperplasia and smooth muscle hyperplasia (Robinson *et al.,* 1996). Additionally it has recently been demonstrated that mucus

hypersecretion persists for long periods after affected horses are moved to an allergen free environment (Dixon *et al.* 1995b).

1.2 Organic Dusts and Occupational Lung Disease in Humans

1.2.1 Background

Organic dust has been defined as dust that originates from plant or animal matter (Heederik *et al.,* 2000). It has been well established that high levels of airborne organic dust occur in many agricultural and industrial environments, including poultry housing (Clark *et al.,* 1983b; Wiegand *et al.,* 1993), farm buildings (Lundholm *et al.,* 1986; Olenchock *et al.,* 1986; Rylander, 1986), various animal houses (Pickrell, 1991; Dutkiewicz *et al.,* 1994), dairy farms (Malmberg, 1990; Louhelainen *et al.,* 1997; Kullman *et al.,* 1998), compost plants (Clark *et al.,* 1983a), equine stables (Crichlow *et al.,* 1980; Webster *et al.,* 1987; Clarke, 1993; McGorum *et al.,* 1998), cotton mills (Rylander, 1987) and saw mills (Douwes *et al.,* 2000a; Mandryk *et al.,* 2000). It is also well recognised that many agricultural and forestry occupations are associated with a high incidence of respiratory symptoms such as those reported amongst swine workers (Larsson *et al.,* 1994; Zejda *et al.,* 1994; Preller *et al.,* 1995a; Schwartz *et al.,* 1995a; Reynolds *et al.,* 1996; Cormier *et al.,* 1997; Wang *et al.,* 1997; Nowak, 1998; Vogelzang *et al.,* 1998; Vogelzang *et al.,* 2000), animal feed workers (Jorna *et al.,* 1994; Smid *et al.,* 1994; Kuchuk *et al.,* 2000), grain workers (Schwartz *et al.,* 1995b), potato processing workers (Zock *et al.,* 1995), poultry workers (Zuskin *et al.,* 1995; Donham *et al.,* 2000), cotton workers (Rylander, 1987; Rylander, 1990; Sigsgaard *et al.,* 1992; Jacobs *et al.,* 1993; Li *et al.,*

1995; Christiani *et al.,* 1999) trout workers (Sherson *et al.,* 1989), sawmill workers (Mandryk *et al.,* 2000) and crop farmers (Monso *et al.,* 2000).

It has recently been suggested that rather than linking clinical disease with a specific environment, symptoms should be related to the relevant pulmonary cellular reactions, particularly inflammation (Rylander, 1992). This has led to the realisation that many of the reported clinical symptoms associated with a particular occupation also occur in workers in other organic dust environments, thus identifying a common link, namely airborne organic dust (Rylander, 1992). In addition to establishing a link between agricultural occupations which result in a high organic dust exposure and respiratory symptoms and/or a reduction in lung function, many studies have also identified an association between certain agricultural occupations and an increase in markers of inflammation in blood (Sigsgaard *et al.,* 1992; Borm *et al.,* 1996; Thorn *et al.,* 1998; Wang *et al.,* 1998; Rylander *et al.,* 1999; Sjogren *et al.,* 1999), nasal lavage fluid (Cormier *et al.,* 1997; Larsson *et al.,* 1997; Wang *et al.,* 1997; Keman *et al.,* 1998; Borm *et al.,* 2000; Douwes *et al.,* 2000b), BALF (Cormier *et al.,* 1997; Larsson *et al.,* 1997; Wang *et al.,* 1997) and sputum (Thorn *et al.,* 1998). Consequently agriculture is considered one of the most hazardous occupations with respect to human health (Kirkhorn and Garry, 2000). Furthermore, the changing patterns of agriculture, such as increases in animal density within confinement houses, has resulted in increased exposure to respiratory hazards in the workplace (Kirkhorn and Garry, 2000).

1.2.2 Organic dust inhalation studies

As part of the recent extensive investigation into the effects of organic dust inhalation, new models for organic-dust induced disease have been established using the criteria developed for disease among workers in cotton mills and swine confinement buildings (Rylander, 1992; Castranova *et al.,* 1996). Controlled acute inhalation studies have been conducted using dry dust challenges in humans (Cavagna *et al.,* 1969; Wang *et al.,* 1996a), pigs (Holst *et al.,* 1994; Urbain *et al.,* 1996b), guinea pigs (Rylander, 1988; Gordon, 1990; Frazer *et al.,* 1993; Gordon, 1994) mice (Ryan *et al.,* 1994; Shvedova *et al.,* 1996) and rabbits (Cavagna *et al.,* 1969), and organic soluble aqueous dust extracts in humans (Clapp *et al.,* 1994; von Essen *et al.,* 1995a; Blaski *et al.,* 1996; Jagielo *et al.,* 1996b; Jagielo *et al.,* 1997; Trapp *et al.,* 1998), mice (Jagielo *et al.,* 1996a; Jagielo *et al.,* 1998; WohlfordLenane *et al.,* 1999) and rabbits (Cavagna *et al.,* 1969). In addition, a few long-term dry dust challenges have been conducted in animal models (Jolie *et al.,* 1999). These models have been successful in reproducing many of the clinical symptoms, and lung dysfunction and inflammation, observed in agricultural workers exposed naturally to organic dusts. In addition, they have improved the understanding of the underlying disease mechanisms.

Inhalation of organic dusts or organic dust extracts results in an increase in the numbers of granulocytes in blood (Jagielo *et al.,* 1997) and BALF (Rylander, 1988; Frazer *et al.,* 1993; Clapp *et al.,* 1994; Ryan *et al.,* 1994; von Essen *et al.,* 1995a; Jagielo *et al.,* 1996a; Jagielo *et al.,* 1996b; Shvedova *et al.,* 1996), an increase in

vascular permeability (Gao *et al.,* 1993) and airway oedema (Gordon, 1990). It also results in an increased concentration and/or expression of inflammatory cytokines (e.g. TNF-alpha, IL-6, IL-1α, IL-1β, IL-8 and IFN-γ) in serum (Shvedova *et al.,* 1996; Wang *et al.,* 1996b), BALF (Clapp *et al.,* 1994; Ryan *et al.,* 1994; Jagielo *et al.,* 1996a; Jagielo *et al.,* 1996b; Shvedova *et al.,* 1996) and/or induced sputum (Park *et al.,* 1998), as well as increased expression of chemokines (e.g. MIP-2) in BALF cells (WohlfordLenane *et al.,* 1999) and increased neutrophil elastase in induced sputum (Park *et al.,* 1998). Both *in vitro* cell culture work and *in vivo* inhalation challenges have provided evidence for the involvement of macrophages, epithelial cells, neutrophils and mast cells in the local pulmonary production of cytokines and mediators in response to organic dust extract (Becker *et al.,* 1999).

1.3 The Role of Endotoxin in the Aetiology of Organic Dust-Induced Disease

It is generally accepted that organic dusts contain a wide variety of components, many of which have been shown to induce either sensitisation or inflammation, when inhaled (Rylander, 1994). However, endotoxins are universally present within all types of organic dusts, and there is increasing evidence that inhaled endotoxin plays a major role in organic dust-induced lung disease.

1.3.1 Endotoxin overview

Endotoxin is perhaps the most important cause of organic dust-induced pulmonary disease (Jacobs, 1997a). It is present in the cell walls of gram-negative bacteria,

which are commonly found in organic dusts (Jacobs *et al.*, 1997). The terms "endotoxin" and "lipopolysaccharide" (LPS) are often used interchangeably, however the term lipopolysaccharide should be reserved to denote chemically pure substances, free from other chemical compounds that can be found in gram-negative bacterial cell walls. The term endotoxin refers to fragments of the gram-negative bacterial cell wall that contain LPS as well as other naturally occurring compounds (Jacobs *et al.*, 1997).

The LPS molecule contains a lipid region (lipid A), and a long covalently linked heteropolysaccharide. The polysaccharide portion is divided into a "core" portion and the O-specific chain, a division made on the basis of chemical composition, structure, mode of biosynthesis and function (Jacobs *et al.*, 1997). The lipid A molecule shows the least variation of all the components of the LPSs in all bacterial families and is primarily responsible for LPS toxicity, which is determined by the presence of 2 D-glucosamine residues that are β-(1-6)-linked, and the phosphoryl groups. Additionally, the location of fatty acids, the number of acyl chains present, the acyl length, and the configuration of the –OH fatty acids are of great importance to endotoxin activity (Rietschel *et al.*, 1987; Rietschel *et al.*, 1990).

Structural variation of the core polysaccharide within a bacterial species tends to be low, with only five known types in the family Enterobacteriaceae (Jacobs *et al.*, 1997). The O-specific chain shows the greatest structural diversity of all the molecular components of LPS (Rietschel *et al.*, 1990). It is composed of 20-40 repeated "oligosaccharide units", the number being dependant on both bacterial strain

characteristics and growth conditions (Jacobs *et al.*, 1997). In addition, the type of monosaccharide and the nature, sequence and type of sugar linkage of the monosaccharide units within the oligosaccharide units vary with each bacterial genus and species (Rietschel *et al.*, 1990).

Like other membrane molecules, LPS is amphipathic, i.e. has both hydrophobic and hydrophilic parts (Taussig, 1984). Thus, in aqueous solution it forms large micelles with the lipid on the inside. Although the lipid A component of LPS determines toxicity, the antiphagocytic effect of LPS can be attributed to the very hydrophilic nature of the O-side chains, thus contributing to virulence (Taussig, 1984). Therefore LPS is a very heterogenous molecule, the biological effects of which are largely dependant on molecular structure.

Various cell types can respond to endotoxins, including polymorphonuclear leucocytes, lymphocytes, epithelial cells, endothelial cells and mast cells, although monocytes/macrophages are the most investigated cell populations which respond to endotoxins (Ulmer, 1997). Each individual cell type reacts in a typical way, however in general these reactions involve mediator production, phagocytosis, proliferation and/or differentiation (Ulmer, 1997). CD14 is the prominent LPS-binding structure on monocytes/macrophages, and interaction of LPS with CD14 is necessary for the specific activation of monocytes or macrophages (Ulmer, 1997). This binding can be catalysed by LPS binding protein (LBP) (Martin *et al.*, 1992), which is traditionally considered to be serum-derived. However, recent work has demonstrated that human

respiratory epithelial cells can also produce this acute phase protein (Dentener *et al.*, 2000). The potential importance of LBP in the lung was highlighted by work which demonstrated an enhanced LPS-induced TNF-alpha gene expression in human and rabbit alveolar macrophages *in vitro* when LPS was complexed with LBP (Martin *et al.*, 1992). In addition, a cell surface co-receptor for CD14, termed the toll-like receptor 4 (TLR 4) has recently been identified which permits LPS-mediated signal transduction (Chow *et al.*, 1999; Ingalls *et al.*, 1999).

1.3.2 Airborne endotoxin in agricultural environments

Not surprisingly, high levels of airborne endotoxin have been detected in environments which have high levels of organic dust, such as the farming industry (Lundholm *et al.*, 1986; Olenchock *et al.*, 1986; Anon, 1989), particularly in pig housing (Clark *et al.*, 1983b; Dutkiewicz *et al.*, 1994; Preller *et al.*, 1995a; Preller *et al.*, 1995b; Schwartz *et al.*, 1995a) and poultry housing (Clark *et al.*, 1983b; Wiegand *et al.*, 1993). Elevated levels of airborne endotoxin have also been detected in other working environments including the cotton industry (Christiani *et al.*, 1993; Li *et al.*, 1995), linen industry (Buick *et al.*, 1994), fibreglass industry (Milton *et al.*, 1995) and potato industry (Zock *et al.*, 1995). Despite this apparent relationship between organic dust and endotoxins and the potentially important biologic effects of organic dusts which contain endotoxins, there is general agreement that the relative amounts of endotoxins in different dusts may vary (Rylander, 1997b). Consequently, several epidemiologic investigations have been conducted in order to determine

whether a relationship existed between endotoxin exposure and disease in exposed subjects.

1.3.3 Correlation between respiratory symptoms and airborne endotoxin exposure

Several studies have identified a correlation between airborne endotoxin exposure and respiratory symptoms, airway inflammatory markers and/or lung dysfunction (Sigsgaard *et al.,* 1992; Rylander and Bergstrom, 1993; Teeuw *et al.,* 1994; Reynolds *et al.,* 1996; Douwes and Heederik, 1997; Wang *et al.,* 1997; Keman *et al.,* 1998; Vogelzang *et al.,* 1998; Donham *et al.,* 2000; Douwes *et al.,* 2000b). In most instances, the airborne endotoxin is in airborne organic dust. However, the role of airborne endotoxin in disease induction is further supported by many studies, which have found that despite a good correlation with endotoxin exposure, respiratory symptoms are poorly correlated or unrelated to the total level of atmospheric dust (Jorna *et al.,* 1994; Smid *et al.,* 1994; Zejda *et al.,* 1994; Schwartz *et al.,* 1995b).

Despite this relationship, these studies did not prove causality, which requires the following requirements to be met: (a) endotoxin must be identified in all environments that cause similar symptoms, (b) endotoxin must be capable of producing the signs and symptoms of the disease in subjects challenged with pure LPS, (c) there must be a demonstrable relationship between the prevalence of the disease and the exposure levels, and (d) there must be a decrease of symptoms after reduction in endotoxin exposure (Rylander, 1997b). The reasons for caution in attributing too much significance to the relationship between endotoxins and clinical

symptoms includes the fact that organic dusts contain many other biologically potent agents, such as bacterial enzymes, tannins, mycotoxins, and β-D-glucan (Rylander, 1997b). It is therefore possible that endotoxin may either act as a surrogate marker for some of these agents, or act in combination with them, either in an additive or synergistic fashion, to result in pulmonary disease.

1.3.4 Endotoxin tolerance

It is well recognised that many of the symptoms experienced by persons working in the cotton industry, who are exposed to cotton dust on a daily basis throughout the working week, are more severe on the first workday of the week (Rylander, 1988; Rylander, 1994). It has been proposed that this reduction in symptom severity throughout the week may reflect a degree of tolerance to inhaled endotoxin, a phenomenon that has recently been demonstrated experimentally in both rats and mice following repeated exposure to inhaled endotoxin (Elder *et al.,* 2000a; Shimada *et al.,* 2000). This information therefore further supports the role of inhaled endotoxin in organic dust induced disease.

1.3.5 Inhalation challenges

1.3.5.1 Endotoxin inhalation challenges

Endotoxin inhalation/instillation challenges have been conducted in humans (Rylander *et al.,* 1989; Sandstrom *et al.,* 1992; Sandstrom *et al.,* 1994; Michel *et al.,* 1995a; Michel *et al.,* 1995b; Rolla *et al.,* 1997) guinea pigs (Snella *et al.,* 1987; Gordon, 1994; Hsieh *et al.,* 1994; Uno *et al.,* 1996; Uno *et al.,* 1997), pigs (Urbain *et*

al., 1996a), rats (Gordon and Harkema, 1993; Ulich *et al.,* 1993; Gordon and Harkema, 1994), hamsters (Lantz *et al.,* 1991) and mice (Ryan *et al.,* 1994; Chignard and Balloy, 2000). These studies invariably show a neutrophil influx into the airways following LPS inhalation (Sandstrom *et al.,* 1992; Caillaud *et al.,* 1996; Michel, 1997), as detected by cytological evaluation of BALF (Sandstrom *et al.,* 1994; Chignard and Balloy, 2000; Larsson *et al.,* 2000) or induced sputum (Nightingale *et al.,* 1998; Thorn and Rylander, 1998b; Michel, 2000). The influx of neutrophils into the airway occurs in a dose-dependant manner (Gordon, 1992; Urbain *et al.,* 1996a; Michel *et al.,* 1997), and may be mediated in part by the intrapulmonary release of cytokines such as IL-1-β and IL-8, the concentration of which correlate with neutrophil numbers following LPS inhalation (Wesselius *et al.,* 1997). An increase in the concentration of TNF-α also appears to be related to neutrophil influx (Ulich *et al.,* 1993), however functional blockade of TNF-alpha in mice failed to prevent a neutrophilic inflammatory response following LPS inhalation (Moreland *et al.,* 2001). In addition, an increased mRNA message for the chemokines MIP-1 and MIP-2 in BALF cells following LPS challenge is supportive of their involvement in neutrophil recruitment (Johnston *et al.,* 1998).

Other markers of inflammation which increase after LPS inhalation include elastase (Nagai *et al.,* 1991; Kawabata *et al.,* 2000), collagenase (Nagai *et al.,* 1991), platelet activating factor (Rylander and Beijer, 1987; Lantz *et al.,* 1991; Dallal and Chang, 1992), fibronectin (Sandstrom *et al.,* 1992; Sandstrom *et al.,* 1994), nitric oxide (NO) (Rolla *et al.,* 1997) and NO metabolites (Toward and Broadley, 2000). Also several studies have demonstrated an increase in airway epithelial and pulmonary

microvascular permeability (Li *et al.,* 1998; Chignard and Balloy, 2000), mucus cell metaplasia (Gordon and Harkema, 1993; Gordon *et al.,* 1996) and increased mucin secretion (Tesfaigzi *et al.,* 2000) following inhaled/instilled endotoxin challenge. In addition to the induction of local pulmonary inflammation, LPS inhalation also results in systemic inflammation (Michel *et al.,* 1992a; Michel *et al.,* 1995a; Michel *et al.,* 1997; Michel *et al.,* 2000) and symptoms [e.g. chest tightness, airway irritation] (Rylander *et al.,* 1989; Michel, 2000), reduced lung function (Rylander *et al.,* 1989; Michel *et al.,* 1992a; Michel *et al.,* 2000) and an increase in bronchial responsiveness/reactivity (Nagai *et al.,* 1991; Vincent *et al.,* 1993; Jarreau *et al.,* 1994; Rylander, 1996; Michel, 2000; Toward and Broadley, 2000).

The mechanisms involved in this LPS induced increase in bronchial responsiveness/reactivity are unclear, however NO, platelet-derived products (Vincent *et al.,* 1993), collagenase and elastase (Nagai *et al.,* 1991), TNF (Uno *et al.,* 1996) and tachykinins (Loeffler *et al.,* 1997) have all been proposed as playing a regulatory role. However, even within a healthy population, an LPS response phenotype exists, whereby LPS inhalation results in the distinct separation of the population into sensitive, intermediate and hyporesponsive individuals with respect to both lung obstruction and *in vitro* LPS-induced cytokine release from blood monocytes and lung macrophages (Kline *et al.,* 1999). It has recently been demonstrated that LPS hyporesponsiveness in humans is related to mutations in TLR 4 (Arbour *et al.,* 2000), a phenomenon which has previously been identified in LPS tolerant/hyporesponsive mice (Hoshino *et al.,* 1999; Qureshi *et al.,* 1999).

1.3.5.2 Similarities between endotoxin and organic dust extract inhalation challenges

Upon comparison of the results of separate endotoxin inhalation challenges and organic dust extract inhalation challenges, it can be concluded that the responses to both challenge systems are similar, namely they are both characterised by a predominantly neutrophilic, obstructive airway disease which is associated with a degree of bronchial hyperreactivity. Although such a comparison suggests a role for inhaled endotoxin in organic dust-induced disease in man, this hypothesis has been further supported by other studies, which have *directly* compared the effects of endotoxin inhalation and organic dust extract inhalation (Clapp *et al.,* 1993; Jagielo *et al.,* 1996b; WohlfordLenane *et al.,* 1999). These studies demonstrated that at equivalent LPS exposure levels, challenge with both soluble LPS and corn dust extract resulted in similar symptoms, changes in airflow, and increases in BALF inflammatory cells and mediators.

1.3.5.3 The role of endotoxin in the response to inhaled organic dust/organic dust extract

A few studies have further highlighted the *specific* role of endotoxin in organic dust-induced disease using mice which were either genetically resistant to endotoxin, or which were rendered endotoxin tolerant by daily injections of increasing doses of LPS (Ryan *et al.,* 1994; Schwartz *et al.,* 1994; George *et al.,* 2001). These mice developed significantly less severe pulmonary inflammation than controls following both acute and chronic inhalation challenge with corn dust extract (Schwartz *et al.,* 1994; George *et al.,* 2001) and exposure to airborne cotton dust (Ryan *et al.,* 1994).

In addition, although both endotoxin-sensitive and endotoxin-resistant mice developed airway hyperreactivity following chronic corn dust extract inhalation challenge, this hyperreactivity persisted only in the endotoxin-sensitive mice (George *et al.,* 2001). In support of these findings, the *in vitro* release of TNFα from alveolar macrophages in response to wool dust leachates was significantly reduced if the macrophages were harvested from mice which were genetically resistant to endotoxin (Brown and Donaldson, 1996). Furthermore, depletion of endotoxin activity from corn dust extract significantly reduced the pulmonary inflammatory response following inhalation challenge in mice (Jagielo *et al.,* 1996a). Similarly, intratracheal pretreatment of mice with the relatively biologically inactive partial agonist of LPS, diphosphoryl lipid A, before exposure to corn dust extract significantly reduced pulmonary inflammation (Jagielo *et al.,* 1998).

The aforementioned evidence indicates that the inflammatory response to inhaled organic dusts is largely attributable to the dust endotoxin activity. However with the exception of Ryan *et al's.* (1994) study, which only evaluated the pulmonary inflammatory and not the functional response to cotton dust exposure, all studies investigating the role of endotoxin have used a soluble extract of organic dust. This may be a criticism since such a model does not permit the evaluation of the role of the particulate components of airborne organic dust in the overall pulmonary inflammatory and functional response.

1.3.6 Interaction between endotoxins and other organic dust components

Despite the overwhelming evidence for the major contribution of inhaled endotoxin in the response to inhaled organic dust, it would be naïve, given the presence of other agents present within these dusts, to consider that endotoxins are solely responsible for pulmonary disease induction. Indeed many other organic dust components have pro-inflammatory properties, including plant tannins, proteins, β-D-glucan, mould spores, bacteria, proteases and mycotoxins (Rylander, 1994; Milanowski *et al.,* 1995a; Milanowski *et al.,* 1995b). Furthermore, there is sufficient evidence that additional endotoxin independent mechanisms exist by which organic dusts can cause pulmonary inflammation (von Essen *et al.,* 1995b). It is therefore likely that many of these components act in an additive fashion, resulting in an inflammatory response in the host, which is related to the relative composition of the inhaled organic dust particles. In addition both *in vitro* and *in vivo* studies have provided evidence to support a complex interaction and/or synergistic relationship between endotoxin and various other organic dust components such as β-D-glucan (Fogelmark *et al.,* 1994; Rylander, 1994; Fogelmark *et al.,* 2001), fungal spores (Shahan *et al.,* 1994) and carbonaceous particles (Oberdorster, 2000).

1.4 The response to inhaled endotoxins/organic dusts in subjects with allergic respiratory disease

1.4.1 The response of human asthmatics to inhaled/airborne grain dust or endotoxin

Despite some conflicting reports in the literature, it is generally accepted that asthmatic subjects can demonstrate higher sensitivity to inhaled LPS than normal subjects (Alexis *et al.,* 2001), and consequently safety guidelines for endotoxin exposure levels are based on values for subjects with histories of atopy or asthma (Rylander, 1997b). Although it is generally accepted that organic dust-induced pulmonary disease represents a non-allergic inflammatory response, primarily to endotoxin, some studies have shown that organic dust exposure results in a significantly greater degree of pulmonary dysfunction in subjects with a history of respiratory allergy compared to healthy subjects (Jacobs *et al.,* 1993). Furthermore, inhalation challenge of asthmatic and non-asthmatic subjects with endotoxin has demonstrated a difference in their response. Asthmatic subjects developed a slight but significant bronchial obstruction and an increase in non-specific bronchial reactivity following inhalation of 22.2μg LPS, but no change in reactivity or pulmonary mechanics was noted in the non-asthmatic group (Michel *et al.,* 1989; Michel *et al.,* 1995a). However, conflicting views exist regarding the association between allergy and the response to endotoxin and organic dust, since other studies have indicated that the lung function response of human subjects to inhaled grain dust extract is independent of pre-existing asthma (Clapp *et al.,* 1994). Furthermore, other studies found that LPS-induced bronchial obstruction is associated with non-specific

responsiveness but not with atopy (Michel *et al.,* 1992b), and that the atopic status of an individual was not a significant determinant of airflow obstruction or lower airway inflammation following organic dust inhalation (Blaski *et al.,* 1996).

1.4.2 Mechanisms of enhanced response of asthmatics to inhaled endotoxin/organic dust

The mechanisms involved in the enhanced response of asthmatics to inhaled grain dust and/or endotoxin are unclear, particularly in light of some conflicting results. In those studies that identified an enhanced response in asthmatics, it is possible that this resulted from a degree of pre-existing subclinical pulmonary disease in these subjects at the time of the endotoxin inhalation challenges and/or organic dust exposures. It is also possible that the organic dust also contained allergens to which the individual was sensitive. Alternatively, an inherent increased sensitivity to endotoxin may exist in atopic/asthmatic subjects. Consistent with this, peripheral blood leucocytes from asthmatics were more susceptible to LPS than those from healthy individuals with respect to sulpholeukotriene release (KrausFilarska *et al.,* 1998). Furthermore, an increased level of constitutive CD14 expression, as demonstrated in atopics, may contribute to an increased LPS sensitivity in asthmatic subjects, as the constitutive sCD14 expression was a good predictor of the magnitude of neutrophil response in induced sputum following LPS inhalation (Alexis *et al.,* 2001).

1.4.3 The role of combined allergen and endotoxin as a determinant of disease severity

In most natural environments, instead of being exposed to single airborne agents, allergic asthmatics are exposed to a combination of allergen and endotoxin (Michel *et al.,* 1991). Studies in humans have shown that endotoxin exposure exacerbates asthmatic symptoms in subjects who are allergic to house dust mites and other allergens (Michel, 1996; Rizzo *et al.,* 1997). Additionally, the concentration of endotoxin in house dust is a more important determinant of asthma severity in house dust mite-sensitized subjects than the concentration of house dust mite allergen (Michel *et al.,* 1991; Michel *et al.,* 1996). Endotoxin exposure has also been shown to increase the risk of wheezing during the first year of life in children with a familial predisposition to asthma or allergy (Park *et al.,* 2001). It has also been speculated that the increased incidence of asthma may not only reflect the increase in environmental allergen concentrations but also increased concentrations of dust components, such as endotoxins, that enhance the response of the lungs to foreign proteins (PlattsMills *et al.,* 1997). There is some evidence to suggest that the increased severity of disease in asthmatics co-exposed to allergen and endotoxin is related primarily to an increase in pulmonary inflammation. For example the pulmonary inflammatory response of ovalbumin (OVA) sensitised mice exposed to a combination of OVA and LPS was significantly greater than that induced by OVA alone (Goldsmith *et al.,* 1999; Tulic *et al.,* 2000). It is interesting to note that this OVA/LPS combination did not increase the degree of airway hyperresponsiveness, and in one study, LPS inhalation abolished the OVA-induced hyperresponsiveness (Tulic *et al.,* 2000).

Sufficient evidence now exists to indicate that airway exposure of atopic asthmatics to combined allergen and endotoxin results in greater pulmonary inflammation and possibly severity of symptoms than those observed with either stimulus alone (Eldridge and Peden, 2000).

1.4.4 Mechanisms of enhanced response to combined allergen and endotoxin stimulus

Several possibilities exist as to the mechanisms by which inhaled endotoxin may enhance the response to inhaled allergen, and thus influence the severity of asthmatic lung disease. Firstly, ragweed antigen inhalation in ragweed allergic asthmatics results in a profound increase in the concentration of the accessory molecules LBP and soluble CD14 in BALF, thus enhancing the capacity of inhaled LPS to activate an inflammatory cascade (Dubin *et al.,* 1996). Although respiratory epithelial cells are now recognised as a potential source of LBP (Dentener *et al.,* 2000), the increased BALF levels of this protein following allergen challenge is likely to result from the allergen induced increase in pulmonary epithelial permeability (Folkesson *et al.,* 1998). In support of the role of LBP, allergen-sensitised LBP-deficient mice failed to develop substantial inhaled allergen-induced airway reactivity compared with allergen sensitised wild type mice (Strohmeier *et al.,* 2001).

In addition, endotoxins can act as adjuvants for delayed hypersensitivity and IgE production, and enhance antigen-specific mediator release (Norn, 1994; Williams and Halsey, 1997). Inhaled endotoxin also down-regulates repeated, antigen exposure-

induced IgE isotype-specific tolerance, thus potentiating allergen-specific airway inflammation (Wan *et al.,* 2000). Furthermore, inhalation of LPS-stimulated allergen (OVA)-specific antibody production in sensitised guinea pigs (Rylander and Holt, 1998). Therefore is possible that as well as allergen potentially increasing the inflammatory response to LPS, LPS may also increase the inflammatory response to allergen. In fact, it is probable that both of these mechanisms occur resulting in a synergistic effect between inhaled allergen and endotoxin.

1.4.5 The possible role of endotoxin in determining the predominant airway inflammatory cell type in asthma and heaves

Despite the proposed similarities between heaves and allergic lung disease in humans, one major difference between these diseases is the predominant inflammatory cell recruited to the airways. Heaves is characterised by an airway neutrophilia (Robinson *et al.,* 1996). In contrast, allergic asthma is characterised by an airway eosinophilia (Coyle *et al.,* 1996), although there is a late neutrophilic response to intra-bronchial allergen challenge that may reflect a response to endotoxin (Hunt *et al.,* 1992). It should be noted however that in the studies by Goldsmith *et al.* (1999) and Tulic *et al.* (2000), which investigated the response to inhaled LPS and allergen in sensitised mice, the predominant inflammatory cell type was the neutrophil. In addition, the neutrophilic response following inhaled allergen challenge in some human asthmatics resulted from endotoxin contamination of the allergen extract (Hunt *et al.,* 1994). Also, the predominantly neutrophilic influx noted in acute, fatal asthma may be due to inhalation of endotoxin-coated mould spores (Sur *et al.,* 1993).

In addition to inducing a direct neutrophilic inflammatory response LPS, may inhibit eosinophil migration following allergen challenge, as demonstrated in the mouse peritoneum (Schimming *et al.,* 1997). Also, inhalation of β-D-glucan, a component of fungal cell walls which is likely to be present in high quantities in mouldy stable dust, decreased the allergen-induced airway eosinophilia associated with OVA inhalation in sensitised guinea pigs (Rylander and Holt, 1998). Furthermore, the β-D-glucan-induced BALF eosinophilia, reported in guinea pigs following a 5week exposure, was absent when animals were co-exposed to LPS (Fogelmark *et al.,* 2001).

Therefore, possible reasons why heaves is characterised by an airway neutrophilia include the fact that dusty stables almost invariably contain high levels of endotoxin (Dutkiewicz *et al.,* 1994). Alternatively, neutrophil recruitment in heaves may result from the fact that active nuclear factor-κB (NF- κB) in bronchial epithelial and BAL cells from heaves-affected horses is mainly p65 homodimers, rather than classical p65-p50 heterodimers (Bureau *et al.,* 2000; Lekeux *et al.,* 2001). The p65 homodimers induce intercellular adhesion molecule-1 (ICAM-1) (Ledebur and Parks, 1995) and IL-8, a potent neutrophil chemo-attractant (Schulte *et al.,* 2000), and not eotaxin, an eosinophilic chemo-attractant which is under p65-p50 control (Matsukura *et al.,* 1999). However there is evidence to suggest that LPS-induced expression of at least ICAM-1 may also be dependent on the binding of p65 homodimers to the same κB binding site, suggesting that the endotoxin content of stable dust may still be partly responsible for the neutrophil influx in heaves (Ledebur and Parks, 1995). In addition, since mast cell derived tryptase directly stimulates IL-8 expression and

upregulates ICAM-1 expression (Cairns and Walls, 1996), mast cell degranulation may also play a critical role in neutrophil recruitment in heaves, following either IgE-mediated mechanisms and/or direct stimulation by endotoxin (Norn *et al.,* 1994) or fungal spores (Larsen *et al.,* 1996).

1.5 Possible interactions between different potential components of stable dust

Certain airborne stable dust components, including proteases, fungal antigens and glucan, may directly induce pulmonary inflammation by non-endotoxin-mediated and non-allergenic mechanisms (Milanowski, 1996; Milanowski, 1997; Iadarola *et al.,* 1998; Milanowski, 1998; Schuyler *et al.,* 1998). In addition, these agents may also potentiate the effects of other dust components such as endotoxin and allergen. For example, fungal spores and endotoxin have a synergistic effect on superoxide anion release from guinea pig BALF cells (Shahan *et al.,* 1994). In addition, other components of organic dusts, including mould spores, bacteria and endotoxins have a synergistic effect on histamine release from human basophil cells via non-immunological mechanisms (Norn *et al.,* 1994). Furthermore, the type of pulmonary inflammation induced by inhalation of a combination of glucan and endotoxin differed histologically from that following inhalation of each individual component, with the former inducing granuloma formation (Fogelmark *et al.,* 1994).

Although little information exists relating to the synergism between organic dust particles and endotoxin, extensive research has been carried out on the potentiating

effects between urban air particles and endotoxin. For example, endotoxin markedly increased cytokine expression of rat and human alveolar macrophages exposed to urban air particles (Dong *et al.,* 1996; Soukup and Becker, 2001), and pre-exposure of rats to LPS, or pre-incubation of rat or human lung derived macrophages with LPS, amplifies lung inflammation and TNF production in response to air particles and ultrafine carbon particles (Imrich *et al.,* 1999a and b; Elder *et al.,* 2000b). Additionally, synergism between air pollutants and allergen has also demonstrated whereby inhalation challenge with leachate of residual oil fly ash (a surrogate of ambient air particles) significantly increased allergen (OVA)-induced airway hyperresponsiveness and inflammation in sensitised mice (Iadarola *et al.,* 1998).

The level of gaseous pollution in equine stables is largely unknown, however levels of ammonia and hydrogen sulphide do tend to rise where deep litter management of bedding is practiced and when there is poor drainage of urine (Clarke, 1987a). Although the safety levels for noxious gases are unknown, it is possible that excessively high levels may result in respiratory inflammation, either directly or indirectly, via interaction with dust components. For example, ozone-pretreatment of bovine alveolar macrophages had an additive effect on TNF release following LPS stimulation (Mosbach *et al.,* 1996). In addition, work on animal models of allergic asthma has shown that exposure to air pollutants such as nitrogen dioxide and ozone may increase levels of serum and pulmonary allergen-specific antibody thus exacerbating immune-mediated lung disease (Gilmour, 1995).

Therefore there is much potential for numerous additive and synergistic interactions to occur, between many different airborne components which may be present within stables. It is probable that what ultimately determines the severity of disease is largely dependent on the relative quantities of these different agents within the airborne environment, in addition to the subjects degree of "sensitivity" to each component. Many of these interactions may involve endotoxin, therefore although the direct response to inhaled endotoxin may be inflammatory, the co-exposure to endotoxin and other agents present in organic dusts may result in an amplified response involving both immunological and non-immunological mechanisms.

1.6 Hypotheses

There are many similarities between equine heaves and organic-dust mediated lung disease in humans and animal models; namely both conditions are characterised by a reversible pulmonary neutrophilia and obstructive lung dysfunction following exposure to certain environments. It is now generally accepted that organic-dust mediated lung disease in humans is due largely to the inhalation of endotoxin. This has been demonstrated firstly by the association between respiratory symptoms in subjects suffering from organic dust-mediated lung disease and the levels of endotoxin exposure, and secondly, by the similarities between the physiological and inflammatory effects of endotoxin and organic dust inhalation.

Upon consideration of the similarities between these two diseases, it is therefore highly probable that endotoxin inhalation contributes to the aetiopathogenesis of

heaves, particularly as equine stables contain relatively high levels of airborne endotoxin. Despite the evidence for an underlying pulmonary hypersensitivity to moulds in heaves, it is probable that endotoxin inhalation contributes to the severity of disease, particularly as an exaggerated response to inhaled endotoxin occurs in some humans with pre-existing allergic respiratory disease.

Consequently, following consideration of the above information, the work detailed in this thesis was initiated to investigate two initial hypotheses:

(1) Exposure to high levels of airborne endotoxin will result in non-specific pulmonary inflammation and dysfunction in both heaves-susceptible and healthy horses. In consideration of the difference in response between healthy human subjects and asthmatics, heaves horses may exhibit a lower response threshold to such challenges.

(2) Exposure to airborne endotoxin will increase the severity of pulmonary inflammation and dysfunction in symptomatic heaves horses that are co-exposed to other components of stable dust.

CHAPTER 2: DOSE-RESPONSE RELATIONSHIP TO INHALED SOLUBLE LIPOPOLYSACCHARIDE (LPS) IN ASYMPTOMATIC HEAVES HORSES AND CONTROLS AND COMPARISON WITH NATURAL ENDOTOXIN EXPOSURE

2.1 Summary

To investigate whether inhaled endotoxin contributes to airway inflammation and dysfunction in horses and to compare the responses of control horses (n=6) and heaves-susceptible horses (n=7), both groups were given inhalation challenges with 20, 200 and 2000μg of soluble *Salmonella typhimurium* Ra60 lipopolysaccharide (LPS). LPS inhalation induced a dose-dependent neutrophilic airway inflammatory response in both groups. Inhalation with 2000μg of LPS also induced detectable lung dysfunction in the heaves group, albeit of mild severity. LPS inhalation did not alter clinical score, tracheal secretion score or airway reactivity in either group. The no-response thresholds were lower for the heaves group (<20μg for airway inflammation; 200 to 2000μg for lung dysfunction) than for the control group (20 to 200μg for airway inflammation; >2000μg for lung dysfunction). To enable comparison of these threshold levels with airborne endotoxin concentrations in stables, horses also received a 5h duration hay/straw challenge, during which the total and respirable airborne endotoxin concentrations were determined. Comparison of the effects of acute LPS inhalation and hay/straw challenges suggest that inhaled endotoxin is not the sole cause of heaves. However, it is likely that it contributes to

airway inflammation, both in heaves horses in concert with other inhalants, and in normal horses when they are exposed to high levels in poor stable environments.

2.2 Introduction

Inhaled endotoxins are an important cause of human pulmonary disease (Jacobs, 1997a), with the severity of pulmonary inflammation and clinical symptoms experienced by subjects exposed to organic dusts being related to the endotoxin concentration of the inhaled dust (Rylander and Bergstrom, 1993; Smid *et al.,* 1994; Zejda *et al.,* 1994; Schwartz *et al.,* 1995b; Vogelzang *et al.,* 1998). Additionally, the severity of human asthma has been related to the level of endotoxin exposure (Michel *et al.,* 1991; Michel, 1996; Michel *et al.,* 1996; PlattsMills *et al.,* 1997; Rizzo *et al.,* 1997), suggesting that inhaled endotoxin may potentiate the inflammatory response to allergens in atopic subjects. In man, considerable efforts have been made to establish no-response threshold levels for inhaled endotoxin (Michel *et al.,* 1997), and to identify safety guidelines for occupational endotoxin exposure (Rylander, 1997b). Since horse stables contain high concentrations of airborne endotoxin (Olenchock *et al.,* 1992; Dutkiewicz *et al.,* 1994; McGorum *et al.,* 1998; Tanner *et al.,* 1998), and given the similarities between heaves and inhaled endotoxin mediated lung disease in other species (McGorum *et al.,* 1998), it is surprising that the role of endotoxin in heaves is unknown. The aims of the present study were (a) to investigate the response of control and heaves horses to increasing doses of inhaled LPS, (b) to determine no-response threshold levels for both control and heaves horses, and (c) to compare no-

response threshold levels of inhaled soluble LPS with airborne endotoxin levels encountered in equine stables.

2.3 Materials and methods

2.3.1 Subjects

2.3.1.1 Heaves horses

The heaves group consisted of 7 horses (3 geldings, 4 mares; median age 17 years, range 8-28; median weight 434 kg, range 323-594) with a history and clinical diagnosis of heaves. The disease status of all subjects was confirmed by hay/straw challenge. A 5h hay/straw challenge (2.3.4.2) induced BALF neutrophilia (>20%), increased volume of tracheal secretions bronchoscopically, and a reduction in PaO_2 in all heaves horses. A more prolonged challenge induced increased maximum transpulmonary pressure ($dPpl_{max}$), isovolumetric and expiratory lung resistance (RL_{ISO} and RL_E, respectively) and work of breathing (W_B), and decreased dynamic compliance (Cdyn). In all cases $dPpl_{max}$ exceeded 15cm H_2O at variable time points following exposure to the hay/straw environment. All of the above clinical and laboratory abnormalities reverted to normal when the heaves horses were moved to a controlled environment (2.3.2.1). These criteria are consistent with the definition of heaves horses as determined by the International Workshop on Equine Chronic Airway Disease (Robinson, 2001).

2.3.1.2 Control horses

The control group consisted of 6 healthy horses with no detectable respiratory tract disorders (all female, median age 6 years, range 4-9; median weight 320 kg, range 316-356). A 5h-hay/straw challenge (2.3.4.2) did not induce detectable pulmonary inflammation or detectable tracheal secretions in control horses. A more prolonged hay/straw challenge did not induce significant lung dysfunction in this group, with the maximum transpulmonary pressure not exceeding 8 cm H_2O.

2.3.2 Stable environment

2.3.2.1 Controlled environment

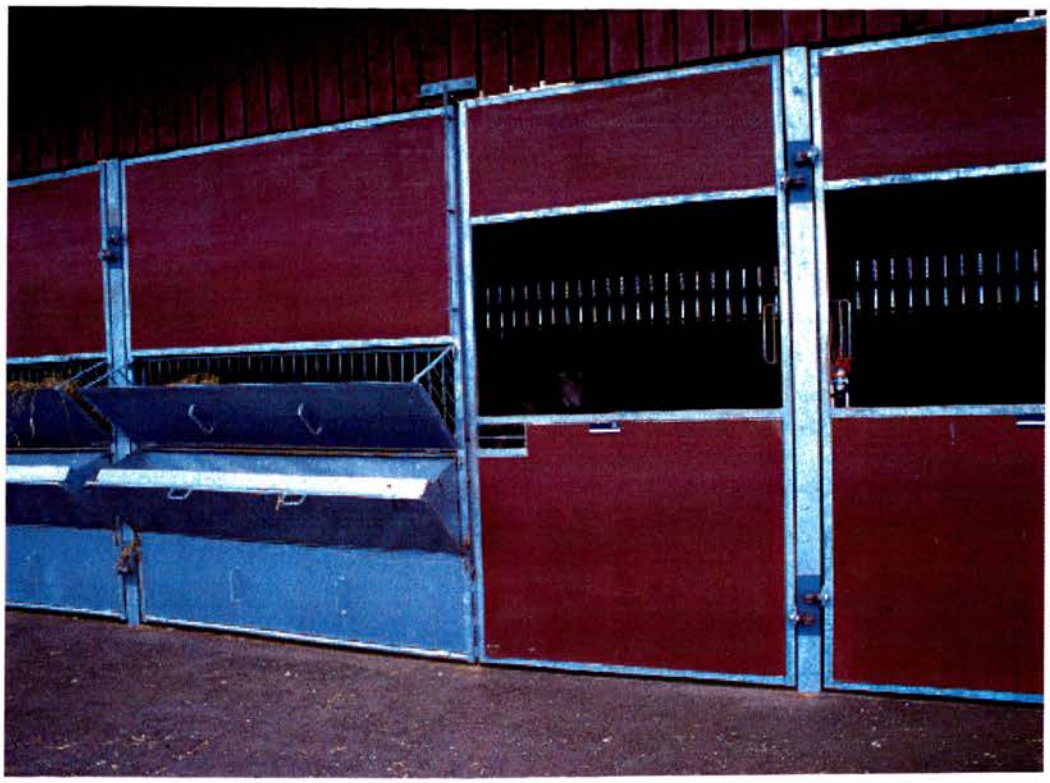
Throughout the study all horses were kept in a low dust environment. This refers to a hay and straw free environment, which would be expected to contain minimal levels of allergens implicated in the aetiology of heaves. This consisted of large (*length* 5m x *width* 4m) ventilated stables (Fig. 2.1) with spaced boarding sections (*height* 1m x *width* 4m) at the top of the back wall (1.75m from ground level) and at the top of the front wall (3m from ground level). The front wall also incorporated a door with a permanently open top section (*width* 1.5m x *height* 1m) and an open grille/feed hopper (*width* 2.5m x *height* 0.5m). Horses in this accommodation were bedded on dry wood shavings and fed haylage and occasionally concentrate feed. Damp bedding and faeces were removed three times daily. These stables were 150m from the closest hay and straw storage area and sheltered from the prevailing wind.

During summer, the horses were occasionally kept at pasture, 300m from the closest hay and straw storage area. Supplementary haylage was provided when necessary.

2.3.2.2 Hay/straw challenge environment

This refers to a poorly ventilated environment in which the horses were exposed to dusty hay and straw. This consisted of a smaller stable (*length* 3.4m x *width* 2.6m) than the controlled environment, which had no wall or roof vents. All doors remained closed during the challenge period. Bedding consisted of deep litter straw, which had accumulated for several weeks, during which time other livestock (including horses and sheep) were kept in the stable. Feeding in this environment consisted of dusty hay with grossly visible fungal contamination. Microscopic examination of the respirable fraction of dust collected following agitation of this hay revealed large numbers of fungal spores, dust mites and dust mite faeces.

Fig 2.1: The well ventilated stables used as the "controlled environment".



2.3.3 Inhalation challenge material

2.3.3.1 Lipopolysaccharide (LPS) solution

All horses received 3 separate increasing doses (20, 200 and 2000μg) of purified *Salmonella typhimurium* Ra60 LPS (kindly donated by Professor Ian Poxton, Department of Medical Microbiology, University of Edinburgh). LPS was diluted from a stock solution (8.89mg/ml) in sterile isotonic saline, immediately prior to nebulisation.

2.3.3.2 Negative (placebo) control

Prior to the LPS inhalation challenges, all horses received an initial control inhalation challenge with sterile isotonic saline (Vetivex, [Sodium Chloride 0.9% w/v], Ivex Pharmaceuticals, Larne, UK) as a placebo and vehicle control challenge.

2.3.4 Inhalation challenges

2.3.4.1 Nebulised inhalation challenges

To facilitate nebulisation, horses were sedated with 20μg/kg romifidine (Sedivet, Boehringer Ingelheim Ltd., Bracknell, Berkshire, UK) and 10μg/kg butorphanol (Torbugesic, Fort Dodge Animal Health, Southampton, UK), intravenously. Although randomisation of the inhalation challenges was considered, the following order was chosen for safety reasons, due to the unknown effects of LPS inhalation in the horse. Several procedures were performed to minimise potential carry-over effects of a preceding challenge on a subsequent challenge. Firstly, inhalation challenges were

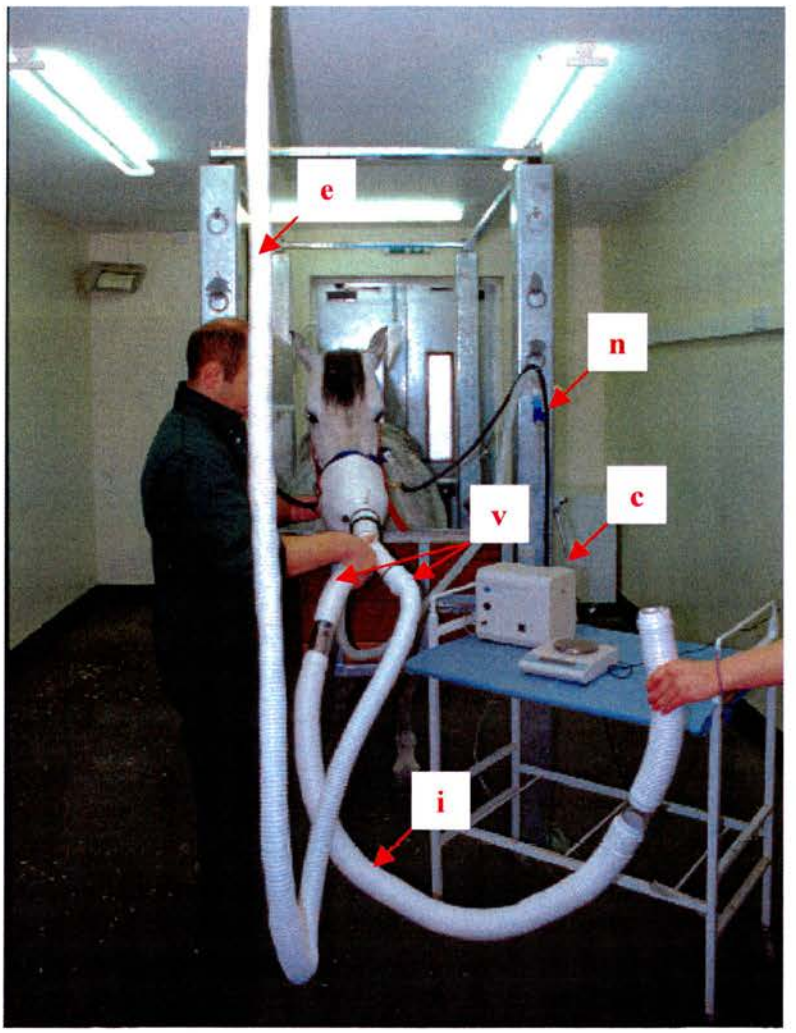
conducted a minimum of 14 days apart. Secondly, all horses were shown to have normal BALF cytology at least 7 days prior to challenges, and normal clinical findings immediately prior to each inhalation challenge. In order to assess any carry-over effects, all baseline lung function, arterial blood gas and venous blood leucocyte data were compared with each other. Additionally, 6 heaves and 4 control horses received a repeat inhalation challenge of 200μg LPS following completion of the other challenges. As well as permitting the assessment of any carry-over effects, comparison of the response to this challenge with that to the original 200μg LPS challenge also determined the repeatability of LPS inhalation challenge.

The aerosol was generated using a compressor (Parimaster, PARI Medical Ltd., West Byfleet, Surrey, UK), with a calibrated output of 7l/min, connected to a nebuliser cup (Sidestream, Medic-Aid Ltd., Bognor Regis, West Sussex, UK) whose manufacturers state that 80% of aerosol is in the respirable range (<5μm). The nebuliser cup contained 2ml of challenge solution. The delivery system is represented in Fig. 2.2.

The challenge solution was nebulised into a section of corrugated tubing (length 185cm, dead space 0.5l), that communicated with the inspiratory arm of the Y-piece delivery system (length 220cm, dead space 4.1l), distal to a one-way inspiratory valve. During inspiration, the aerosol was inhaled via the one-way valve and an airtight facemask. To account for aerosol losses within the corrugated tubing, pilot studies showed it was necessary to aerosolise 1.05ml solution from the nebuliser cup to achieve delivery of 1ml to the inspiratory arm of the delivery device. Any further losses between the inspiratory arm and the subject nostrils were not accounted for.

During expiration, expired breath passed via a one-way valve on the expiratory arm of the delivery system and was vented to the external environment.

Fig. 2.2: Delivery system used for nebulised inhalation challenges, showing compressor (c), neuliser cup (n), inspiratory (i) and expiratory (e) arms of Y-piece delivery system, with the position of the one way valves marked (v).



2.3.4.2 Hay/straw challenges

For the hay/straw challenge, horses were housed for 5h in the hay/straw challenge environment previously described (2.3.2.2). This environment had previously been

shown to induce airway inflammation, clinical signs and lung dysfunction in heaves horses (McGorum *et al.,* 1993c). During this challenge, time zero (t-0) represented the time when the horse entered the stable.

2.3.5 Dust collection

During the hay/straw challenge, total and respirable stable dusts were collected using a personal sampler (AFC124 High Flow Personal Sampler, Casella Ltd., Kempston, UK) and total and respirable sampling heads (Casella Ltd., Kempston, UK), onto 25mm diameter, 0.8μm pore size, cellulose acetate filters (Millipore, Bedford, USA). Respirable dust refers to dust particles of sufficiently small aerodynamic size (<7μm) and shape to allow deposition within the lower airways. The sampling heads were attached on either side of the subjects head collar, approximately 15cm from the nostrils (Figs. 2.3-2.5).

Fig. 2.3: Personal samplers (s) were attached on either side of a girth strap and connected to dust sampling heads (h).

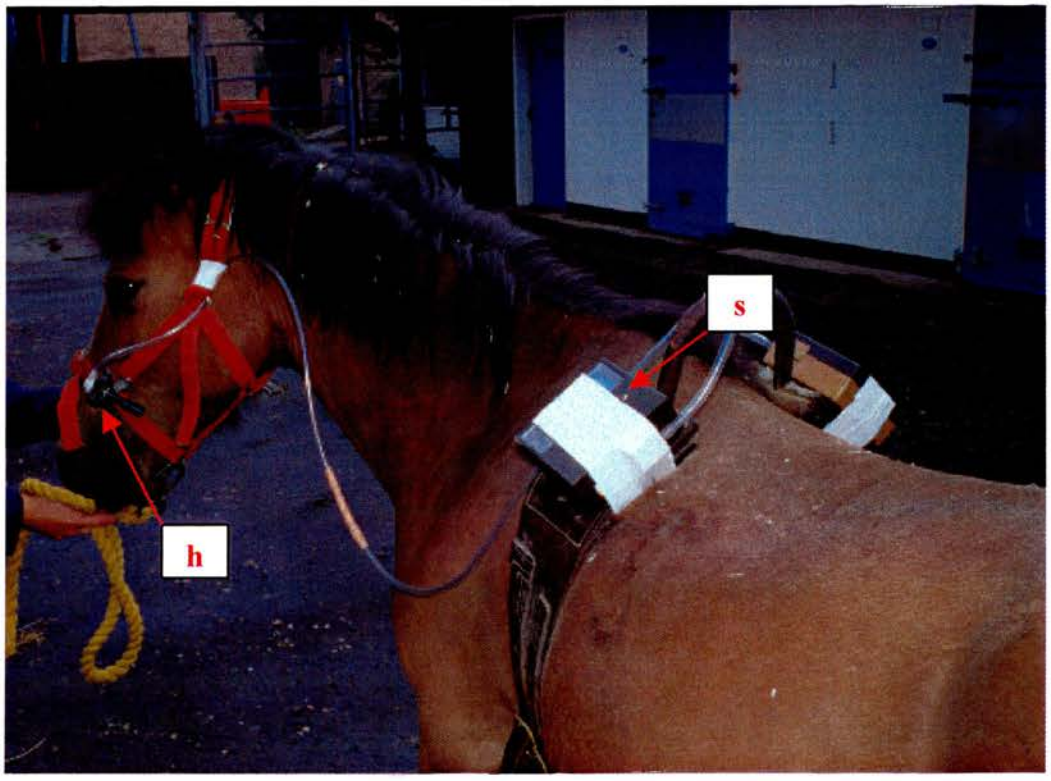


Fig. 2.4: A sampling head designed for collection of total airborne dust (t) was attached to to the right side of the head-collar in order to collect from the horses breathing zone

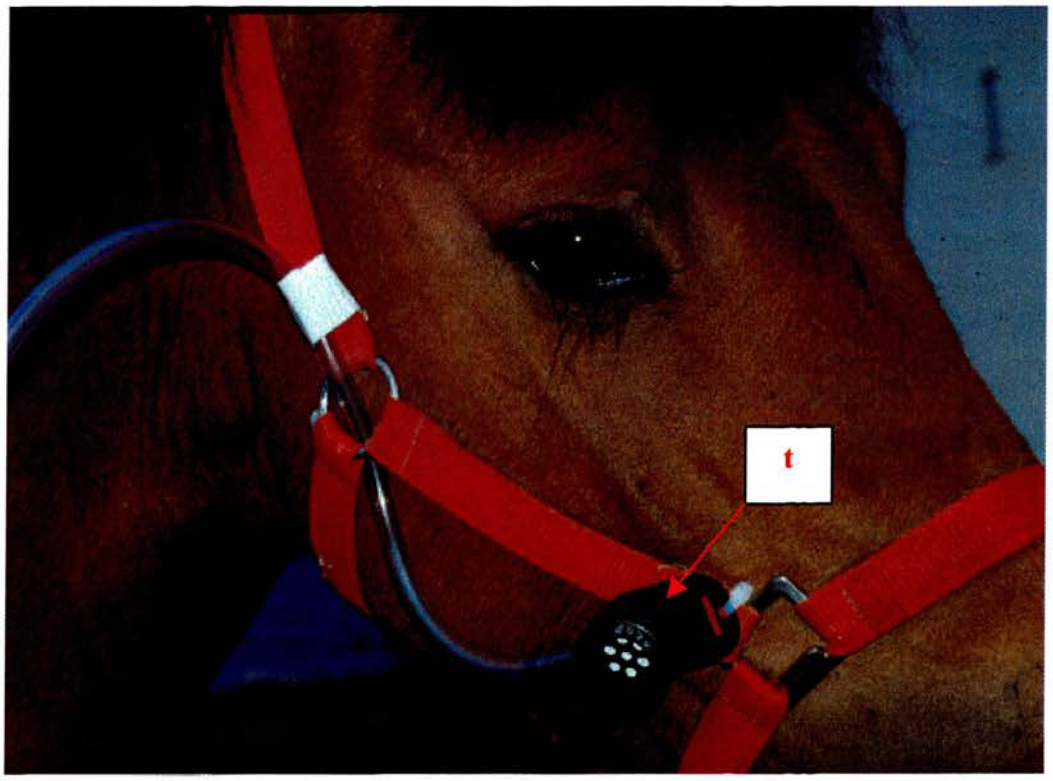
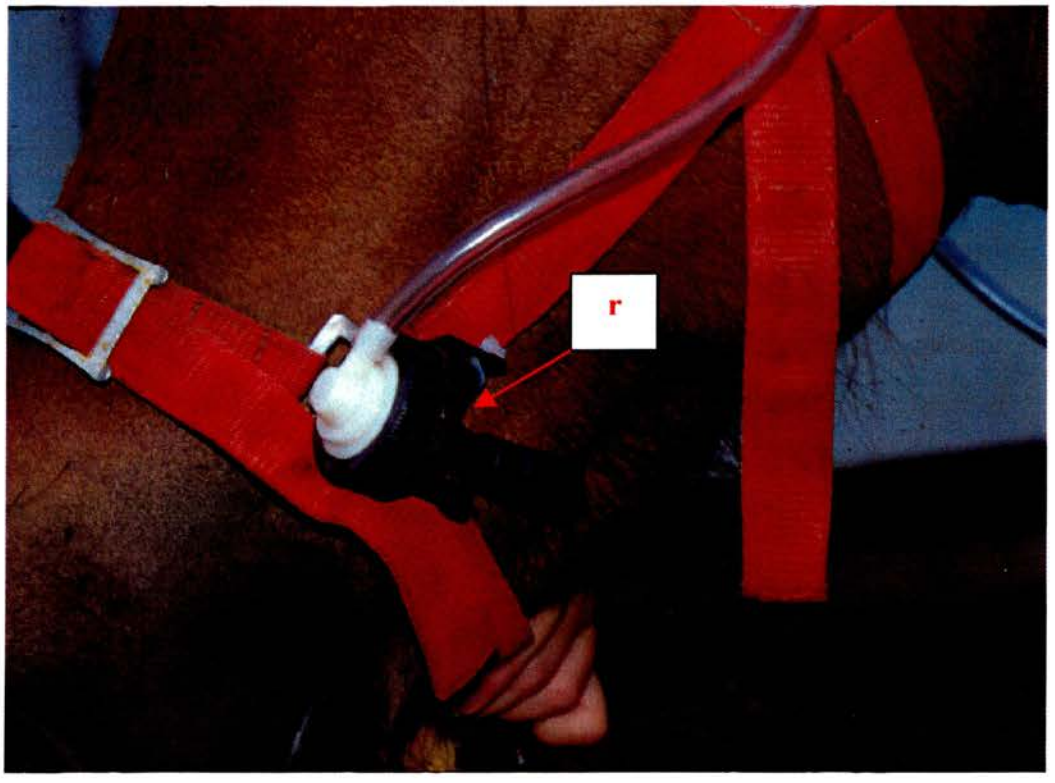


Fig. 2.5: A sampling head designed for collection of respirable airborne dust (r) was attached to to the left side of the head-collar in order to collect from the horses breathing zone



This ensured the collection of airborne dust present in the subjects breathing zone, thus providing a more accurate indication of the amount inhaled of dust compared with remote sampling (Woods *et al.,* 1993). The sampling period was 5h, and the pump flow rate (2 l/min) was calibrated before and at the end of each sampling period using a calibrated rotameter (Casella Ltd., Kempston, UK).

2.3.6 Analysis of dust

2.3.6.1 Calculation of airborne dust concentration

The mass of collected dust was determined by weighing the filters before and after dust collection. To minimise the effects of variable moisture content on filter mass, filters were conditioned overnight in a partially open container in the laboratory at room temperature prior to weighing (Anon 1993). The dust concentration per m^3 of air sampled was then calculated using the following equation:

$$\text{airborne dust } (mg/m^3) = \text{dust collected (mg)} \times \frac{1000 \text{ (i.e. number of litres in } 1m^3)}{600 \text{ (i.e. litres collected over 5h @ 2l/min)}}$$

After weighing, sample filters were stored in individual sterile universal containers at -20°C, prior to endotoxin analysis.

2.3.6.2 Measurement of endotoxin in airborne dust

The endotoxin content of filters was determined using an endotoxin specific *Limulus* amoebocyte lysate assay (Endospecy, Seikagaku Co, Tokyo, Japan). Samples were

prepared by adding 5ml and 10ml of sterile water (water for injection, Animalcare Ltd., Dunnington, York, UK) to the respirable dust and total dust fractions, respectively. Containers were rotated end-over-end, at room temperature for 1h to elute endotoxin. To remove particulate matter, which could interfere with the assay, the eluates were centrifuged at 1600g for 15min. Following centrifugation, the supernatant was decanted, transferred to another sterile container and frozen at –80°C until analysis.

For analysis, all reagents, samples and standards were brought to room temperature. Samples and standards were mixed vigorously for 30s with a vortex mixer. Serial dilutions of the samples were then made in order to ensure a final sample concentration that did not exceed the endotoxin standard (*E.coli* 0111:B4) provided with the kit. Serial dilutions (1:1, 1:2, 1:4 and 1:8) of the standard were also made to provide a standard curve. 10µl of standard, sample or distilled endotoxin free water (negative control) was pipetted into a sterile 96 well microplate. 100µl of *Limulus* amoebocyte lysate substrate solution was immediately added to each well. The microplate was then incubated at 37°C for 30min, following which the reaction was stopped by the addition of 200µl of 0.6M acetic acid to each well. The absorbance of the resulting colour reaction was read photometrically (Microplate Autoreader, Bio-Tek Instruments Inc., Winooski, VT, USA) at 405nm, and compared to a standard curve, prepared during each analysis. All samples were analysed in duplicate and the mean value calculated. Analysis was repeated if (a) the paired values differed from their mean by >10% of the mean, (b) either of the paired values exceeded the value of undiluted standard solution, or (c) either of the paired values were less than the value

obtained from a 1:8 dilution of the standard solution. In addition, the sterile water used to dilute the samples was tested as a sample and compared with the endotoxin-free water provided with the kit in order to confirm that it was not contaminated with endotoxin.

The endotoxin concentration per m^3 of air sampled was then calculated using the following equation:

$$\text{Airborne endotoxin (ng/ m}^3\text{)} = \text{endotoxin per sample (ng)} \times \text{inverse of dilution factor} \times \frac{1000}{600}$$

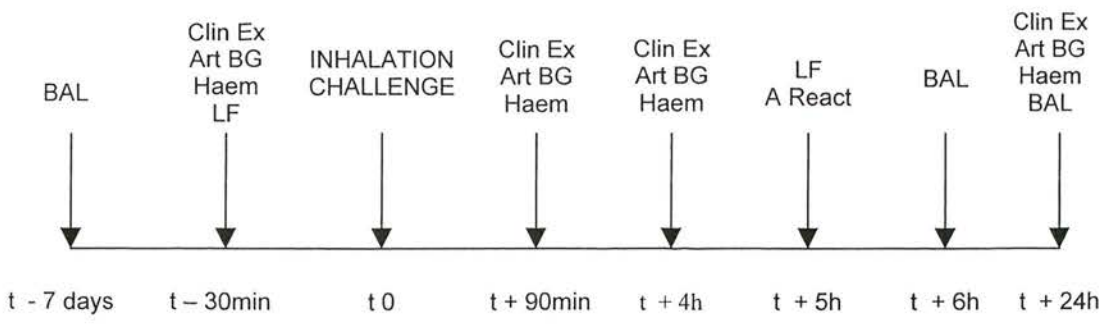
In addition, the endotoxin content of the dust was calculated using the following equation:

$$\text{Endotoxin content of dust (ng/mg)} = \frac{\text{endotoxin per sample (ng)} \times \text{inverse of dilution factor}}{\text{dust collected (mg)}}$$

2.3.7 Monitoring the response to challenges

The timing and method of the assessment of the response to challenges is summarised in Fig. 2.6.

Fig. 2.6: Study design. For the 5h hay/straw challenges, the horses entered the stable at t=0.
BAL = bronchoalveolar lavage; LF = lung function evaluation; Clin Ex = clinical examination; Art BG = Arterial blood gas analysis; Haem = haematological analysis; A React = airway reactivity evaluation.



2.3.7.1 Clinical examination

The clinical response to challenge was assessed using the clinical scoring system summarised in Table 2.1.

Table 2.1: Clinical scoring system

CLINICAL VARIABLE	RESPONSE	SCORE
COUGH	Present Absent	0 1
NASAL DISCHARGE	Present Absent	0 1
DYSPNOEA	Absent Mild Moderate Severe	0 1 2 3
RESPIRATORY RATE	< 20 breaths/min 20 – 30 breaths/min > 30 breaths/min	0 1 2
THORACIC AUSCULTATION	Normal sounds Increased normal sounds Adventitious sounds Marked adventitious sounds	0 1 2 3
PULSE RATE	< 50 beats/min 50 – 70 beats/min > 70 beats/min	0 1 2
RECTAL TEMPERATURE	Normal Elevated (> 39.5°C)	0 1
TOTAL SCORE		13

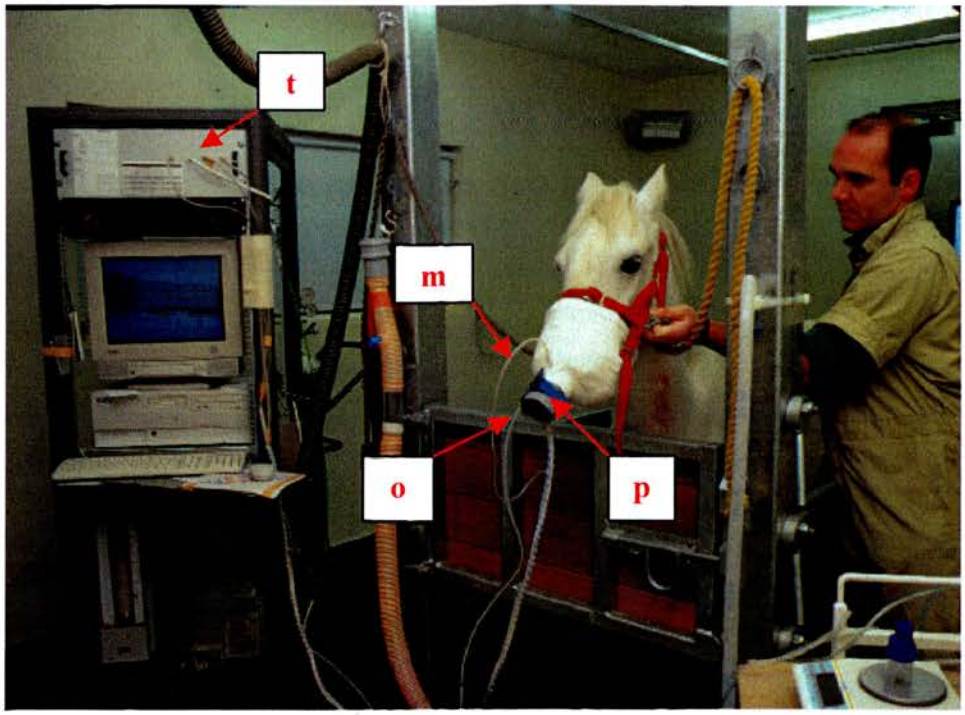
2.3.7.2 Arterial blood gas analysis

Arterial blood samples were collected by carotid puncture using a 21gauge 4cm needle into heparinised plastic syringes (Arterial Blood Sampler, Bayer Ltd., Halstead, Essex, UK). Samples were analysed either immediately or following less than 30min storage at 4°C, for PaO_2, $PaCO_2$ and arterial pH, using an AVL Opti CCA blood gas analyser (AVL Medical Instruments UK Ltd, Stone, Staffs), at an altitude of 170m. Blood gas tensions data were corrected to the rectal temperature of the horse.

2.3.7.3 Pulmonary mechanics testing

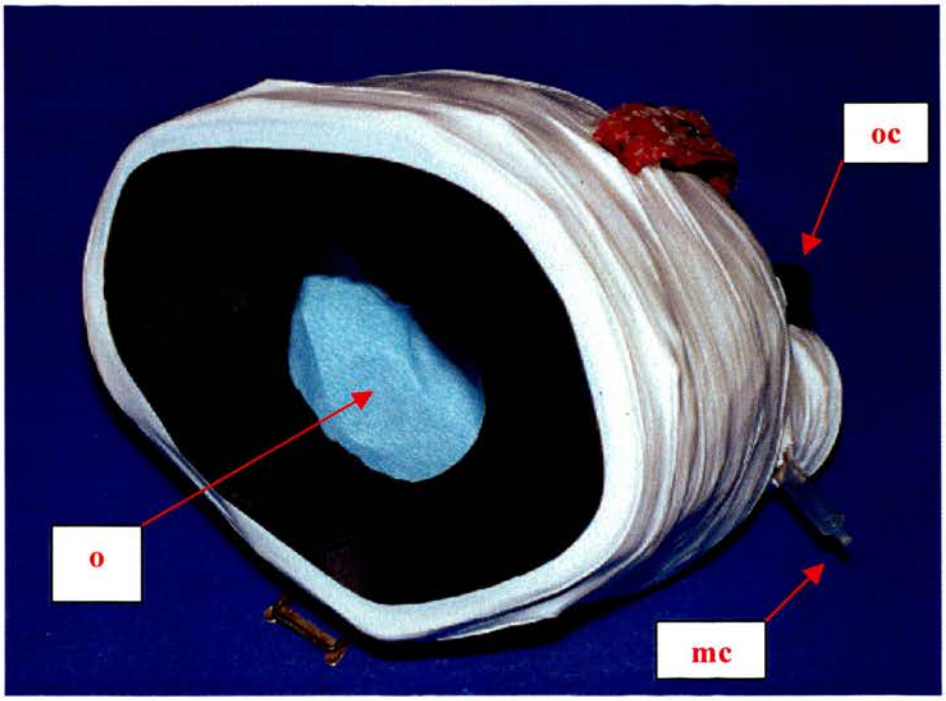
All horses unfamiliar with this procedure were given 2 training periods prior to the collection of data in order to minimise anxiety, which may influence lung function (Deegan and Klein 1985). All measurements were performed on standing unsedated horses restrained in stocks only with a headcollar (Fig. 2.7). No sedation was administered immediately prior to or during pulmonary mechanics testing to avoid any drug induced effects on pulmonary function (Reitmeyer *et al.,* 1986; Broadstone *et al.,* 1992; Lavoie *et al.,* 1992). Sedation was however administered immediately following baseline measurements to ensure subject safety and compliance during the nebulised challenge. Therefore when assessing the pulmonary function responses to challenge, a period of 5h had elapsed following the intravenous administration of a sedative drug. Recording commenced immediately after the horses were connected to the apparatus, and 2 separate 60s periods of data were collected.

Fig. 2.7: Horse restrained in stocks, wearing an airtight facemask with attached pneumotachograph (p) connected to pressure transducer (t). The oesophageal (o) and mask catheters (m) are also connected to pressure transducers (t).



Respiratory flow was measured using a heated pneumotachograph (A. Fleish No.4, Bilthoven, Holland), mounted on an airtight facemask and connected to 2 pressure transducers, the output of which were conditioned, amplified as necessary, and converted from analog to digital form, using appropriate hardware (National Instruments Co., Austin, Texas). Custom-designed computer software (Labview, National Instruments Co., Austin, Texas) was used to facilitate integration of the flow signal to yield volume. The part of the facemask into which the horse's muzzle was inserted was composed of taught flexible rubber with a central opening (12cm x 12cm) (Fig. 2.8). The flexible rubber margins of the opening conformed to the contours of the horse's face caudal to the position of the lip commisures, thus providing an airtight seal. The mask was secured by means of a woven strap, which passed behind the horse's poll.

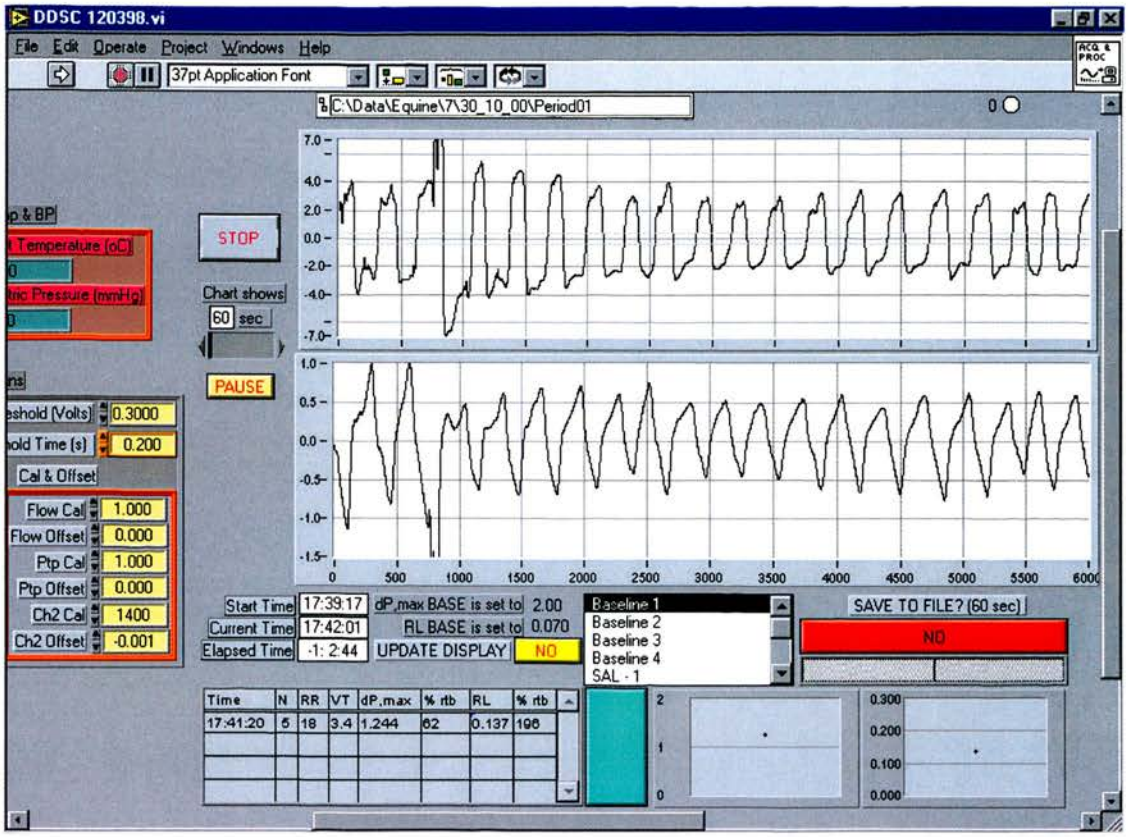
Fig. 2.8: Facemask with attachment for mask catheter (mc), portal for oesophageal catheter (oc) and opening in rubber seal, into which the horse's muzzle was placed (o).



An oesophageal balloon catheter, consisting of a latex condom secured over the end of a polythene catheter (length 2550mm, O.D. 4mm, I.D. 2mm, ARCO, Linlithgow, UK), which had a series of spirally arranged holes distally, and a mask catheter (O.D. 4mm, I.D. 2mm, ARCO), were connected to separate pressure tranducers to permit measurement of transpulmonary pressure (Ptp). The output of the transducers were conditioned, amplified as necessary, and converted from analog to digital form, using appropriate hardware (National Instruments Co., Austin, Texas, USA). The oesophageal catheter was positioned at a point within the thoracic oesophagus, initially estimated by holding the tubing at the side of the horse, and then repositioned to a point which gave the greatest recording of Ptp, without any recorded artefacts consistent with pulsatile/rhythmic cardiac movement. The catheter was passed to the same position for each individual horse. The catheter balloon assembly was filled with 4ml air, this volume being within the range of high compliance of the balloon (Appendix 2.1).

The output of the data acquisition equipment was directed to an electronic flow and pressure time trace, permitting real time assessment of the quality of data recorded (Fig. 2.9). In addition, raw data was electronically transferred to a spreadsheet format (Excel, Microsoft Co., Redmond, WA, USA), which recorded all flow and pressure data over time for both 60s periods of data collection. Measurements were performed at an altitude of 170m above sea level.

Fig. 2.9: Example of flow and pressure traces, which permitted real-time assessment of the quality of data recorded.

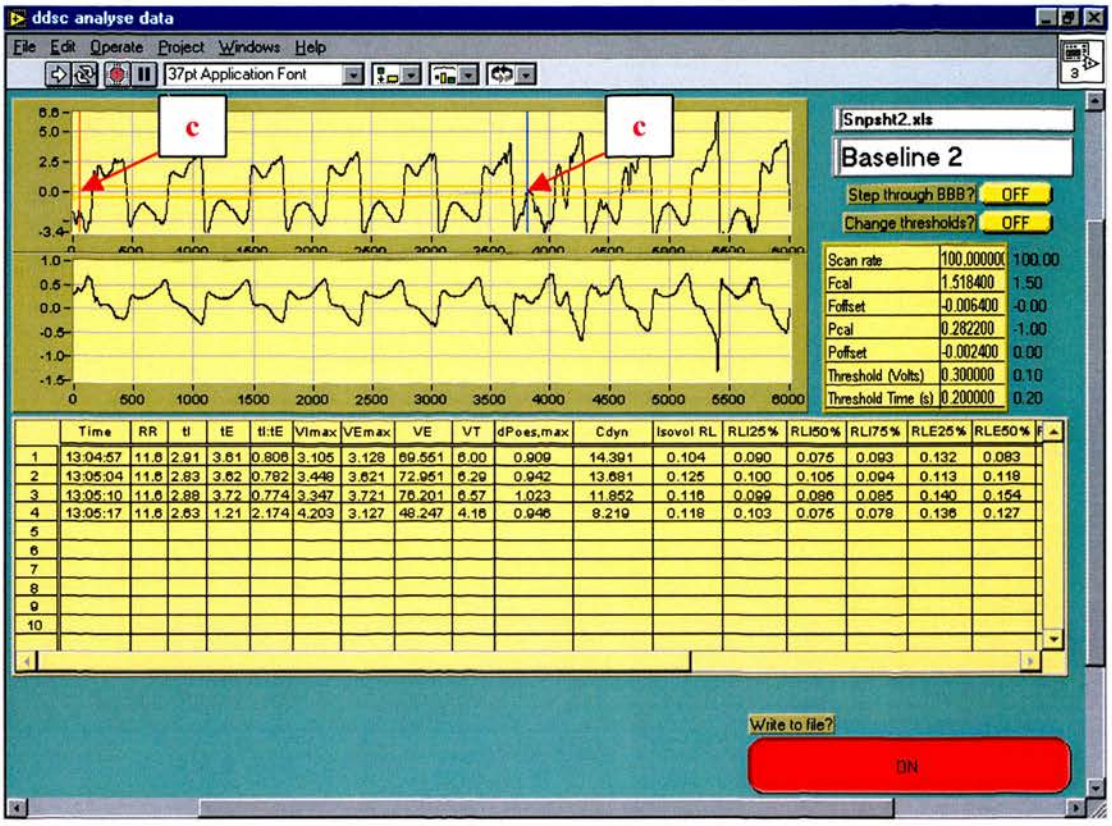


Flow and pressure calibrations were made using, respectively a rotating vane flow meter (Rotameter KDG Mobrey 2000, KDG Mobrey Ltd., Crawley, Sussex, England, UK) and a water manometer (Fisher Scientific, Loughborough, Leicesteshire, UK). All outputs were linear over the working ranges (Appendices 2.2 and 2.3). The frequency response characteristics of the flow and pressure recording systems were phase matched up to 8Hz (Appendix 2.4), using standard techniques (Macklem 1974).

Following collection of both 60s periods of data, the recorded electronic traces were visually assessed. From each period, a series of consecutive breaths, devoid of

artefacts, were selected for further analysis (Fig. 2.10). A minimum of 6 total representative breaths was selected for analysis.

Fig.2.10: Example of use of electronic callipers (c) to select representative breaths devoid of artefacts for further analysis.



For each breath, custom designed software (Labview, National Instruments Co., Austin, Texas) was used to derive the following lung function indices from flow, Ptp and tidal volume (V_T): dynamic compliance (Cdyn); maximum transpulmonary pressure change ($dPpl_{max}$); isovolumetric lung resistance (RL_{iso}); lung resistance at 25%, 50% and 75% inspired volume ($RL_{I25\%}$, $RL_{I50\%}$, $RL_{I75\%}$, respectively); lung resistance at 25%, 50% and 75% expired volume ($RL_{E25\%}$, $RL_{E50\%}$ and $RL_{E75\%}$,

respectively); total, resistive and elastic work of breathing (Wb_t, Wb_{res} and Wb_{el}, respectively), expiratory and inspiratory resistive work of breathing (WB_{Eres} and WB_{Ires}, respectively), inspiratory total work of breathing (WB_{Itot}), maximum inspiratory and expiratory flow (V'_{Emax} and V'_{Imax}, respectively), respiratory rate (RR), time for expiration and inspiration (T_E and T_I, respectively) and the ratio of T_I to T_E (T_I:T_E). A mean of the values obtained from the selected breaths was calculated to give a single value for the baseline measurement and for the 5h response measurement.

Airway reactivity was assessed immediately following collection of the 5h lung function data. To ensure safety and subject co-operation, horses were sedated for this procedure by the intravenous administration of 20μg/kg romifidine (Sedivet, Boehringer Ingelheim Ltd., Bracknell, Berkshire, UK) and 10μg/kg butorphanol (Torbugesic, Fort Dodge Animal Health, Southampton, UK). Two minutes following the administration of sedative, subjects received a 1min duration aerosol challenge with saline, using the method described above, followed immediately by 2min of lung function data recording. Data obtained throughout this 2min period were used to calculate baseline measurements. This process (1min aerosol challenge and 2min data collection) was then repeated during and following nebulisation of doubling concentrations of methacholine chloride (Sigma Aldrich Co. Ltd., Poole, Dorset, UK) dissolved in saline, starting with 0.4mg/ml. The increasing concentrations (0.4, 0.8, 1.6, 3.2, 6.4, 12.5, 25 and 50mg/ml) of methacholine chloride solution were prepared from a stock solution of 100mg/ml, immediately prior to the assessment of airway reactivity. Airway reactivity was assessed by measuring the concentration of inhaled

methacholine chloride solution required to reduce the dynamic compliance to 70% of the baseline value recorded following saline inhalation (PCCdyn70). PCCdyn70 was used to assess airway reactivity, as in the majority of cases the 30% reduction in this variable preceded any significant increase in lung resistance following methacholine chloride inhalation (*personal observation*). The data acquisition software permitted real time calculations of mean dynamic compliance values calculated over a 20s recording period, however final PCCdyn70 values were calculated following the analysis of selected breaths as previously described.

2.3.7.4 Tracheal secretion scoring

The volume of tracheal secretions detected during endoscopy was graded 0-5 as previously described (Dixon *et al.* 1995) as summarised in Table 2.2.

Table 2.2: Tracheal secretion grading system (as described by Dixon *et al.* [1995a])

GRADE	ENDOSCOPIC FINDINGS
0	No respiratory secretions seen
1	A few droplets of respiratory secretions present
2	Small pool of respiratory secretions present
3	Moderate pool of respiratory secretions present
4	Large pool of respiratory secretions present
5	Very large pool (> 20ml) of respiratory secretions present

2.3.7.5 Bronchoalveolar lavage fluid (BALF) collection

Transendoscopic bronchoalveolar lavage (BAL) was performed under sedation using intravenous 20μg/kg romifidine and 10μg/kg butorphanol. Horses were also

restrained with a headcollar and nose twitch. A 14mm diameter endoscope (Olympus CF Type 200HL, 1.7m working length, Olympus Optical Co. Ltd., Tokyo, Japan) introduced via the rhino-pharyngeal route into the larynx and trachea, was passed distally until it "wedged" in a third or fourth generation bronchus. The t-7d and 6h BALF samples were both collected from the right accessory lung lobe, and the 24h BALF samples were collected from the left ventral segment. Room temperature sterile saline (300ml) was instilled via the biopsy channel of the endoscope into the occluded bronchus and immediately aspirated using sterile 60ml plastic syringes. Instillation and recovery of BALF took less than 45s. The collected BALF was immediately processed.

2.3.7.6 Bronchoalveolar lavage fluid (BALF) processing

Total unfiltered BALF cell counts were performed using a haemocytometer (Neubauer haemocytometer, Fischer Scientific UK Ltd., Loughborough, Leics.). Duplicate cytospin glass slide preparations, prepared by centrifuging 100µl BALF at 300rpm (10g) for 3min (Shandon Cytospin 3, Shandon Scientific Ltd., Runcorn, Cheshire) were air-dried and stained using a Leishmans stain (Fischer Scientific UK Ltd., Loughborough, Leics.). Differential counts of 500 nucleated cells were made on both slides by light microscopy (Leica Microsystems UK Ltd., Milton Keynes) under x1000 magnification, and a mean value for each cell type was calculated. Absolute BALF cell counts were determined for each cell type by multiplying the cell ratio (%) by the total BALF cell count /100.

The remaining BALF was immediately centrifuged at 600g for 5min, after which the supernatant was decanted into another sterile plastic universal container. The remaining cell pellet was resuspended in the residual supernatant retained by surface tension. A pre-calculated volume of lysis buffer (Buffer RLT, Quiagen, Crawley, W. Sussex, UK), containing 10µl/ml beta mercaptoethanol (Sigma-Aldrich Co. Ltd., Poole, Dorset, UK) was added to the re-suspended cell pellet resulting in a concentration of 8.6 x 10^6 cells/ml lysis buffer. This lysed cell sample was then aliquoted into 350µl volumes, each containing the lysed contents of 3 x 10^6 cells, and immediately frozen at -80°C. The decanted supernatant was further centrifuged at 1600g for 15min, and the resulting supernatant was decanted, aliquoted and frozen at -80°C.

2.3.7.7 Venous blood collection and analysis

Venous blood was collected by jugular venipuncture into plain, heparinised and ethylenediaminetetraacetic acid (EDTA) coated vacutainers. The heparinised samples were immediately centrifuged at 1600g for 10min, and the plasma was aliquoted into sterile eppendorfs, and stored at -80°C. Following fibrin clot formation in the plain vacutainers, the samples were centrifuged at 1600g for 10min, and the serum was aliquoted into sterile eppendorfs, and stored at -80°C. The samples containing EDTA were submitted for a total and differential leucocoyte count, performed using an electronic cell counter (Baker System 9120 plus CP, Biochem Immunosystems, Allentown, PA, USA) and microscopic examination of a blood smear, respectively.

2.4 Statistical analysis

Non-parametric tests were used, as the data were either not normally distributed and/or the groups of compared data did not have equal variance. The effects of each challenge were determined primarily by performing within-group analyses.

To check for the presence of any carry-over effects of one challenge on a subsequent challenge, pre-challenge (baseline) measurements of arterial blood gases, lung mechanics and peripheral blood leucocytes were compared using a Friedman test, and when significant, a Wilcoxon Rank Sum test was performed on paired data.

To check for any effects of challenge where pre-challenge measurements were made at t-30min (arterial blood gas analyses, peripheral blood leucocyte and neutrophil counts, and lung mechanics), the post-challenge values were expressed as % of baseline value, except for clinical scores where actual values were used. As saline was the vehicle for LPS delivery, the effect of LPS challenge was assessed by pairing and subtracting post-LPS (% of baseline value) and post-saline (% of baseline value) data. Where no pre-challenge data was collected (BALF cytology), comparisons were made with saline (placebo) challenge data at equivalent time points. A Friedman test was performed on sets of paired data to reduce the risk of type 1 errors, and when significant, a Wilcoxon Rank Sum test was performed on paired data.

Between group (heaves *vs.* controls) analyses were performed for BALF neutrophil numbers, using the Mann Whitney test.

Significance was assumed if $P<0.05$. Following correction for any effect of saline inhalation, changes from baseline are expressed as increase/decrease or change in median values, with 95% confidence interval for the difference in median, calculated for non-parametric data as described by Campbell and Gardner (1994).

The two separate 200μg LPS inhalation challenges were compared using a Wilcoxon Rank Sum test, and as an indication of repeatability, the differences in paired values were plotted against their mean as described by Bland and Altman (1986). Good repeatability was assumed if the calculated differences in paired values fell within 2 standard deviations of the mean of the differences (British Standards Institution, 1979). Results are expressed as median and range.

2.5 Results

2.5.1 Dust and endotoxin exposure during hay/straw challenge

Total and respirable airborne dust endotoxin concentrations, and dust endotoxin content, in the hay/straw challenge stable are given in Table 2.3. The estimated biologically active endotoxin exposure received during the 5h challenge was calculated using the following formula:

$$D(ng) = E^{a}(ng/m^{3}) \times V(m^{3}/h) \times T(h) \times C$$

D = 5h exposure to biologically active endotoxin
E^{a} = airborne endotoxin concentration (calculated either from the total or respirable dust fraction)
V = average ventilation rate of both groups (total 13 horses) immediately following hay/straw challenge (3.1 m^{3}/h)
T = duration of challenge (h)
C = correction factor of 3 for approximate three-fold underestimation of biologically active endotoxin content in organic dust by the *Limulus amoebocyte* lysate method (Rylander *et al.* 1989).

Table 2.3: Total and respirable airborne dust endotoxin concentrations, and dust endotoxin content, in the hay/straw challenge stable.

	TOTAL DUST	RESPIRABLE DUST
Airborne dust concentration (mg/m^3)	2.83 (0.83-6.83)	0.50 (0.17-0.83)
Endotoxin content of dust (ng/mg)	56.00 (31.40-163.92)	11.86 (4.53-98.22)
Airborne endotoxin concentration (ng/m^3)	160.00 (86.88-580.56)	3.95 (1.75-61.39)
5h endotoxin exposure (μg)	2.48 (1.35-9.00)	0.06 (0.03-0.95)
5h biologically active endotoxin exposure (μg)	7.44 (4.04-27.00)	0.18 (0.08-2.85)

2.5.2 Response to challenge

2.5.2.1 Clinical examination

All horses had a clinical score of zero prior to all challenges. When compared with baseline values, no significant increase in clinical scores was detected in either group following any of the challenges (Appendix 2.5).

2.5.2.2 Arterial blood gases and pH analyses

Raw data for arterial blood gas measurements are presented in Appendix 2.6. There was no significant difference in the baseline blood gas indices prior to each of the challenges, indicating a lack of any carry-over effects. The percent change in arterial blood gases and pH measurements from baseline is presented in Table 2.4. Following correction for saline inhalation, LPS challenges did not significantly alter arterial pH, PaO_2 or $PaCO_2$ when compared with baseline values in either group. In the heaves group, hay/straw challenge reduced PaO_2 at 90min (decrease in median 6%, 95% confidence interval (CI) 1-17; $P<0.05$), and increased arterial pH at 4h (increase in median 0.2%, 95% CI 0.1-0.6; $P<0.05$). $PaCO_2$ was reduced in the control group at 24h following hay/straw challenge (decrease 9%, 3-15; $P<0.05$).

Table 2.4: The percent (%) of baseline values for arterial blood gases and pH measurements (median and range) in heaves (n=7) and control (n=6) horses at 1.5, 4 and 24h following inhalation challenge with saline, 20, 200 and 2000μg LPS, and mouldy hay/straw challenge.

		SALINE		20μg LPS		200μg LPS		2000μg LPS		HAY/STRAW	
		CONTROLS	HEAVES	CONTROLS	HEAVES	CONTROLS	HEAVES	CONTROLS	HEAVES	CONTROLS	HEAVES
pH	1.5h	100.0 99.8-100.8	99.9 (99.2-100.3)	99.9 (99.2-100.1)	100.0 (95.9-100.4)	99.6 (99.2-99.9)	99.9 (99.5-100.3)	99.9 (99.1-100.4)	100.1 (99.9-100.4)	100.1 (99.7-100.6)	100.5 (99.7-101.0)
	4h	100.0 (99.9-100.1)	100.1 (99.2-100.3)	100.1 (99.2-100.1)	100.0 (95.9-100.4)	100.0 (99.2-100.6)	100.0 (99.5-100.5)	99.9 (98.9-100.3)	100.1 (99.5-100.6)	99.9 (99.7-100.5)	100.1 (100.0-100.9)
	24h	100.1 (99.9-100.7)	99.6 (99.5-100.8)	99.9 (99.5-101.1)	99.9 (95.9-100.7)	99.6 (98.8-100.0)	100.3 (98.9-100.9)	99.8 (99.2-100.3)	100.4 (99.7-100.8)	99.8 (99.1-100.0)	100.0 (99.2-100.5)
pCO2	1.5h	103.9 (97.0-112.5)	97.9 (83.0-109.5)	102.2 (95.5-112.5)	97.8 (90.9-106.7)	106.0 (98.8-116.4)	106.7 (93.0-116.3)	102.3 (97.1-111.9)	103.7 (94.0-116.2)	100.0 (88.9-105.0)	100.0 (91.4-112.2)
	4h	101.4 (89.2-106.7)	97.8 (81.1-104.7)	100.0 (95.8-105.0)	95.1 (91.1-100.0)	100.9 (89.6-113.2)	102.4 (95.6-110.3)	105.6 (89.4-111.9)	106.8 (86.3-108.1)	97.1 (89.1-105.0)	95.2 (85.1-109.8)
	24h	100.0 (89.6-110.0)	95.2 (84.9-109.3)	99.8 (95.6-117.5)	100.0 (91.1-104.5)	101.4 (91.7-115.2)	97.6 (92.3-115.4)	99.8 (82.2-109.5)	95.5 (78.0-102.3)	90.6 (85.3-97.5)	92.4 (85.1-107.3)
pO2	1.5h	98.0 (91.5-117.2)	100.0 (85.3-118.6)	107.9 (93.1-113.0)	102.0 (97.5-119.5)	104.6 (78.0-113.4)	104.3 (86.2-106.5)	109.6 (101.8-	102.1 (89.2-127.6)	102.4 (88.6-118.8)	96.9 (81.0-99.0)
	4h	108.2 (97.1-114.0)	99.0 (87.9-109.0)	105.8 (92.1-117.3)	104.1 (90.9-124.5)	100.4 (81.3-122.4)	103.5 (93.5-106.5)	105.1 (90.4-111.8)	103.3 (94.9-107.5)	101.0 (91.4-129.9)	93.9 (86.4-101.0)
	24h	101.0 (94.3-105.0)	104.3 (88.7-121.3)	100.1 (94.9-108.9)	107.4 86.4-120.7)	107.3 (81.4-132.3)	106.3 (92.7-111.8)	98.2 (89.8-109.0)	102.2 (90.8-130.3)	108.8 (100.0-144.3)	97.4 (89.3-110.3)

2.5.2.3 Lung mechanics and airway reactivity

Raw data for lung function measurements are presented in Appendix 2.7. There was no significant difference in the baseline lung function measurements prior to each of the challenges, indicating a lack of any carry-over effects. The percent change in lung function measurements from baseline is presented in Table 2.5. Following correction for saline inhalation, LPS inhalation challenges had no significant effect on lung function of controls. The heaves group had significantly increased $RL_{E50\%}$ (increase in median: 106%, 95% CI 18-2017; $P<0.05$) and $RL_{E75\%}$ (increase in median 116%, 95% CI 34-595; $P<0.05$) at 5h following inhalation of 2000μg when compared with baseline values (Table 2.5, Fig. 2.11 and 2.12).

Fig. 2.11: Percent (%) of baseline $RL_{E50\%}$ in heaves horses (n=7) at 5h following inhalation challenge with saline, 20, 200 and 2000μg LPS minus percent of baseline $RL_{E50\%}$, at 5h following inhalation challenge with saline. * = statistical outlier.

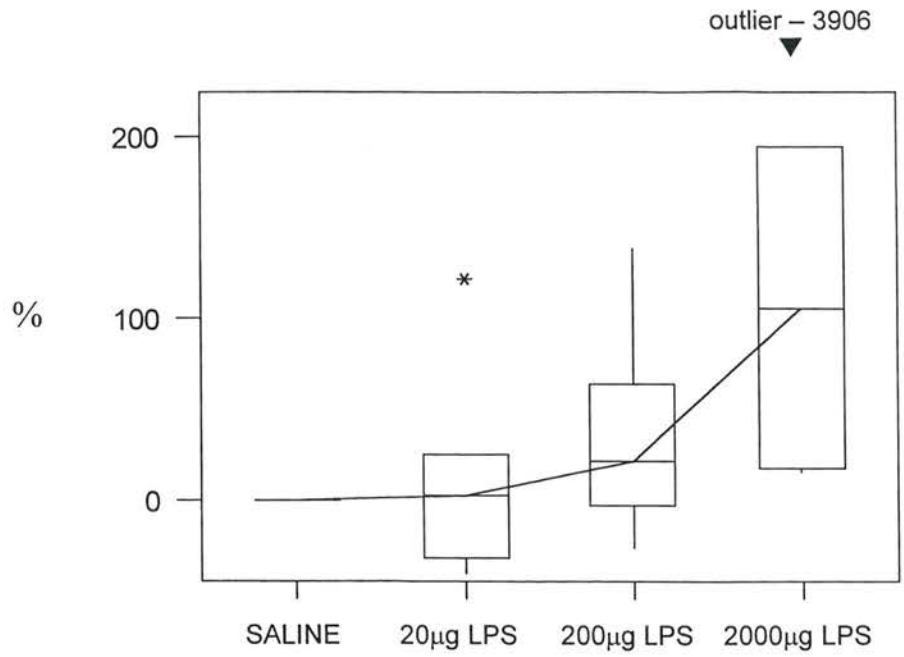
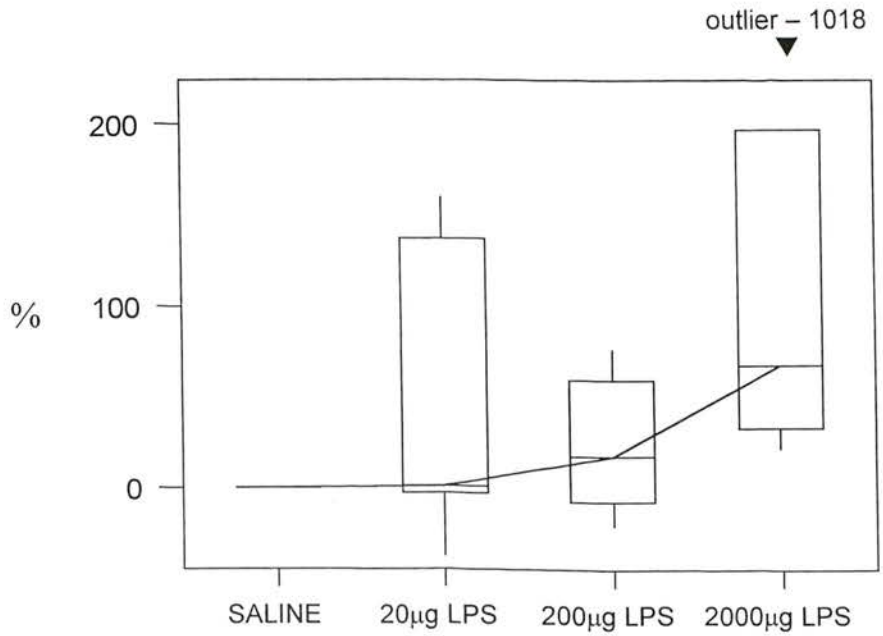


Fig. 2.12: Percent (%) of baseline $RL_{E75\%}$ in heaves horses (n=7) at 5h following inhalation challenge with saline, 20, 200 and 2000μg LPS minus percent of baseline $RL_{E75\%}$, at 5h following inhalation challenge with saline.



PCCdyn values following challenge are presented in Appendix 2.8. None of the challenges altered airway reactivity in either group when compared with saline inhalation challenge. There was no significant difference in airway reactivity between the first (median 6.9mg/ml, range 1.5-19.4) and second (5.2mg/ml, 2.8-17.1) 200μg LPS inhalation challenges. There was good agreement of PCCdyn70 between these 2 challenges, with all the calculated differences in paired values falling within 2 standard deviations of the mean of the differences.

Table 2.5(a): Percent (%) of baseline lung function measurements (median and range) in heaves (H; n=7) and control (C; n=6) horses at 5h following inhalation challenge with 20, 200 and 2000μg LPS, and mouldy hay/straw challenge (H/S).

		Cdyn	dPpl	RL_{iso}	RR	VT	Wb	$RL_{E25\%}$	$RL_{E50\%}$	$RL_{E75\%}$	$RL_{I25\%}$	$RL_{I50\%}$	$RL_{I75\%}$
SALINE	C	68.4 (47.1-157.1)	134.3 (104.2-162.2)	137.0 (85.3-180.3)	91.5 (54.4-106.5)	110.3 (97.7-165.4)	109.1 (78.9-450.0)	71.6 (26.9-151.1)	81.0 (43.8-166.5)	164.0 (132.0-217.6)	148.6 (115.9-190.5)	125.3 (102.8-152.9)	120.1 (106.2-141.9)
	H	96.4 (57.9-104.9)	111.6 (60.0-260.6)	95.9 (76.9-197.3)	90.1 (48.6-110.6)	113.2 (67.2-200.7)	70.8 (35.8-172.0)	66.1 (0.0-125.3)	69.3 (42.7-115.0)	129.4 (77.0-593.2)	102.5 (84.9-590.5)	89.4 (70.5-446.9)	99.2 (29.0-232.9)
20μg LPS	C	76.7 (46.3-128.8)	137.9 (69.8-159.8)	133.3 (74.4-174.2)	86.4 (75.1-132.4)	111.2 (84.0-146.7)	132.2 (6.0-2550.0)	110.7 (0.0-2900.0)	104.3 (9.9-11100.0)	174.2 (38.5-391.5)	164.2 (70.7-293.5)	129.3 (77.3-164.1)	123.0 (108.7-147.0)
	H	67.0 (51.3-100.6)	129.1 (89.7-169.9)	108.6 (63.9-160.3)	100.7 (62.2-126.3)	94.0 (87.9-151.1)	86.6 (51.1-169.3)	70.9 (0.8-185.5)	70.0 (60.6-211.1)	133.1 (59.3-620.9)	133.6 (66.5-419.5)	107.1 (74.6-400.0)	105.8 (78.3-154.5)
200μg LPS	C	128.6 (78.8-144.9)	103.0 (85.0-118.2)	112.3 (86.9-153.6)	90.3 (64.2-108.2)	114.2 (83.5-136.5)	137.9 (97.7-1200.0)	108.6 (0.0-241.0)	136.1 (90.4-298.0)	90.2 (78.6-155.9)	110.1 (71.7-154.9)	104.1 (74.0-144.0)	100.0 (65.1-191.9)
	H	93.7 (49.2-114.8)	106.6 (78.4-126.7)	111.5 (66.7-167.4)	100.0 (84.8-125.1)	107.1 (78.2-144.0)	89.6 (62.5-372.5)	99.0 (63.0-138.4)	98.9 (71.6-125.6)	103.2 (32.3-119.8)	121.6 (71.2-136.4)	90.7 (50.3-106.5)	116.1 (65.2-161.5)
2000μg LPS	C	84.5 (54.6-100.5)	122.6 (106.1-177.2)	139.8 (97.3-207.4)	100.0 (51.1-121.4)	99.6 (95.6-125.4)	136.4 (0.0-410.0)	203.0 (0.0-950.0)	113.0 (35.1-240.0)	132.0 (94.0-439.0)	116.4 (83.1-310.0)	134.9 (84.3-181.6)	120.9 (62.5-185.2)
	H	111.8 (69.9-124.4)	119.5 (103.0-354.9)	140.5 (111.5-708.7)	101.1 (63.8-144.1)	102.9 (78.6-127.8)	138.1 (82.4-15500.0)	148.8 (86.0-4000.0)	138.0 (99.4-1116.7)	95.9 (74.3-403.1)	153.0 (60.2-343.2)	157.3 (89.2-500.0)	149.9 (98.8-439.0)
H/S	C	104.1 (36.5-165.7)	80.3 (56.3-177.8)	101.6 (77.4-146.9)	89.9 (79.1-148.0)	79.4 (68.1-121.6)	139.2 (36.7-1250.0)	155.7 (35.8-3750.0)	195.4 (46.1-4950.0)	78.2 (33.7-116.9)	93.6 (51.0-102.9)	86.4 (71.1-109.7)	95.7 (31.4-121.4)
	H	97.6 (44.1-154.9)	89.5 (61.3-178.6)	71.1 (39.7-245.2)	118.8 (67.2-152.6)	94.0 (84.4-104.3)	108.3 (10.6-210.3)	73.2 (28.8-204.8)	88.1 (42.1-206.6)	58.9 (34.9-243.8)	62.8 (38.1-248.2)	84.2 (34.5-230.2)	85.3 (40.8-193.3)

Table 2.5(b): Percent (%) of baseline lung function measurements (median and range) in heaves (H; n=7) and control (C; n=6) horses at 5h following inhalation challenge with 20, 200 and 2000μg LPS, and mouldy hay/straw challenge (H/S).

		T_E	T_I	$T_I:T_E$	V'_E	V'_{Emax}	V'_{Imax}	Wb_{el}	Wb_{res}	Wb_{Eres}	Wb_{Ires}	Wb_{Itot}
SALINE	C	105.3 (90.7-175.9)	107.5 (92.0-179.8)	100.3 (40.1-253.8)	100.7 (89.8-103.3)	93.5 (79.5-116.5)	173.5 (119.5-194.8)	137.8 (110.3-204.7)	98.3 (6.7-222.4)	160.5 (134.3-199.4)	174.2 (130.6-183.7)	95.4 (89.1-116.8)
	H	103.4 (83.1-219.9)	111.8 (86.3-263.8)	101.5 (41.3-110.5)	104.7 (43.8-117.9)	116.0 (43.3-137.9)	131.6 (48.3-646.1)	95.3 (48.2-505.7)	86.2 (42.0-439.0)	112.0 (58.5-545.3)	118.1 (51.9-597.2)	101.2 (56.1-140.4)
20μg LPS	C	120.0 (80.1-130.8)	119.4 (72.5-140.1)	100.1 (66.4-115.6)	98.7 (84.6-136.5)	99.8 (85.0-126.0)	163.0 (59.0-435.4)	144.6 (114.3-167.5)	104.2 (43.3-170.4)	162.0 (59.4-331.0)	162.9 (59.1-375.5)	97.1 (86.3-153.0)
	H	102.1 (80.6-177.9)	93.4 (76.8-241.2)	93.1 (75.9-166.1)	99.2 (84.1-121.9)	112.2 (49.1-124.6)	162.2 (90.6-400.2)	105.5 (71.7-172.2)	86.5 (55.9-150.6)	120.7 (75.1-304.1)	146.8 (84.6-355.3)	111.6 (73.7-140.5)
200μg LPS	C	118.7 (92.7-149.2)	111.8 (86.2-166.4)	98.6 (90.9-116.6)	100.8 (74.8-114.2)	107.3 (69.7-117.1)	111.0 (82.3-148.9)	115.5 (88.6-174.8)	141.7 (77.5-1143.3)	109.1 (88.1-141.9)	103.6 (97.7-134.3)	106.4 (80.7-121.1)
	H	100.2 (81.3-120.3)	100.0 (74.7-113.5)	96.5 (70.5-167.2)	97.4 (90.6-129.4)	113.8 (71.6-129.1)	132.6 (72.0-203.6)	117.1 (52.7-151.0)	109.7 (49.3-179.0)	115.0 (56.6-163.2)	123.4 (65.2-184.8)	113.0 (97.2-121.9)
2000μg LPS	C	99.5 (81.1-187.3)	99.7 (90.3-189.6)	98.8 (95.4-109.8)	96.8 (55.1-122.3)	99.1 (52.9-105.1)	136.5 (86.9-173.0)	123.6 (93.8-178.2)	134.5 (21.3-287.3)	126.1 (108.1-153.6)	129.6 (101.2-162.8)	89.3 (60.6-107.6)
	H	100.5 (65.6-117.3)	99.6 (71.4-124.5)	96.8 (87.4-157.3)	109.5 (62.3-143.2)	89.0 (76.9-106.9)	122.7 (49.8-142.2)	147.9 (98.9-477.9)	149.7 (54.9-654.5)	161.7 (93.1-352.2)	145.2 (67.8-220.6)	96.3 (62.2-114.6)
H/S	C	106.9 (67.7-127.7)	105.4 (64.4-129.1)	93.9 (85.4-114.1)	96.9 (58.7-100.6)	91.4 (53.5-106.8)	62.5 (33.3-423.6)	79.0 (38.5-139.0)	102.7 (23.7-281.0)	59.8 (37.9-106.8)	61.8 (38.3-206.5)	98.9 (55.9-114.3)
	H	100.0 (70.5-171.9)	87.8 (61.8-123.8)	91.5 (53.0-114.3)	116.6 (63.5-140.5)	94.1 (68.6-160.0)	83.6 (44.4-202.3)	82.7 (41.7-169.9)	94.1 (57.7-167.0)	70.9 (31.2-196.4)	76.1 (38.3-181.8)	89.9 (72.2-170.2)

2.5.2.4 Tracheal secretion score

Compared with saline challenge, LPS challenges did not significantly increase tracheal secretion score at 6h in either group, however 2 horses in the heaves group had increased tracheal secretions following inhalation of 200 and 2000μg LPS. Heaves horses had significantly ($P<0.05$) increased tracheal secretions score after hay/straw challenge, when compared with saline (Table 2.6).

Table 2.6 Tracheal secretion scores (median and range) in heaves (n=7) and control (n=6) horses, 6h following inhalation challenge with saline, 20, 200 and 2000μg LPS, and hay/straw challenge.

	SALINE	20μg LPS	200μg LPS	2000μg LPS	HAY/STRAW
CONTROLS	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
HEAVES	0 (0-0)	0 (0-1)	0 (0-2)	0 (0-3)	2 (1-3)

2.5.2.6 BALF cytology

Examination of BALF revealed neutrophils, macrophages, lymphocytes, ciliated columnar epithelial cells, non-ciliated cuboidal epithelial cells, lymphocytes, eosinophils, mast cells and "basophiloid cells".

Pooled pre-challenge BALF cytology for both groups (n=13) is presented in Table 2.7. There was no significant difference in the pre-challenge BALF cytology prior to each of the challenges, indicating a lack of any carry-over effects. Saline inhalation did not significantly alter BALF cytology in either group. The BALF neutrophil

counts and ratios at 6h and 24h after each challenge are presented in Appendix 2.9, and summarised in Table 2.8.

Table 2.7: Baseline total and differential BALF cell counts (x10^5/ml) (median and range) at t-7d in both control (C; n=4) and heaves horses (H; n=6) prior to inhalation challenge with 20, 200 and 2000μg LPS and mouldy hay/straw challenge (H/S). TCC = total cell count.

		TCC	Lymphocytes	Macrophages	Neutrophils	Mast cells	Basophiloid cells	Eosinophils	Epithelial cells
20μg LPS	C	3.30 (2.40-4.60)	1.42 (1.00-2.78)	1.35 (1.07-2.30)	0.03 (0.01-0.06)	0.14 (0.08-0.19)	0.00 (0.00-0.14)	0.01 (0.00-0.03)	0.00 (0.00-0.01)
	H	4.35 (2.90-9.10)	2.14 (1.15-4.50)	2.18 (1.42-4.22)	0.08 (0.04-0.25)	0.18 (0.07-0.38)	0.00 (0.00-0.01)	0.00 (0.00-0.01)	0.00 (0.00-0.00)
200μg LPS	C	5.35 (3.70-7.00)	2.50 (1.52-5.24)	2.03 (0.92-2.84)	0.06 (0.02-0.18)	0.20 (0.10-0.33)	0.01 (0.00-0.03)	0.03 (0.02-0.55)	0.00 (0.00-0.00)
	H	4.98 (1.50-7.20)	2.95 (0.69-4.02)	1.81 (0.63-2.93)	0.12 (0.03-0.15)	0.08 (0.03-0.38)	0.01 (0.00-0.03)	0.00 (0.00-0.03)	0.00 (0.00-0.03)
2000μg LPS	C	3.90 (2.40-7.30)	1.81 (0.80-4.38)	1.75 (1.16-2.29)	0.07 (0.03-0.08)	0.09 (0.02-0.33)	0.00 (0.00-0.07)	0.04 (0.00-0.17)	0.00 (0.00-0.00)
	H	4.35 (1.80-9.70)	2.23 (0.48-5.25)	1.94 (1.21-4.08)	0.08 (0.04-0.18)	0.07 (0.03-0.12)	0.02 (0.00-0.06)	0.00 (0.00-0.01)	0.00 (0.00-0.01)
H/S	C	2.05 (1.00-2.40)	0.70 (0.30-0.98)	1.08 (0.64-1.50)	0.02 (0.01-0.03)	0.10 (0.05-0.15)	0.01 (0.00-0.03)	0.00 (0.00-0.00)	0.00 (0.00-0.00)
	H	4.15 (2.40-9.10)	1.76 (1.36-5.66)	2.19 (0.72-3.01)	0.06 (0.03-0.13)	0.11 (0.03-0.27)	0.00 (0.00-0.01)	0.00 (0.00-0.12)	0.00 (0.00-0.00)

Table 2.8: BALF neutrophil counts (x10^5/ml) and ratios (%) (median and range) in control (n=6) and heaves (n=7) horses at 6 and 24h following inhalation challenge with saline, 20, 200 and 2000μg LPS and mouldy hay/straw challenge (H/S).

	BALF neutrophil count (x10^5/ml)				BALF neutrophil ratio (%)			
	Heaves		Controls		Heaves		Controls	
	6h	24h	6h	24h	6h	24h	6h	24h
SALINE	0.06 (0.03-0.20)	0.08 (0.04-0.16)	0.06 (0.01-0.17)	0.04 (0.02-0.60)	2.3 (0.6-4.5)	1.7 (1.1-11.5)	1.3 (0.2-3.2)	1.7 (0.6-17.7)
20μg LPS	0.28 (0.18-0.53)	0.20 (0.13-1.04)	0.09 (0.01-0.17)	0.03 (0.02-0.18)	6.1 (5.6-7.2)	4.7 (2.4-23.6)	1.7 (0.3-6.2)	1.8 (0.6-2.6)
200μg LPS	1.45 (0.42-2.22)	0.77 (0.26-1.26)	0.57 (0.08-2.59)	0.39 (0.27-3.43)	23.2 (10.5-41.8)	13.7 (6.1-21.9)	13.9 (3.1-28.4)	12.3 (5.7-41.3)
2000μg LPS	3.25 (0.57-4.34)	1.28 (0.76-6.24)	1.44 (0.52-2.70)	1.69 (1.12-2.70)	34.2 (28.4-65.8)	33.4 (17.8-39.6)	36.7 (10.9-64.2)	37.9 (19.0-51.0)
H/S	2.05 (0.74-9.83)	0.67 (0.27-1.41)	0.17 (0.01-0.40)	0.09 (0.06-0.22)	36.0 (21.0-60.7)	17.6 (5.4-31.5)	7.0 (0.3-11.2)	4.3 (1.9-7.4)

LPS induced a dose-dependent BALF neutrophilia in both groups (Figs. 2.13-2.16). In the heaves group, when compared with saline inhalation, absolute BALF neutrophil count was significantly ($P<0.05$) increased at both 6 and 24h after 20μg, 200μg and 2000μg LPS inhalation. These significant increases were also seen in the BALF neutrophil ratio with the exception of the 24h values following 20μg LPS inhalation.

In controls, BALF neutrophil count and ratio were significantly ($P<0.05$) increased at 6h after inhalation of 200μg and 2000μg LPS, and at 24h after inhalation of 2000μg LPS. BALF neutrophil count and ratio were significantly ($P<0.05$) increased in the heaves group at 6h (Figs. 2.13 and 2.14) and 24h after hay/straw challenge. No increase in BALF neutrophil count was seen in the control group at 6h or 24h after hay/straw challenge (Fig. 2.15), however a slight, yet significant ($P<0.05$) increase in BALF neutrophil ratio was noted in this group at 6h (Fig. 2.16). Absolute BALF neutrophil count was significantly greater ($P<0.01$) at both 6h and 24h in the heaves group compared with the control group, following inhalation of 20μg LPS and after hay/straw challenge (Fig. 2.17). In addition, the heaves group also had a significantly greater BALF neutrophil ratio following 20μg LPS inhalation ($P<0.05$ at 6h, $P<0.01$ at 24h) and hay/straw challenge ($P<0.01$) (Fig 2.18).

Fig. 2.13: BALF neutrophil counts (x10^5/ml) in heaves (n=7) horses at 6h following inhalation challenge with saline, 20, 200 and 2000μg LPS and mouldy hay/straw challenge (H/S).

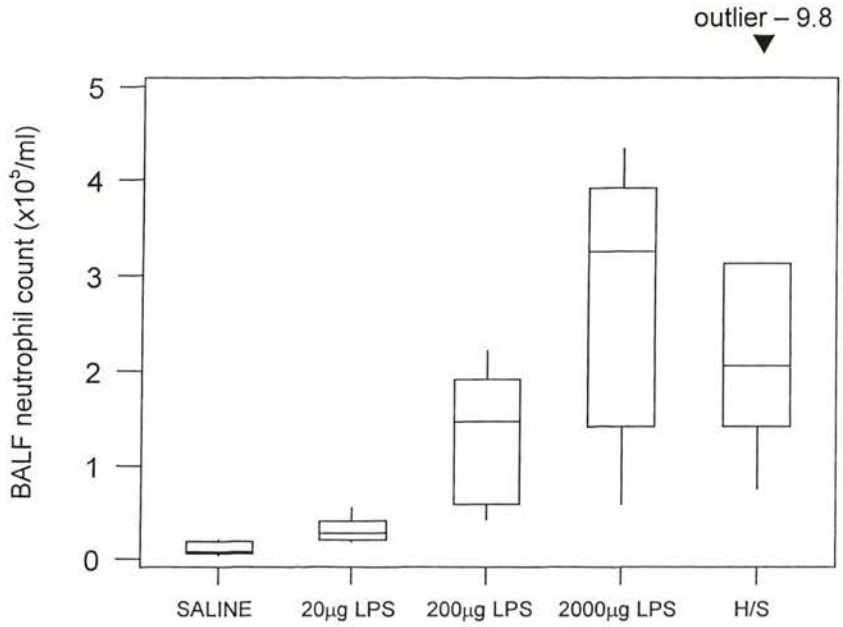


Fig. 2.14: BALF neutrophil ratio (%) in heaves (n=7) horses at 6h following inhalation challenge with saline, 20, 200 and 2000μg LPS and mouldy hay/straw challenge (H/S). * = statistical outlier.

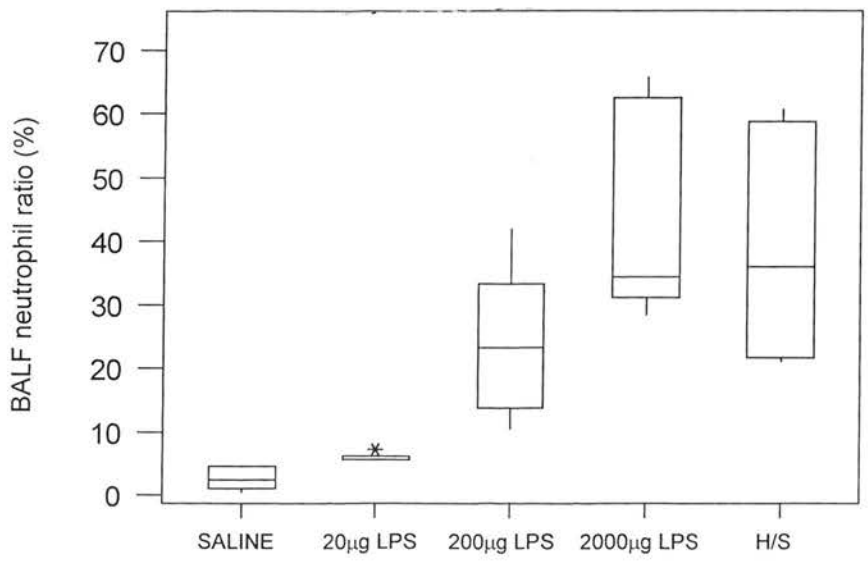


Fig. 2.15: BALF neutrophil counts (x10^5/ml) in control (n=6) horses at 6h following inhalation challenge with saline, 20, 200 and 2000μg LPS and mouldy hay/straw challenge (H/S).

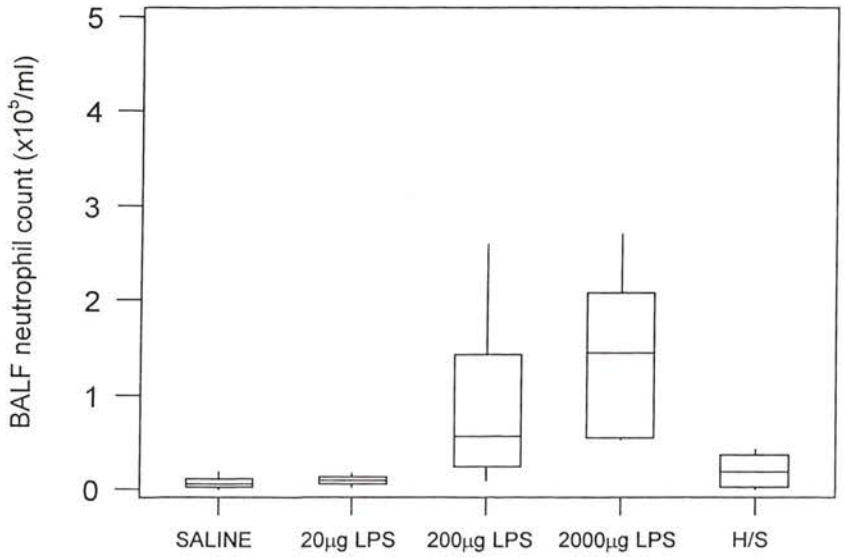


Fig. 2.16: BALF neutrophil ratio (%) in control (n=6) horses at 6h following inhalation challenge with saline, 20, 200 and 2000μg LPS and mouldy hay/straw challenge (H/S).

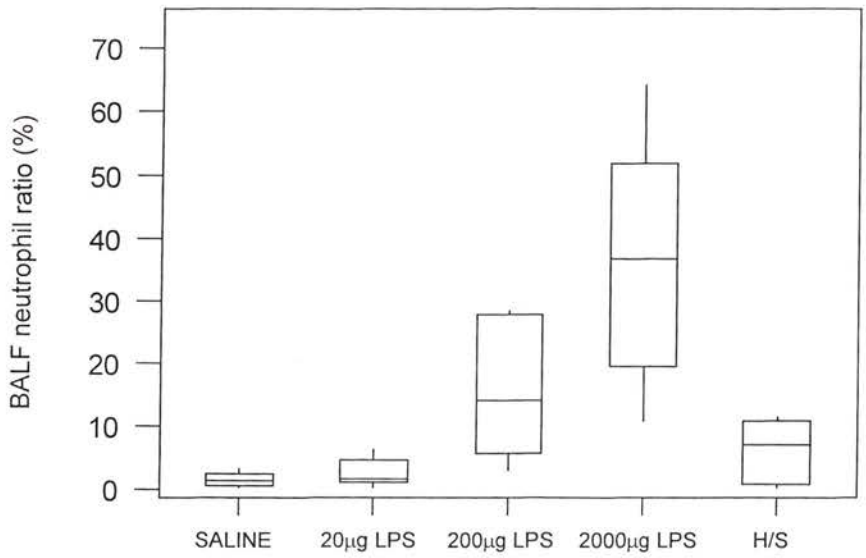


Fig. 2.17: BALF neutrophil counts (x10^5/ml) in heaves (n=7) and control (n=6) horses at 6h following inhalation challenge with saline, 20, 200 and 2000μg LPS and mouldy hay/straw challenge (H/S). + = heaves; O = controls.

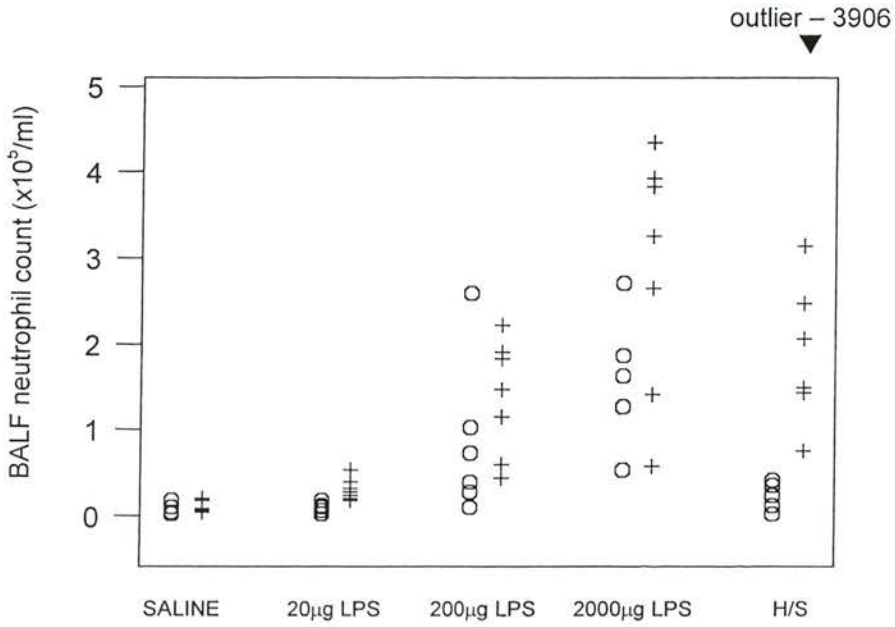
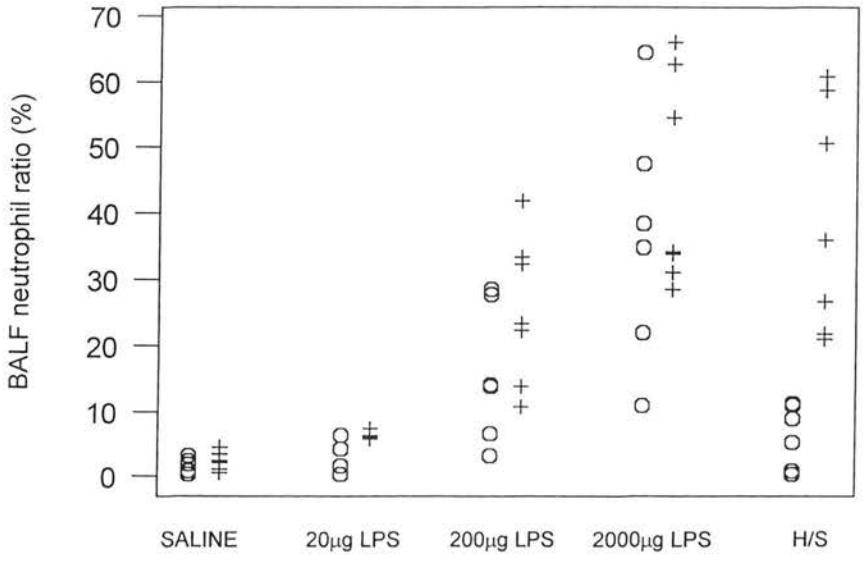
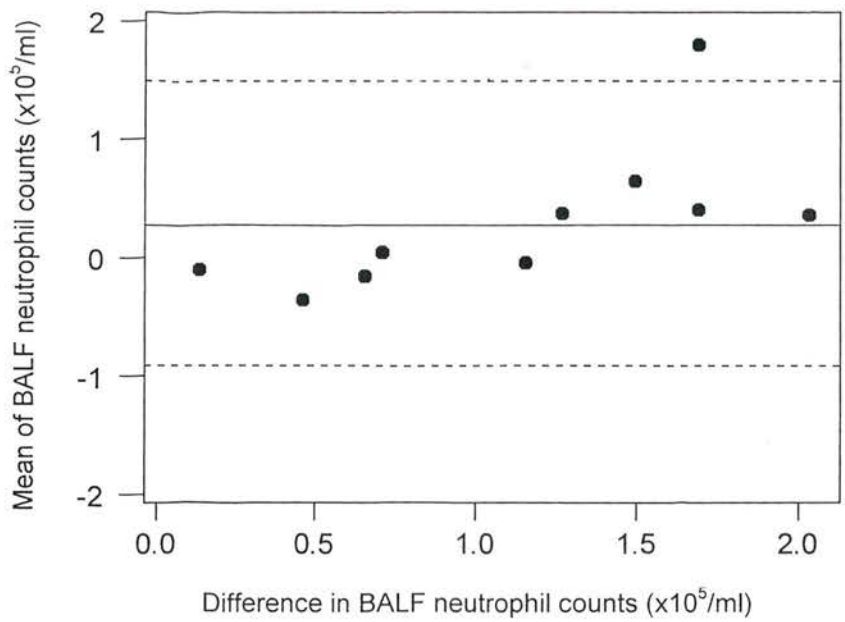


Fig. 2.18: BALF neutrophil ratio (%) in heaves (n=7) and control (n=6) horses at 6h following inhalation challenge with saline, 20, 200 and 2000μg LPS and mouldy hay/straw challenge (H/S). + = heaves; O = controls.



There was no significant difference in BALF neutrophil counts between the first (1.3 x 10^5/ml, 0.3-2.2), and the second (0.9 x 10^5/ml, 0.6-1.5) 200μg LPS inhalation challenges. Nine of the 10 calculated differences in paired values fell within 2 standard deviations of the mean of the differences. As the data point falling out with this range was a clear statistical outlier (out with the lower limit defined as: first quartile minus 1.5 x [third quartile minus first quartile]), repeatability was considered good (Bland and Altman 1986) (Fig. 2.19).

Fig. 2.19: Difference between BALF neutrophil counts (x10^5/ml) plotted against the mean of the BALF neutrophil counts (x10^5/ml) in heaves (n=6) and control (n=4) horses at 6h following both 200μg LPS inhalation challenges. Solid line = mean of the differences; dotted line = mean of the differences +/- 2 standard deviations of the differences.



Due to the influence of a BALF neutrophilia on the ratio of other BALF cell types, other BALF cell types were considered only as absolute numbers. The absolute number of other BALF cell types at 6h following all challenges is summarised in Table 2.9.

Table 2.9: Total and differential BALF cell counts ($x10^5$/ml) (median and range) in heaves (H; n=7) and control (C; n=6) horses at 6h following inhalation challenge with saline, 20, 200 and 2000μg LPS and mouldy hay/straw challenge (H/S).

		TCC	Lymphocytes	Macrophages	Mast cells	Basophiloid cells	Eosinophils
SALINE	C	3.85 (2.00-9.60)	1.59 (0.99-6.22)	1.98 (0.73-2.80)	0.25 (0.06-0.39)	0.02 (0.00-0.22)	0.03 (0.00-0.06)
	H	3.80 (1.30-5.60)	2.09 (0.80-3.33)	1.58 (0.34-2.96)	0.13 (0.09-0.19)	0.01 (0.00-0.11)	0.01 (0.00-0.01)
20μg LPS	C	3.50 (1.90-11.10)	1.37 (0.62-6.22)	1.54 (0.33-3.94)	0.12 (0.05-0.32)	0.03 (0.00-0.09)	0.01 (0.00-0.58)
	H	4.60 (2.90-7.40)	2.45 (1.88-4.83)	1.68 (0.70-2.57)	0.07 (0.06-0.30)	0.01 (0.00-0.12)	0.00 (0.00-0.01)
200μg LPS	C	3.95 (2.70-9.40)	1.87 (1.17-3.02)	1.89 (0.51-4.18)	0.10 (0.04-0.28)	0.02 (0.01-0.10)	0.01 (0.00-0.21)
	H	4.90 (4.00-8.20)	2.37 (1.26-3.83)	1.06 (0.61-2.48)	0.07 (0.02-0.40)	0.01 (0.00-0.15)	0.00 (0.00-0.03)
2000μg LPS	C	4.05 (1.50-7.30)	1.11 (0.68-3.69)	0.91 (0.17-1.76)	0.08 (0.00-0.23)	0.02 (0.00-0.07)	0.03 (0.01-0.34)
	H	6.60 (1.70-12.40)	1.78 (0.68-5.39)	0.77 (0.23-3.94)	0.05 (0.01-0.17)	0.01 (0.00-0.05)	0.01 (0.00-0.10)
H/S	C	2.55 (2.10-4.60)	0.89 (0.61-3.09)	1.26 (0.66-1.89)	0.11 (0.01-0.12)	0.02 (0.00-0.12)	0.01 (0.00-0.02)
	H	6.20 (2.80-16.20)	2.30 (0.60-4.26)	1.44 (0.90-3.16)	0.10 (0.08-0.12)	0.01 (0.00-0.08)	0.00 (0.00-0.02)

Reduced absolute BALF macrophage ($P<0.05$) and mast cell ($P<0.05$) numbers followed 2000μg LPS inhalation only in controls at 6h (Figs. 2.20 and 2.22), with heaves horses showing a similar but non-significant reduction (Figs. 2.21 and 2.23) when compared with saline inhalation. None of the challenges induced significant changes in BALF total cell count, or absolute lymphocyte, epithelial cell, basophiloid or eosinophil counts. There was no difference between the 6h and 24h BALF total or

absolute cell counts following all challenges, although there was a trend towards a reduction in all cell types at 24h (Figs. 2.24 and 2.25).

Fig. 2.20: BALF macrophage count ($x10^5$/ml) in control (n=6) horses at 6h following inhalation challenge with saline and 2000μg LPS.

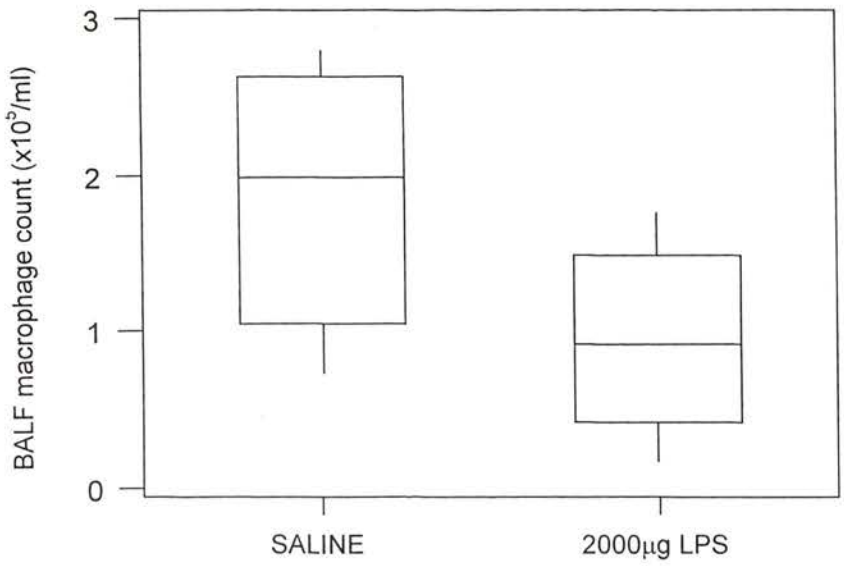


Fig. 2.21: BALF macrophage count ($x10^5$/ml) in heaves (n=7) horses at 6h following inhalation challenge with saline and 2000μg LPS.

Fig. 2.22: BALF mast cell count (x10^5/ml) in control (n=6) horses at 6h following inhalation challenge with saline and 2000μg LPS.

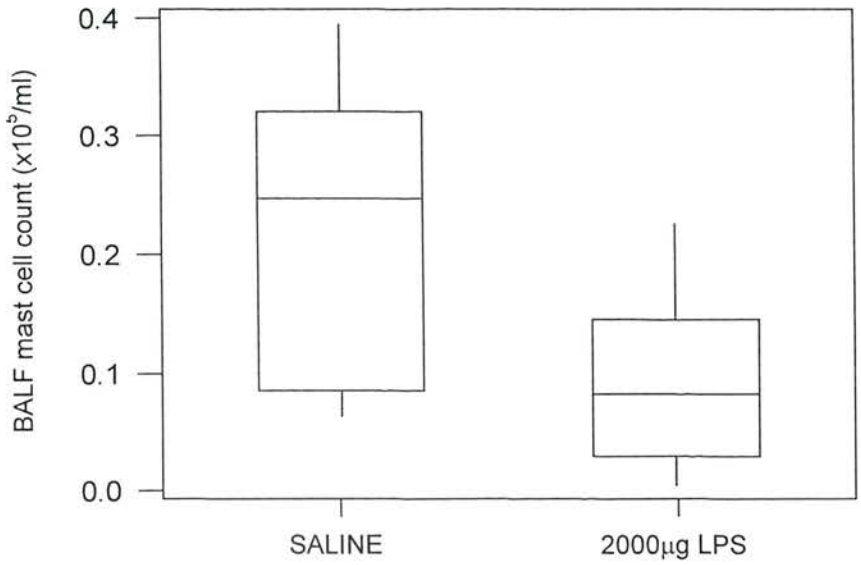


Fig. 2.23: BALF mast cell count (x10^5/ml) in heaves (n=7) horses at 6h following inhalation challenge with saline and 2000μg LPS.

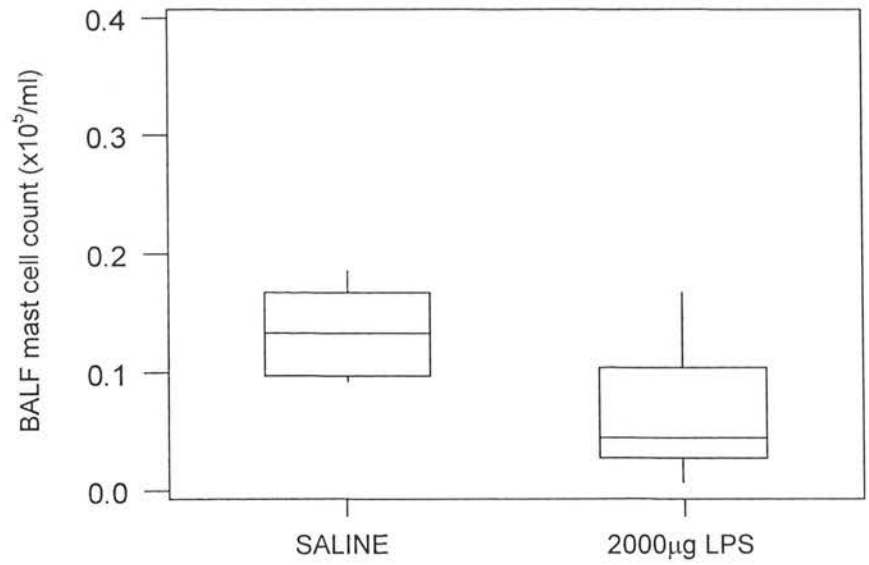


Fig. 2.24: BALF neutrophil count ($x10^5$/ml) in heaves (n=7) horses at 6 and 24h following inhalation challenge with saline, 20, 200 and 2000μg LPS and hay/straw challenge. * = 2000μg LPS, Δ = hay/straw challenge, x = 200μg LPS, + = 20μg LPS, O = saline.

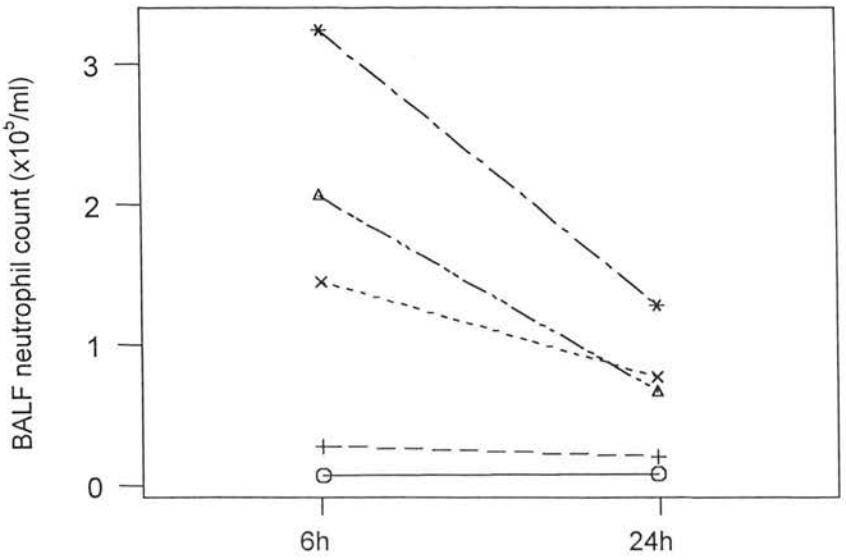
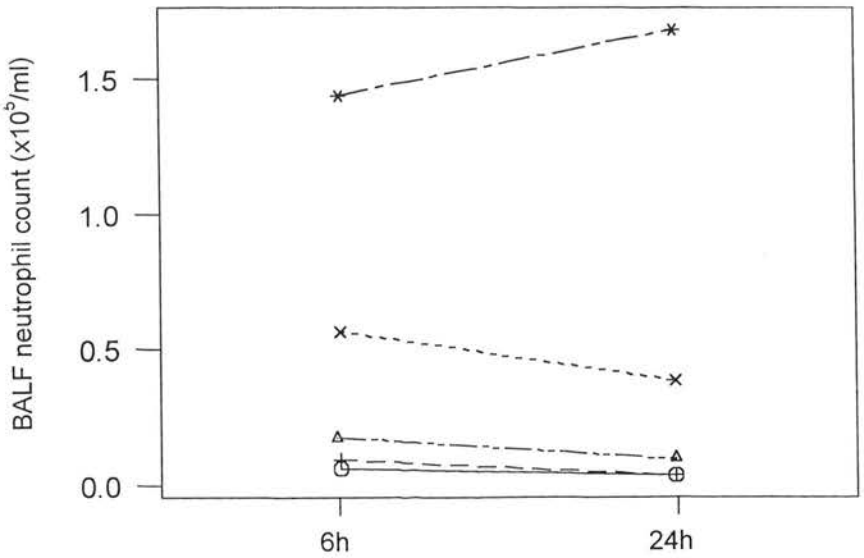


Fig. 2.25: BALF neutrophil count ($x10^5$/ml) in control (n=6) horses at 6 and 24h following inhalation challenge with saline, 20, 200 and 2000μg LPS and hay/straw challenge. * = 2000μg LPS, Δ = hay/straw challenge, x = 200μg LPS, + = 20μg LPS, O = saline.



2.5.2.6 Haematological analyses

Raw data for peripheral blood total leucocyte and neutrophil counts are presented in Appendix 2.10. There was no significant difference in the baseline peripheral blood total leucocyte or neutrophil values prior to each of the challenges, indicating a lack of detectable carry-over effects. The percent of baseline peripheral blood total leucocyte and neutrophil values is presented in Table 2.10. Following correction for the effects of saline inhalation, challenge with 2000μg LPS significantly reduced peripheral blood total leucocyte counts at 4h when compared with baseline in both groups (heaves: reduction in median 14%, 95% CI 5-24; $P<0.05$; controls: reduction in median 23%, 95% CI 12-36; $P<0.05$).

Control horses also had a significant, but minor, reduction in peripheral blood total leucocyte counts at 90min following inhalation of 200μg LPS (reduction in median 8%, 95% CI 0-17; $P<0.05$). Compared with baseline values, hay/straw challenge significantly increased peripheral blood total leucocyte counts in the controls at 90min (increase in median 13%, 95% 1-24; $P<0.05$). A marked and significant increase in peripheral blood neutrophil counts was also noted in the heaves group at 24h following hay/straw challenge (increase in median 34%, 95% CI 13-75; $P<0.05$).

Table 2.10: Percent (%) of baseline peripheral blood total leucocyte (WBC) and neutrophil (PMN) numbers (median and range) in heaves (n=7) and control (n=6) horses at 1.5, 4 and 24h following inhalation challenge with saline, 20, 200 and 2000μg LPS, and mouldy hay/straw challenge.

		SALINE		20μg LPS		200μg LPS		2000μg LPS		HAY/STRAW	
		CONTROLS	HEAVES	CONTROLS	HEAVES	CONTROLS	HEAVES	CONTROLS	HEAVES	CONTROLS	HEAVES
WBCs	1.5h	97.9 (89.4-108.2)	95.2 (85.9-105.0)	90.4 (83.5-103.2)	100.0 (80.0-110.3)	86.0 (79.3-107.7)	96.6 (77.4-120.9)	93.3 (77.0-107.8)	98.1 (78.8-111.1)	112.7 (101.3-123.6)	102.2 (84.9-112.7)
	4h	112.1 (102.8-118.8)	104.8 (95.7-115.1)	101.1 (89.5-112.8)	105.9 (86.8-115.2)	94.6 (87.0-116.5)	101.8 (79.8-126.9)	93.9 (67.2-101.9)	89.0 (77.5-105.0)	111.3 (96.9-121.2)	103.3 (93.8-112.7)
	24h	142.7 (96.5-166.7)	124.5 (98.9-137.0)	124.7 (103.4-151.7)	112.7 (93.9-197.1)	106.9 (75.0-123.1)	117.1 (92.4-158.2)	118.6 (68.8-132.9)	114.8 (91.3-202.8)	128.8 (94.6-148.4)	125.3 (83.9-150.5)
PMNs	1.5h	120.7 (89.3-163.3)	102.3 (89.1-106.4)	103.1 (71.9-130.0)	104.1 (91.6-151.7)	91.6 (69.4-105.7)	104.8 (78.2-137.3)	86.7 (68.1-149.7)	102.0 (67.3-143.7)	108.8 (82.3-156.8)	96.3 (88.7-112.6)
	4h	125.9 (78.0-164.8)	113.4 (100.2-137.7)	107.8 (82.5-121.3)	107.0 (96.0-116.5)	94.4 (74.8-146.1)	104.9 (85.2-169.9)	92.7 (50.7-106.9)	112.7 (77.5-125.9)	110.3 (63.7-148.6)	114.3 (89.9-122.9)
	24h	177.8 (112.9-316.7)	129.0 (107.4-151.2)	163.2 (112.4-273.9)	131.9 (58.5-349.8)	118.7 (89.7-156.2)	137.0 (81.2-184.2)	130.8 (80.3-147.6)	148.9 (84.5-145.3)	159.4 (84.9-233.8)	130.0 (102.1-214.0)

2.6 Discussion

The systemic and pulmonary effects of inhalation with soluble LPS in control and asymptomatic heaves horses are reported for the first time. Consistent with endotoxin inhalation studies in man and other species (Gordon, 1992; Schwartz *et al.,* 1994; Urbain *et al.,* 1996a; Michel *et al.,* 1997), inhalation of 20, 200 and 2000μg soluble *Salmonella typhimurium* Ra60 LPS induced a dose-dependent airway neutrophilia, with BALF neutrophil numbers increasing approximately 50-fold in heaves horses at 6h after the highest dose (2000μg) challenge. Pulmonary recruitment of neutrophils may have been induced by a variety of alveolar macrophage derived cytokines, including tumour necrosis factor-alpha, interleukin 1 (IL-1), IL-6 and IL-8, which are present in increased concentrations in BALF following endotoxin inhalation in other species (Derochemonteixgalve *et al.,* 1991; Ryan *et al.,* 1994; Schwartz *et al.,* 1994; Jagielo *et al.,* 1996a; Jagielo *et al.,* 1996b; Ulmer, 1997; Wesselius *et al.,* 1997).

After inhalation of 2000μg LPS, absolute BALF macrophage and mast cell numbers were significantly reduced in controls, and non-significantly reduced in the heaves group. Reduced BALF macrophage numbers have been reported following LPS inhalation in other species, possibly due to LPS-induced macrophage apoptosis (Michel *et al.,* 1997), or to migration of macrophages from the lung following antigenic stimulation or for clearance of apoptotic neutrophils (Brazil, 2000) and possibly other apoptotic inflammatory cells. The significance of the reduction in BALF mast cell numbers is unclear, but may be artefactual and reflect failure to identify (and count) degranulated mast cells on cytospin preparations, as endotoxin

has been shown in rat skin to directly stimulate mast cell degranulation (Iuvone *et al.*, 1999).

Peripheral blood total leucocyte counts were reduced at 4h following inhalation of 2000μg LPS in both groups, consistent with a combined systemic and pulmonary response. This reduction probably reflects margination and pulmonary recruitment of leucocytes, as occurs following LPS inhalation in guinea pigs (Fogelmark *et al.*, 1992). As found in this study, the circulating leucocyte response to inhaled endotoxin was similar in healthy and asthmatic humans, whereas only the asthmatic group had significant lung dysfunction (Michel *et al.*, 1992a; Michel *et al.*, 1995a). There was no significant alteration in peripheral blood neutrophil counts in either group, at the time points studied, in contrast to hay/straw challenge, which induced a peripheral blood neutrophilia in the heaves group at 24h.

LPS inhalation challenges had no significant effect on clinical score in either group. This is not surprising, since many of the clinical symptoms reported by humans following LPS inhalation, including chest tightness, headaches, joint pain and tiredness (Rylander *et al.*, 1989; Rylander *et al.*, 1999) are subjective, and would therefore be difficult to detect in horses.

Heaves horses showed a significant deterioration in lung function (as determined by lung mechanics testing), only after inhalation of 2000μg LPS. Similarly in healthy humans, high doses (>80-200μg) of inhaled LPS are required to produce combined

restrictive and obstructive lung dysfunction, albeit moderate and inconsistent (Cavagna *et al.,* 1969; Rylander *et al.,* 1989; Michel *et al.,* 1995a; Michel, 1997). While the increased $RL_{E50\%}$ and $RL_{E75\%}$ noted in the heaves group is consistent with an obstructive component, the relative insensitivity of pulmonary mechanics testing in the horse (performed on tidal breathing) may have prevented detection of mild restrictive dysfunction. As in humans, where lung dysfunction is more pronounced in atopics and asthmatics (Michel *et al.,* 1989; Rylander, 1996), control horses had no significant lung dysfunction, even after inhalation of 2000μg LPS.

The difference between the 2 groups with respect to lung mechanics cannot be explained solely by the possible atopic status of the heaves group. The effects of endotoxin are inflammatory in nature, and not IgE-mediated (Michel, 1997), and studies in humans have demonstrated that the bronchial obstruction induced by LPS inhalation is associated with non-specific responsiveness but not with atopy (Michel *et al.,* 1992b). However, the exaggerated lung dysfunction noted in the heaves group may reflect a degree of undetected pre-existing airway inflammation in this group, despite them having been maintained in a dust free environment for several weeks prior to the challenges, thus magnifying the response to inhaled LPS. No alteration in airway reactivity was detected in either group, in contrast to the increased airway reactivity noted at 6h after endotoxin inhalation in human asthmatics (Michel *et al.,* 1989; Michel *et al.,* 1992a). Failure to detect increased airway reactivity may be due to insufficient dose of LPS, or to attenuation of the methacholine induced

bronchconstriction by the bronchodilatory effects of the α2-agonist drug (Broadstone *et al.,* 1992) used to sedate horses for this procedure.

LPS challenges did not significantly increase tracheal mucus score in either group at the 6h or 24h time points, although the heaves group did show a trend for increased mucus volume with increasing doses of LPS. In other species, inhaled endotoxin induces (Gordon and Harkema, 1994; Gordon *et al.,* 1996), or is correlated with, airway mucus hypersecretion (Rylander *et al.,* 1999).

The role of inhaled endotoxin in human occupational respiratory diseases is well documented (Douwes and Heederik, 1997; Jacobs, 1997a), and the necessity for dose-response experiments as a prerequisite to establishing no-response safety thresholds has been recognised (Michel *et al.,* 1997). As a result of this series of inhalation challenges, comparisons can be made between the levels of airborne endotoxin detected in equine environments and the minimal threshold exposures of soluble LPS required for induction of lung inflammation and dysfunction in the horse. However the lack of information on the deposition of inhaled aerosolised solution compared with inhaled organic dust in the horse greatly reduces the accuracy of any such comparisons. The response threshold of LPS for inducing airway inflammation was lower in the heaves group (20μg), than controls (200μg), and the magnitude of BALF neutrophilia was, albeit insignificantly, more marked in heaves horses than controls. The reason for this difference in LPS response between the 2 groups is unclear. However human studies have identified a similar phenomenon

when asthmatics are compared with healthy controls, whereby both the atopic status and the increased neutrophilic response to LPS inhalation in asthmatics was associated with the constitutive expression of CD14, the principal receptor in mediating LPS responses (Alexis *et al.,* 2001).

The response thresholds for lung dysfunction in the heaves (2000μg LPS) and control (>2000ug LPS) groups were higher than the response thresholds for inflammation as assessed by BALF cytology. Consistent with this finding, in other species, markers of inflammation (e.g. BALF and/or sputum neutrophils, myeloperoxidase, tumour necrosis factor-alpha, eosinophil cationic protein and lactate dehydrogenase) were more sensitive indices of the effects of inhaled endotoxin than lung dysfunction (Gordon, 1992; Herbert *et al.,* 1992; Michel *et al.,* 1997).

In this study, the 5h duration hay/straw challenge was estimated to result in an exposure to biologically active endotoxin equivalent to 0.18 (0.08-2.85)μg as calculated from *respirable* dust and biologically active endotoxin equivalent to 7.44 (4.04-27.00)μg, as calculated from *total* dust. These exposures are mostly lower than the thresholds for lung inflammation and dysfunction in both groups. Similarly, in man, while the role of endotoxin in occupational lung disease is well recognised, with respect to the induction of clinical symptoms, the threshold exposure for soluble LPS (20μg) (Michel *et al.,* 1989; Michel *et al.,* 1995a) exceeds that for endotoxin present in airborne organic dust (1.8-3.0μg) collected from swine housing environment (Larsson *et al.,* 1994). This apparent discrepancy may be explained by several factors

that limit direct comparison of the threshold exposures for inhaled endotoxin in acute experimental LPS inhalation challenges and in organic dust exposure, as follows:

(a) Other agents present in stable dust, such as moulds and glucans, may potentiate the response to endotoxin (Fogelmark *et al.,* 1994; Hunt *et al.,* 1994). Pre-exposure of human asthmatics to allergen potentiates their response to LPS (Martin *et al.,* 1992) by increasing vascular permeability and extravasation of LPS binding protein and soluble CD14 receptors from the pulmonary circulation (Dubin *et al.,* 1996). Consequently, guidelines for safe environmental levels are based on values for persons with histories of atopy or asthma (Rylander, 1997b). Possibly, concomitant mould allergen exposure could increase LPS responsiveness to a greater extent in heaves horses than in controls.

(b) The biologically active endotoxin content of the stable dust may have been underestimated by the *Limulus* amoebocyte lysate assay used in this study, since this method detects mainly soluble endotoxin and underestimates the biologically active particulate endotoxin (Rylander *et al.,* 1989). While the recommended correction factor of 3 (Rylander *et al.,* 1989) was applied when calculating the biologically active endotoxin content of stable dust, this correction factor may be incorrect given the probable variation in the proportions of soluble and particulate endotoxin in dusts from different sources. The method and duration of elution of endotoxin from the filters may have had an effect on amount of "biologically available" endotoxin detected by the *Limulus* amoebocyte lysate assay. Although there is no consensus regarding the most effective method for extraction of endotoxin from filters, the

method employed in the current study was chosen to reflect those methods most frequently described in the literature (Jacobs, 1997b). An example of the influence of extraction methods is the reported sevenfold difference in detected endotoxin when 0.05% Tween is used as a diluent (Douwes *et al.*, 1995), presumably resulting in the disruption of micelles, thus resulting in the exposure of the lipid A component of LPS.

(c) Short duration challenges, as used for soluble LPS inhalation, may produce less effect than longer duration exposure (e.g. 5h hay/straw challenge). Similarly with intravenous LPS challenges, slow infusion is frequently preferred to bolus administration, owing to the ability of the mononuclear phagocyte system to neutralise circulating endotoxins (Urbain *et al.,* 1996a). As the increased content of stored mucosubstances in rat airways following endotoxin inhalation is duration-dependant (Gordon and Harkema, 1994; Gordon *et al.,* 1996), this may explain why there was significant mucus hypersecretion following hay/straw challenge but not following LPS inhalation.

(d) The acute LPS and hay/straw challenges likely differed with respect to the efficacy of delivery and deposition of aerosol and dust particles, the anatomical site in which they were deposited, and the mechanisms and rate by which they were cleared. While in the order of 7% of the LPS aerosol generated by the jet nebuliser may have been deposited in the lung (Votion *et al.,* 1997), the proportion of respirable and total airborne stable dust reaching the lungs during the hay/straw challenge cannot be determined. Therefore, without a detailed knowledge of the aerodynamic prperties of

the challenge aerosol (particulate or aqueous) the exposure level in the breathing zone may not accurately reflect the "dose" delivered to the target cells within the lung. Finally, it is unclear whether the endotoxin concentration in respirable or total airborne stable dust should be considered when making a comparison with the threshold exposure of soluble LPS. In the hay/straw challenge the majority of endotoxin in respirable stable dust likely reached the lower airways, however the endotoxin in the non-respirable fraction may have caused inflammation and dysfunction of the larger airways (Jacobs, 1997b) and may have contributed to the response to challenge.

(e) It could be argued that there was a difference between the LPS present within the organic dust (comprised a variety of LPS types) and that present within the LPS solution used in the LPS inhalation challenges (*S. typhimurium* Ra60 mutant), with respect to virulence within the lung. Given the inability to produce a pure endotoxin mix which is representative of those types of LPS encountered in equine stables, the choice of the *S. typhimurium* Ra60 mutant LPS was considered appropriate. It was considered likely that Enterobacteriaceae largely contribute to endotoxin present on airborne dust in equine stables. Although the shortened polysaccharide chain of the *S. typhimurium* Ra60 mutuant is more representative of respiratory tract-derived LPS than gastrointestinal-derived LPS (Makela and Stocker, 1984), it did contain a complete core oligosaccharide plus lipid A. This structure is responsible for a major part of the biological activity of LPS and is shared by many Enterobacteriaceae including all *Escherichia coli* and *Salmonella* species (Prof. IR Poxton, personal communication). However, truncation of the polysaccharide chain may have resulted

in an alteration of its virulence due to a reduction in phagocytosis in the lung (Taussig, 1984). One major advantage of the LPS used in the inhalation challenges was the homogenous nature of the preparation, compared with the very heterogenous nature of wild type LPS and even some commercially available preparations (Prof. IR Poxton, personal communication).

Despite the aforementioned problems in comparing endotoxin levels in the hay/straw challenge and the threshold exposure for soluble LPS inhalations, several observations suggest that inhaled endotoxin was not the sole cause of lung inflammation and dysfunction in the heaves group following hay/straw challenge. Firstly, the total estimated exposure of endotoxin encountered in the hay/straw challenge (7.44μg) was *markedly* lower than the threshold exposure of soluble LPS (200-2000μg) required to induce a similar degree of BALF neutrophilia. Secondly, the hay/straw challenge did not induce a BALF neutrophilia in controls, while inhalation of ≥200μg LPS induced BALF neutrophilia in both groups. Thirdly, in contrast to LPS inhalation, hay/straw challenge significantly increased tracheal mucus score in the heaves group.

However, it is likely that endotoxin *per se* causes airway inflammation in horses in stables with very poor air hygiene, since *respirable* endotoxin concentrations in such stables may be as high as 3437ng/m^3 (Dutkiewicz *et al.,* 1994). A 5h exposure to this concentration would equate to an LPS exposure of 160μg, which exceeds the threshold exposure of LPS which causes inflammation in horses with asymptomatic

heaves (20μg), and possibly that which causes inflammation in control horses (between 20 and 200μg).

In this study, as in human studies (Michel *et al.,* 1997), all horses were given LPS in increasing rather than randomised doses. This order was selected for safety reasons, given the absence of data on the effects of acute LPS inhalation in horses, and given the potential for significant individual-dependent variability in the response (Michel *et al.,* 1992b). It could be argued that randomisation of challenge order may have minimised potential carry-over effects from prior challenges. Carry-over effects could include potentiation due to persistence of inflammation, early-phase tolerance (Ulmer, 1997), and late-phase (occurring after several weeks) tolerance due to production of anti-endotoxin antibodies (Johnston and Greisman, 1985; Ulmer, 1997). Daily exposure of rats to LPS did result in a gradual reduction in chemokine and neutrophil concentration in recovered BALF (Shimada *et al.,* 2000) However, the excellent repeatibility of inflammatory (BALF neutrophilia) and functional (PCCDyn70) changes following repeated 200ug LPS challenge, suggests that carry-over effects were insignificant in this study. Further, since early-phase tolerance to inhaled endotoxin lasts no more than 2 days (Johnston and Greisman, 1985), it was unlikely to have influenced the response to subsequent challenges that were separated by at least 2 weeks.

In conclusion, inhaled endotoxin induces neutrophilic airway inflammation and dysfunction in horses. While this study suggests that inhaled endotoxin is not the sole cause of heaves, it suggests that it may contribute to disease aetiopathogenesis.

Acceptance of the dose-response data described is a prerequisite to the development of acceptable endotoxin exposure levels for horse accommodation. Control or heaves-affected horses housed in stables with poor air hygiene may be exposed to airborne endotoxin levels exceeding the threshold dose levels that induce airway inflammation. While further work is required to determine the effect of inhaled endotoxin on subclinical pulmonary dysfunction and thus on exercise performance, potentially detrimental effects may be minimised by optimising air hygiene in stables.

CHAPTER 3: COMPARISON OF TWO DIFFERENT HAY/STRAW CHALLENGE SYSTEMS WITH RESPECT TO DISEASE INDUCTION IN ASYMPTOMATIC HEAVES HORSES AND AIRBORNE DUST AND ENDOTOXIN EXPOSURE

3.1 Summary

To determine whether the pulmonary inflammatory response of heaves-susceptible animals to organic dust challenge was related to airborne endotoxin exposure, 6 heaves horses were exposed to 2 different hay/straw challenge environments. Both challenges consisted of a 5h exposure to dusty hay and straw, however one of the challenge environments used hay with obvious visible mould contamination. The severity of airway disease, as indicated by the BALF neutrophilia and arterial hypoxaemia, was significantly different despite airborne dust and endotoxin concentrations in the horses' breathing zones being similar in both challenges. Furthermore, the environment that induced the greater disease severity had a higher airborne concentration of β-D-glucan, albeit non-significantly. This likely reflected the greater degree of visible mould contamination of the hay used during this challenge. Inhaled endotoxin appears, therefore, not to be the main determinant of disease severity. As the BALF neutrophilia was greater in horses exposed to mouldy hay, mould exposure may be a more important determinant of disease severity.

3.2 Introduction

The experimental LPS dose-response inhalation experiments detailed in Chapter 2 established that the induction of heaves in susceptible horses does not appear to be entirely due to inhaled endotoxin (Chapter 2). However several problems were highlighted regarding the comparison of endotoxin exposure in an environment containing high levels of organic dust and during soluble LPS nebulisation. These included the following: (a) the presence of other potentially pro-inflammatory or allergenic agents present in stable dust, such as moulds and glucans, which may potentiate the response to endotoxin (Fogelmark *et al.,* 1994; Hunt *et al.,* 1994); (b) the probable underestimation of the biologically active endotoxin content of stable dust using the *Limulus* amoebocyte lysate assay (Rylander *et al.,* 1989); (c) the different duration of the two challenges, and (d) the differences between the two challenge systems with respect to the efficacy of delivery and deposition of aerosol and the mechanisms and rate of particle clearance.

In addition, the finding that inhaled soluble LPS did induce some of the features of heaves, and that heaves susceptible horses responded to a lower concentration of LPS than controls, indicates that further investigation of the role of endotoxin in this disease is warranted. Numerous studies in human occupational environments containing high concentrations of organic dust have established that the incidence of respiratory disease is correlated to the level of endotoxin exposure (Sigsgaard *et al.,* 1992; Teeuw *et al.,* 1994; Milton *et al.,* 1995; Preller *et al.,* 1995a; Schwartz *et al.,* 1995a; Schwartz *et al.,* 1995b; Reynolds *et al.,* 1996; Keman *et al.,* 1998; Donham *et al.,* 2000) often more so than to the level of dust exposure (Rylander and Bergstrom,

1993; Zejda *et al.,* 1994). In addition, several studies have shown that the clinical severity of asthma in house dust mite-sensitive humans was correlated with endotoxin exposure, yet poorly correlated with exposure to house dust mite allergens (Michel *et al.,* 1991; Michel *et al.,* 1996; Rizzo *et al.,* 1997).

To overcome the problems associated with a comparison of the endotoxin exposure during an acute challenge (soluble LPS inhalation) with a more chronic challenge (5h hay/straw challenge), comparisons were made between 2 different hay/straw challenges of equal duration. Heaves susceptible horses received two separate 5h hay/straw challenges, which differed only with respect to the source of hay used for feeding. Both hay sources were dusty, however only one had visual evidence of mould contamination. The systemic and pulmonary responses of heaves horses to both challenges were measured and compared. These responses were than related to the airborne dust, endotoxin and β-D-glucan (an indicator of airborne fungal content) exposure to establish whether the severity of disease in heaves-susceptible horses was related to specific dust components.

3.3 Materials and methods

3.3.1 Subjects

Six horses (3 geldings, 3 mares; median age 17 years, 8-28; median weight 434 kg, 323-594) with a history and clinical diagnosis of heaves were used. The disease status of all subjects was confirmed by mouldy hay/straw challenge (2.3.4.2).

3.3.2 Challenges

3.3.2.1 Challenge environments

Hay/straw challenge B (H/S A): Six heaves horses were housed for 5h in a small (3.7 x 3.7m) poorly ventilated stable with the doors and air vents closed. Horses were bedded on deep litter straw, and fed a mixture of good quality and dusty hay.

Hay/straw challenge A (H/S B): For comparison, the same 6 heaves horses were housed for 5h in an environment identical to that above, with the exception that the dusty hay used for feeding contained visible mould growth. This hay/straw challenge has been shown to induce heaves only in susceptible horses (McGorum *et al.,* 1993c), and is described previously (2.3.2.2).

3.3.2.2 Challenge protocol

All 6 horses received H/S A challenge first, followed by H/S B. Several procedures were performed to minimise potential carry-over effects of H/S A challenge on H/S B challenge. Firstly, challenges were conducted a minimum of 14 days apart. Secondly, all horses were shown to have normal BALF cytology at least 7 days prior to challenges, and normal clinical findings immediately prior to each inhalation challenge. In order to assess any carry-over effects, the 2 sets of baseline lung function, arterial blood gas and pH, and venous blood leucocyte values were compared.

3.3.3 Stable dust collection

Throughout the 5h duration of both hay/straw challenges, total and respirable dust was collected from the horse's breathing zones, as described previously (2.3.5).

3.3.4 Analysis of dust

3.3.4.1 Calculation of airborne dust concentration

The collected dust was weighed (*2.3.6.1*) and sample filters were then stored in individual sterile universal containers at -20°C, prior to endotoxin and β-D-glucan analyses.

3.3.4.2 Measurement of endotoxin in airborne dust

The endotoxin content of filters was determined using an endotoxin specific *Limulus* amoebocyte lysate assay as previously described (*2.3.6.2*).

3.3.4.3 Measurement of β-D-glucan in airborne dust

The β-D-glucan content of filters collected from the respirable dust samplers was determined using a glucan-specific *Limulus* amoebocyte lysate assay (Gluspecy, Seikagaku Co, Tokyo, Japan). Samples were prepared as described for endotoxin analysis. For analysis, all reagents, samples and standards were brought to room temperature. Samples and standards were mixed vigorously for 30s with a vortex mixer. Serial dilutions of the samples were then made in order to ensure a final sample concentration that did not exceed the β-D-glucan standard provided with the

kit. Serial dilutions (1:1, 1:2, 1:4 and 1:8) of the standard were also made to provide a standard curve. 50µl of standard, sample or β-D-glucan-free distilled water (negative control) was pipetted into a sterile 96 well microplate. 50µl of *Limulus* amoebocyte lysate substrate solution was then quickly added to each well. The microplate was then incubated at 37°C for 30min and the reaction was stopped by adding 200µl of 0.6M acetic acid to each well. The absorbance of the resulting colour reaction was read photometrically (Microplate Autoreader, Bio-Tek Instruments Inc., Winooski, VT, USA) at 405nm, and compared to a standard curve, prepared during each analysis. All samples were analysed in duplicate and the mean value calculated. Analysis was repeated if (a) the paired values differed from their mean by >20% of the mean, (b) either of the paired values exceeded the value of undiluted standard solution, or (c) either of the paired values was less than the value obtained from a 1:8 dilution of the standard solution.

The β-D-glucan concentration per m^3 of air sampled was then calculated using the following equation:

$$\text{Airborne β-D-glucan (ng/ m}^3\text{)} = \text{β-D-glucan per sample (ng)} \times \text{inverse of dilution factor} \times \frac{1000}{600}$$

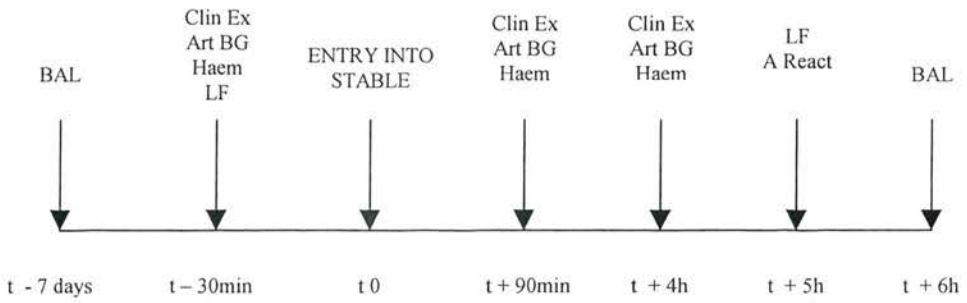
In addition, the β-D-glucan content of the dust was calculated using the following equation:

$$\text{β-D-glucan content of dust (ng/mg)} = \frac{\text{β-D-glucan per sample (ng)} \times \text{inverse of dilution factor}}{\text{dust collected (mg)}}$$

3.3.5 Monitoring the response to challenges

The response to challenges was assessed using clinical scoring, arterial blood gases and pH analyses, venous blood haematology, lung mechanics, airway reactivity and BALF cytology as previously described (2.3.7). The timing of the assessment of the response to challenges is summarised in Fig. 3.1.

Fig. 3.1: Study design.
BAL = bronchoalveolar lavage; LF = lung function evaluation; Clin Ex = clinical examination; Art BG = Arterial blood gases and pH analyses; Haem = haematological analysis; A React = airway reactivity evaluation.



3.4 Statistical analysis

Responses to the 2 hay/straw challenges and the airborne concentration of dust, endotoxin and β-D-glucan of the 2 challenges were compared by performing within-group, paired analyses. Where pre-challenge measurements were made at t-30min (arterial blood gases and pH analyses, and lung mechanics) the post-challenge values were expressed as % of baseline value, except for clinical scores where actual values were used. A Wilcoxon Rank Sum test was performed on paired sets of data; namely

horses' response data collected from identical time points, or airborne dust, endotoxin and β-D-glucan concentrations within the challenge stable.

To check for the presence of any carry-over effects of the first challenge on the second challenge, pre-challenge (baseline) measurements of arterial blood gases and pH, lung mechanics and blood leucocytes were compared using a Wilcoxon Rank Sum test. Significance was assumed if $P<0.05$.

3.5 Results

3.5.1 Dust, endotoxin and β-D-glucan exposure

There was no significant difference in airborne dust, endotoxin or β-D-glucan exposure between the 2 hay/straw challenges (Table 3.1). The respirable dust endotoxin concentration was non-significantly ($P=0.059$) higher during H/S A (Fig. 3.2), and the respirable dust β-D-glucan concentration was non-significantly ($P=0.059$) higher during H/S B (Fig 3.3).

Table 3.1: Airborne respirable and total dust, endotoxin and β-D-glucan concentrations during H/S A (n=6) and H/S B (n=6) challenges (median and range).

	H/S A	H/S B
Total dust concentration (mg/m^3)	1.2 (0.7-1.7)	1.5 (0.5-1.9)
Respirable dust concentration (mg/m^3)	0.1 (0.1-0.6)	0.2 (0.1-0.3)
Airborne endotoxin concentration (ng/m^3) [calculated from total dust fraction]	156 (36-412)	138 (87-427)
Airborne endotoxin concentration (ng/m^3) [calculated from respirable dust fraction]	6.9 (2.9-38.7)	3.6 (1.8-4.3)
Dust endotoxin concentration (ng/mg) [calculated from total dust fraction]	83 (30-145)	67 (37-150)
Dust endotoxin concentration (ng/mg) [calculated from respirable dust fraction]	32.0 (7.8-71.3)	9.0 (5.3-25.8)
Airborne β-D-glucan concentration (ng/m^3) [calculated from respirable dust fraction]	3.5 (2.3-6.2)	8.5 (3.2-16.8)
Dust β -D-glucan concentration (ng/mg) [calculated from respirable dust fraction]	24.6 (10.3-36.0)	42.6 (15.8-168.4)

Fig. 3.2: Airborne endotoxin concentration (ng/m^3) in respirable dust during hay/straw challenges H/S A (n=6) and H/S B (n=6).

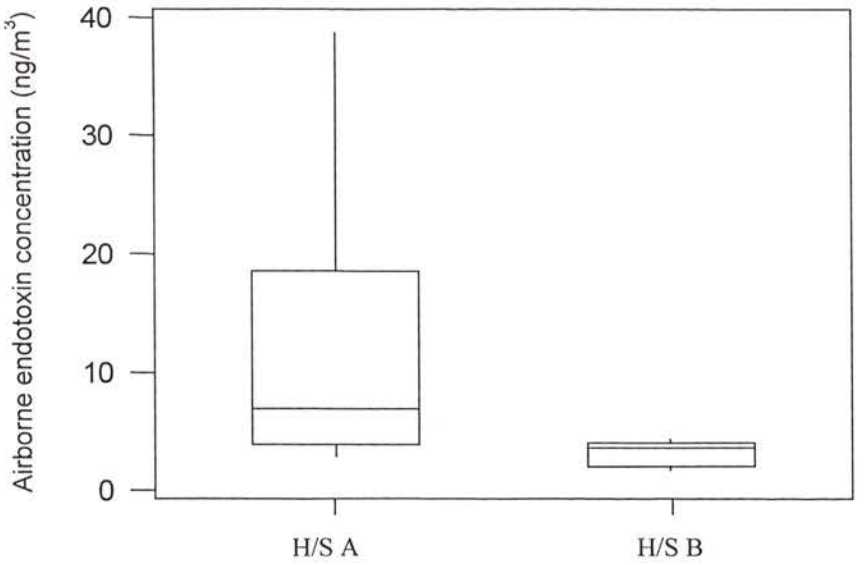
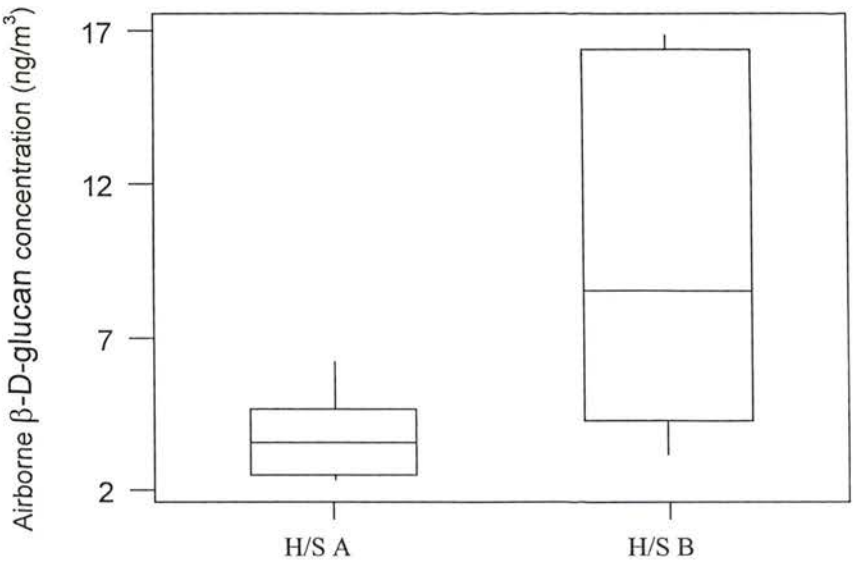


Fig. 3.3: Airborne β-D-glucan concentration (ng/m^3) in respirable dust during hay/straw challenges H/S A (n=6) and H/S B (n=6).



3.5.2 Response to challenges

3.5.2.1 Clinical examination

All horses had a clinical score of zero prior to all challenges. When compared with baseline values, no significant increase in clinical scores was detected in either group following H/S A or H/S B (Appendix 3.1).

3.5.2.2 Arterial blood gas analysis

Raw data for arterial blood gases and pH measurements are presented in Table 3.2. There was no significant difference in the baseline blood gas indices prior to each of the challenges, indicating a lack of detectable carry-over effects. H/S B induced a

significant ($P<0.05$) arterial hypoxaemia at 90 min (median decrease in PaO_2 4%, 95% CI 1-19), with a similar but non-significant ($P=0.059$) reduction at 4h post challenge.

Table 3.2: Arterial blood gases (mmHg) and pH measurements (median and range) in heaves (n=6) horses at 0, 1.5, 4 and 24h following hay/straw challenges H/S A and H/S B.

		pH	$PaCO_2$	PaO_2
H/S A	0h	7.41 (7.39-7.44)	40 (38-46)	104 (91-108)
	1.5h	7.42 (7.41-7.45)	41 (33-47)	94 (84-130)
	4h	7.42 (7.39-7.44)	41 (37-45)	94 (90-105)
	24h	7.41 (7.37-7.45)	39 (33-43)	107 (89-117)
H/S B	0h	7.39 (7.37-7.45)	42 (35-50)	103 (94-114)
	1.5h	7.42 (7.37-7.47)	43 (32-51)	94 (85-106)
	4h	7.42 (7.39-7.46)	43 (36-47)	94 (86-107)
	24h	7.39 (7.35-7.41)	40 (32-46)	98 (89-111)

3.5.2.3 Lung mechanics and airway reactivity

Raw data for lung mechanics measurements are presented in Appendix 3.2. PCCdyn values following challenge are presented in Appendix 3.3. There was no significant difference in baseline lung function measurements between the 2 challenges, indicating a lack of detectable carry-over effects. Neither of the challenges altered lung function when compared with baseline values, and the challenges did not significantly differ from one another with respect to airway reactivity.

3.5.2.4 BALF cytology

The BALF cytology data following both challenges are presented in Appendix 3.4, and summarised in Table 3.3. H/S B induced a significantly ($P<0.05$) higher BALF neutrophil count and ratio than H/S A at 6h (Table 3.3, Figs. 3.4 and 3.5). There was no difference between H/S A and H/S B with respect to numbers of other BALF cell types.

Table 3.3: Total and differential BALF cell counts ($x10^5$/ml) (median and range) in heaves (n=6) horses at 6h following hay/straw challenges H/S A and H/S B. TCC = total BALF cell count; Lymph = lymphocytes.

	TCC	Lymph	Macrophages	Neutrophils	Mast cells	Basophiloid cells	Eosinophils	Epithelial cells
H/S A	5.05 (2.10-9.80)	2.53 (0.86-4.32)	1.82 (0.78-4.65)	0.45 (0.34-0.70)	0.18 (0.08-0.38)	0.01 (0.00-0.05)	0.01 (0.00-0.01)	0.00 (0.00-0.00)
H/S B	6.40 (2.80-16.20)	1.87 (0.60-4.26)	1.73 (0.90-3.16)	1.97 (0.74-9.83)	0.10 (0.08-0.12)	0.01 (0.00-0.08)	0.00 (0.00-0.02)	0.00 (0.00-0.00)

Fig. 3.4: BALF neutrophil counts ($x10^5$/ml) in heaves (n=6) horses at 6h following hay/straw challenges H/S A and H/S B.

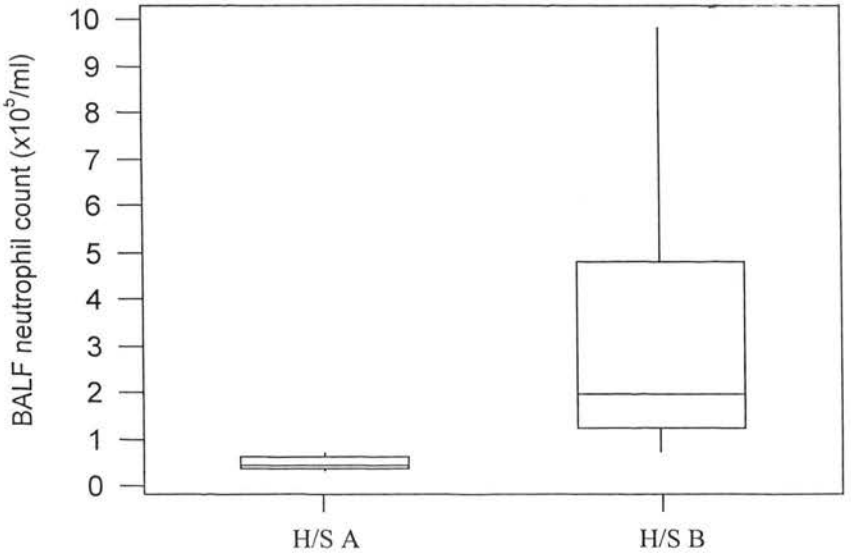
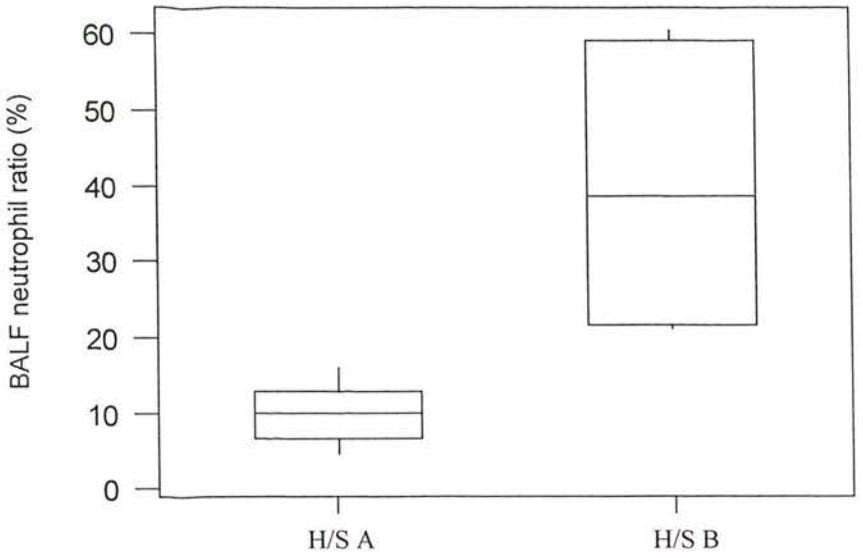


Fig. 3.5: BALF neutrophil ratio (%) in heaves (n=6) horses at 6h following hay/straw challenges H/S A and H/S B.



3.6 Discussion

The results of this study suggest that the pulmonary response of heaves-susceptible horses to housing in a dusty environment is not *solely* related to the magnitude of airborne endotoxin exposure. This is consistent with the findings of the study described in Chapter 2, whereby the pulmonary functional and inflammatory responses of heaves horses to high concentrations of inhaled LPS was not significantly different from that of controls. Despite the pulmonary neutrophilic inflammation being significantly greater following H/S B challenge, there was no significant difference between the 2 challenge systems with respect to airborne concentration of total or respirable dust, or the endotoxin and β-D-glucan concentration of these 2 dust fractions within the horses breathing zone. In fact, the

H/S A challenge resulted in a non-significantly greater endotoxin exposure as calculated from the respirable dust fraction, yet induced only minor pulmonary neutrophilic inflammation and no alteration in lung mechanics or arterial blood gas tension compared with H/S B challenge.

In addition, H/S B resulted in a non-significantly greater exposure to the mould cell wall component (1-3)-β-D-glucan as calculated from the respirable dust fraction. As airborne (1-3)-β-D-glucan has been shown to reflect mould exposure (Douwes *et al.,* 1998; Rylander *et al.,* 1998; Chew *et al.,* 1999; Dillon *et al.,* 1999; Mandryk *et al.,* 2000; Wouters *et al.,* 2000), this finding supports previous studies that have identified an association between mould exposure and clinical signs in heaves-susceptible horses (McPherson and Thomson, 1983; Thomson and McPherson, 1984; Derksen *et al.,* 1988; McGorum *et al.,* 1993c; Robinson *et al.,* 1996). Furthermore, as several human studies have identified an association between respiratory symptoms or airway responsiveness and (1-3)-β-D-glucan exposure (Rylander, 1997a and c; Rylander, 1998; Thorn *et al.,* 1998; Thorn and Rylander, 1998a; Rylander *et al.,* 1999), it is possible that as well as reflecting the degree of mould exposure, (1-3)-β-D-glucan inhalation may in itself result in respiratory inflammation and dysfunction. The pro-inflammatory effects of (1-3)-β-D-glucan have been demonstrated both *in vivo*, by means of inhalation studies in humans and laboratory animals (Fogelmark *et al.,* 1992; Rylander and Fogelmark, 1994; Fogelmark *et al.,* 1997; Schuyler *et al.,* 1998; Beijer *et al.,* 1999; Sigsgaard *et al.,* 2000; Fogelmark *et al.,* 2001), and *in vitro* following challenge of lung derived macrophages (Milanowski *et al.,* 1995b; Ljungman *et al.,* 1998).

The dust concentrations measured during both challenges were similar yet lower than previously reported levels in which personal samplers were used to collect from the breathing zone of horses housed in a confined hay and straw environment (Bartz and Hartung, 1993; Woods *et al.,* 1993; McGorum *et al.,* 1998). The greater airborne dust concentration in the study by McGorum et al (1998) is somewhat surprising considering the better ventilation provided in that study in which the top door of the stable remained open during the sampling period.

Despite this finding, the endotoxin concentrations were greater in the current study compared with those reported by McGorum *et al.* (1998) in which the dust collection procedure was identical. This likely reflects the higher endotoxin content of the dust in the current study, a finding that is supported by other studies in which a poor correlation between dust levels and endotoxin levels (Kullman *et al.,* 1998; Douwes *et al.,* 2000a) has been reported. It is possible that the actual endotoxin content of the dust was greater than that measured using the *Limulus* amoebocyte lysate assay due to its reported underestimation of particulate endotoxin which would have been pelletted by centrifugation prior to analysis (Rylander *et al.,* 1989). However this underestimation would have applied to both H/S A and H/S B challenges to a similar degree, considering the similar respirable dust levels measured in both systems.

Numerous human studies have identified an association between pulmonary function, inflammation or symptoms and endotoxin exposure in workers exposed to organic dusts (Sigsgaard *et al.,* 1992; Rylander and Bergstrom, 1993; Teeuw *et al.,* 1994;

Milton *et al.,* 1995; Preller *et al.,* 1995a; Schwartz *et al.,* 1995a; Schwartz *et al.,* 1995b; Vogelzang *et al.,* 1998). In many cases either a poor association, or no association at all, has been identified between respiratory symptoms and dust exposure (Rylander and Bergstrom, 1993; Jorna *et al.,* 1994; Smid *et al.,* 1994; Zejda *et al.,* 1994; Wang *et al.,* 1996a). However other studies have identified an association between pulmonary function and dust levels (Christiani *et al.,* 1999), and no correlation between pulmonary function and endotoxin exposure (Allermann and Poulsen, 2000). The reason for the disagreement between some of these studies is unclear, however it may reflect the complex nature of organic dusts (Kullman *et al.,* 1998), many components of which have been implicated in a range of respiratory diseases (Lacey, 1993).

It has already been established that endotoxin inhalation in the horse results in pulmonary inflammation and dysfunction (Chapter 2), however the level of soluble LPS exposure required to induce these effects greatly exceeds the degree of endotoxin exposure encountered in both H/S A and H/S B challenges. Although there are limitations in making such direct comparisons, the lack of association between endotoxin exposure and pulmonary inflammation in the current study may simply reflect the fact that the level of endotoxin exposure was below the response threshold for the horse. However in both challenges the airborne endotoxin concentration greatly exceeded the recommended safety levels for human workers (Rylander, 1997b).

Although the greater severity of pulmonary inflammation detected following H/S B challenge appeared to relate to the higher level of mould exposure, it may also have been partly related to endotoxin exposure. It is possible that exposure to moulds in H/S B challenge resulted in a greater inflammatory response to co-inhaled endotoxin. Co-exposure to endotoxin and either purified (1-3)-β-D-glucan or allergen has been reported to induce an exaggerated inflammatory pulmonary response (Fogelmark *et al.,* 1994; Wan *et al.,* 2000), and it is likely that a complex interaction exists between the microbial components of organic dusts with respect to their combined pro-inflammatory properties (Fogelmark *et al.,* 2001). In order to investigate this possibility further, a third challenge system would have been required in which horses were exposed to airborne mould in the absence of endotoxin. However as endotoxins are prevalent in many forms of organic dust (Jacobs, 1997a), such a dry dust challenge system would be difficult to create. As inhalation models can be used to determine which components of a mixture are the most important for inducing the observed adverse outcome (Thorne, 2000), further studies require an inhalation challenge system which is representative of some or all of the components of hay dust and which permits the selective manipulation of those components. This would further the understanding of the relative contribution of each allergenic or pro-inflammatory agents present in hay dust to the overall pulmonary inflammatory and functional response.

In summary, this chapter support the findings of Chapter 2, namely that inhaled endotoxin does not appear to be solely responsible for the pulmonary inflammatory and functional response of heaves-susceptible horses to organic dust exposure.

However, it remains possible that inhaled endotoxin contributes to this overall pulmonary response when inhaled in concert with other organic dust components such as mould spores.

CHAPTER 4: DOSE-RESPONSE RELATIONSHIP TO INHALED SOLUBLE *ASPERGILLUS FUMIGATUS* EXTRACT IN ASYMPTOMATIC HEAVES HORSES

4.1 Summary

Previous studies showed a shortfall in response of heaves horses to inhaled mould extract compared with natural disease (McGorum *et al.,* 1993c). To investigate whether insufficient dose delivery was responsible for this shortfall, the response of 6 heaves horses to inhalation of saline (placebo), and 3 doses of soluble *Aspergillus fumigatus* extract was assessed. Inhalation challenge with 0.5, 1.6 and 5mg *A. fumigatus* extract significantly increased BALF neutrophil ratios compared with saline. Only 1.6 and 5mg *A. fumigatus* extract inhalation caused significant lung dysfunction compared with saline. There was no significant difference in the pulmonary inflammatory or functional response to 1.6 and 5mg extract inhalation. A good agreement was found between the response to these 2 doses with respect to airway neutrophil numbers and lung function, indicating that a plateau was attained for both measured responses. As the magnitude of the response was less than that of natural disease, this study therefore supports a role for other inhalants, in addition to the soluble components of *A. fumigatus,* in the aetiopathogenesis of heaves.

4.2 Introduction

The results presented in both Chapters 2 and 3 indicate that inhaled endotoxin is not the sole inhalant responsible for the response of heaves susceptible horses during

dusty hay/straw exposure. Indeed, the results of the challenge experiments presented in Chapter 3 are more supportive of a role for inhaled moulds in disease aetiopathogenesis. However considering the myriad of inhalants to which horses are exposed when housed in dusty environments, it is possible that endotoxin plays a contributing role when co-presented to the lung along with other inhalants, such as moulds.

Mould extract inhalation challenges have been used previously in the investigation of heaves (Derksen and Robinson, 1981; Derksen *et al.,* 1988; McGorum *et al.,* 1993c). Although such studies have implicated a hypersensitivity to inhaled moulds in disease pathogenesis (McPherson *et al.,* 1979; Derksen *et al.,* 1988; McGorum *et al.,* 1993c), little attention has been given to the additional role of other inhaled components. Although experimental mould extract (*Aspergillus fumigatus, Faenia rectivirgula*) inhalation results in pulmonary neutrophilic inflammation and dysfunction, consistent with the natural disease, the magnitude in response is less than that observed following dusty hay/straw exposure (McGorum *et al.,* 1993c). While one suggested reason for this shortfall in response is that there was insufficient dose delivery during the experimental inhalation challenges, no dose-response inhalation challenges have been reported. To establish whether insufficient dose delivery is responsible for this response shortfall following mould extract inhalation, a series of dose-response inhalation experiments was conducted in 6 heaves horses, using a soluble *A. fumigatus* extract.

4.3 Materials and methods

4.3.1 Subjects

6 horses (3 geldings, 3 mares; age 17 years, 8-28; weight 434 kg, 323-594) with a history and clinical diagnosis of heaves were used as previously described (3.3.1). The disease status of all subjects was confirmed by mouldy hay/straw challenge as previously described (2.3.4.2). All horses were kept in a low dust environment (2.3.2.1) throughout the duration of the study.

4.3.2 *Aspergillus fumigatus* extract

Soluble *Aspergillus fumigatus* extract (AFE) prepared from both the cellular (somatic) and extracellular (culture filtrate) components of *A. fumigatus* culture was kindly donated by Dr. John Edwards, MRC Immunology Lab., Sully Hospital, Penarth, Wales. Doses of 0.5mg, 1.6mg and 5mg AFE were used in the inhalation challenges, being prepared from a stock solution of 10mg/ml and diluted in physiologic saline immediately prior to use. A constant 1ml volume of challenge solution was delivered to the facemask for all challenges.

4.3.3 Inhalation challenges

4.3.3.1 Inhalation challenge protocol

To facilitate subject compliance, horses were sedated with intravenous 20μg/kg romifidine and 10μg/kg butorphanol immediately prior to each inhalation challenge. The aerosol was generated and delivered as previously described (2.3.4.1). The order

of challenges in all horses was constant i.e. 5mg followed by 0.5mg, followed by 1.6mg. To minimise potential carry-over effects of a preceding challenge on subsequent challenges, inhalation challenges were conducted a minimum of 14 days apart and all horses were shown to have normal clinical findings immediately prior to each inhalation challenge. In order to assess any carry-over effects, all baseline lung function and arterial blood gases and pH values were compared statistically.

4.3.3.2 Positive (hay/straw exposure) and negative (saline) control challenges

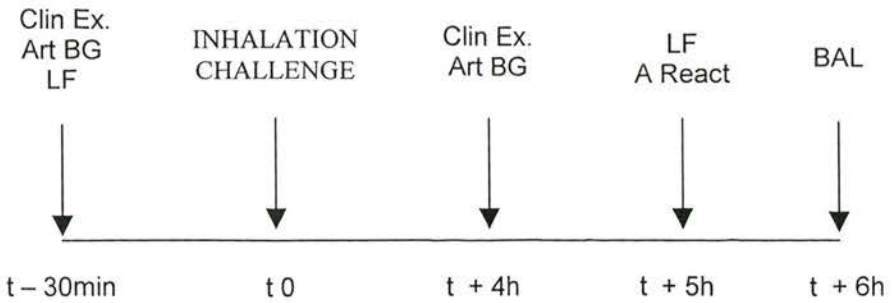
To compare the AFE responses with those of placebo and a conventional hay/straw challenge, comparisons were made with saline inhalation and a 5h-duration conventional hay/straw challenge in the same horses, as previously described (2.3.4.2).

4.3.3 Monitoring the response to challenges

The method and timing of assessment of response to each challenge is summarised in Fig. 4.1. Responses to the AFE, hay/straw and saline challenges (Chapter 2) were assessed using clinical scoring, lung mechanics, airway reactivity, blood gases and pH analyses and BALF cytology, as previously described (2.3.7).

Fig. 4.1: Study design.

BAL = bronchoalveolar lavage; LF = lung function evaluation; Clin Ex = clinical examination; Art BG = Arterial blood gases and pH analysis; A React = airway reactivity evaluation.



4.4 Statistical analysis

As the data were either not normally distributed and/or the groups of compared data did not have equal variance, non-parametric tests were used. The effects of each challenge were determined by performing within-group analyses.

To check for the presence of any carry-over effects of one challenge on a subsequent challenge, pre-challenge (baseline) measurements of arterial blood gases and pH and lung mechanics were compared using a Friedman test, and when significant, a Wilcoxon Rank Sum test was performed on paired data.

To check for any effects of challenge, where pre-challenge measurements were made at t-30min (arterial blood gas analyses and lung mechanics), the post-challenge values were expressed as % of baseline value, except for clinical scores where actual values were used. As saline was the vehicle for AFE delivery, the effect of AFE challenge was assessed by pairing and subtracting post-AFE (% of baseline value) and post-saline (% of baseline value) data. Where no pre-challenge data was collected (BALF cytology), comparisons were made with saline (placebo) challenge data at an equivalent time point. A Friedman test was performed on sets of paired data to reduce the risk of type 1 errors, and when significant, a Wilcoxon Rank Sum test was performed on paired data.

Significance was assumed if $P<0.05$. Following correction for any effect of saline inhalation, changes from baseline are expressed as increase/decrease or change in median values (% baseline following AFE challenge minus % baseline following

saline), with 95% confidence interval for the difference in median, calculated for non-parametric data (Campbell and Gardner 1994). Results in tables are expressed as median and range.

To assess the level of agreement in neutrophil response to 1.6 and 5mg AFE challenges, the differences in paired values were plotted against their mean (Bland and Altman 1986), for BALF neutrophil counts. Good agreement was assumed if the calculated differences in paired values fell within 2 standard deviations of the mean of the differences (British Standards Institution, 1979).

4.5 Results

4.5.1 Dose-response to AFE inhalation challenge.

4.5.1.1 Clinical examination

All horses had a clinical score of zero prior to all challenges. When compared with baseline values, no significant increase in clinical scores was detected at 4h following any of the AFE challenges (Appendix 4.1).

4.5.1.2 Arterial blood gases and pH analyses

Raw data for arterial blood gases and pH measurements are presented in Appendix 4.2. There was no significant difference in the baseline blood gas or pH indices prior to any of the inhalation challenges, indicating a lack of detectable carry-over effects.

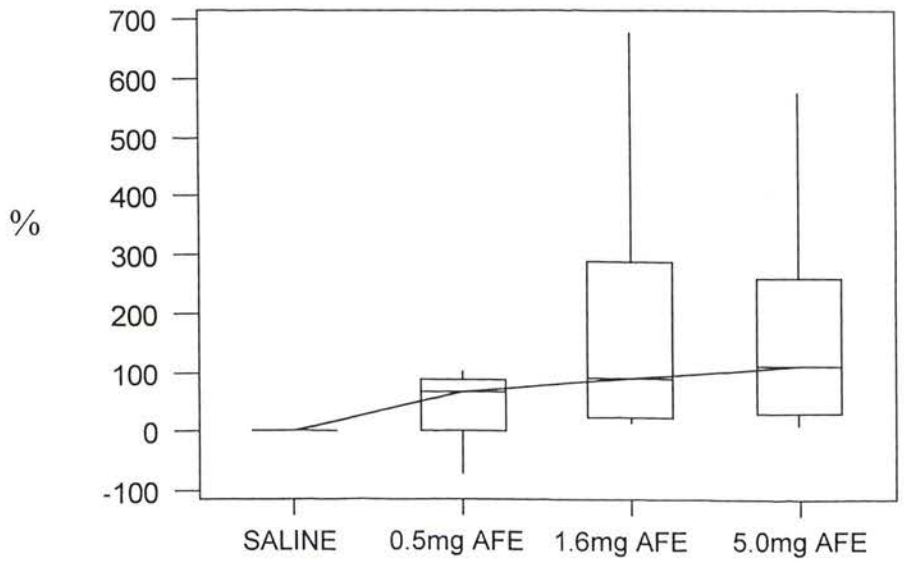
When compared with baseline values, no significant change in blood gas or pH values was detected at 4h following any of the AFE challenges.

4.5.1.3 Lung mechanics and airway reactivity

Raw data for lung mechanics measurements are presented in Appendix 4.3. With the exception of respiratory rate and inspiratory resistive work of breathing (Wb_{Ires}), there was no significant difference in the baseline lung mechanics measurements prior to each of the challenges, indicating a lack of detectable carry-over effects. The percentage change in lung mechanics measurements from baseline is presented in Table 4.1. PCCdyn values following challenge are presented in Appendix 4.4.

Following correction for any effects of saline inhalation, a dose dependant alteration in lung mechanics was detected following AFE challenge (Fig. 4.2). Both 1.6mg and 5mg, but not 0.5mg, AFE inhalation resulted in a significant ($P<0.05$) increase in $R_{LE25\%}$ (1.6mg - increase in median 94%, 95% CI 14-678; 5mg - increase in median 114%, 95% CI 12-578) at 5h.

Fig. 4.2: Percent (%) of baseline $RL_{E25\%}$ in heaves horses (n=6) at 5h following inhalation challenge with saline, 0.5, 1.6 and 5mg AFE minus percent of baseline $RL_{E75\%}$, at 5h following inhalation challenge with saline.



There was no significant difference between the 1.6mg and the 5mg FE challenges with respect to $R_{LE25\%}$. In addition, as all of the 6 calculated differences in $R_{LE25\%}$ values for the 1.6 and 5mg AFE challenges fell within 2 standard deviations of the mean of the differences and the mean of the differences approximated zero (Fig. 4.3), agreement was considered good (Bland and Altman 1986). Both of these findings suggested that there was a plateau in the lung function response following 1.6 and 5mg AFE inhalation. None of the AFE doses induced a significant alteration in airway reactivity at 5h.

Fig. 4.3: Difference plotted against mean of percent (%) of baseline $RL_{E25\%}$ in heaves horses (n=6) at 5h following inhalation challenge with 1.6 and 5mg AFE minus percent of baseline $RL_{E75\%}$, at 5h following inhalation challenge with saline.
Solid line = mean of the differences; dotted line = mean of the differences ± 2 standard deviations of the differences.

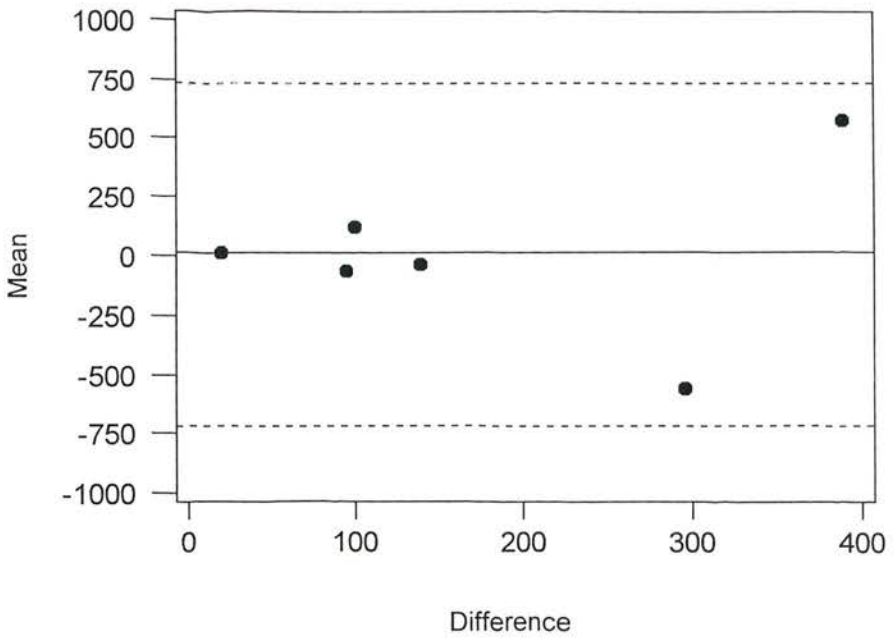


Table 4.1 (a and b): Percent (%) of baseline lung mechanics measurements (median and range) in heaves (n=6) horses at 5h following inhalation challenge with saline, 0.5, 1.6 and 5.0mg AFE. For statistical analyses, see 4.4.

(a)

	Cdyn	dPpl	RL_{iso}	RR	VT	Wb	$RL_{E25\%}$	$RL_{E50\%}$	$RL_{E75\%}$	$RL_{I25\%}$	$RL_{I50\%}$	$RL_{I75\%}$
SALINE	96.85 (75.80-104.88)	108.13 (59.98-188.49)	88.70 (76.88-197.35)	93.02 (62.35-110.58)	111.33 (67.19-165.78)	95.82 (28.95-146.50)	62.14 (35.80-172.00)	64.98 (0.00-125.25)	70.33 (50.66-114.96)	124.76 (76.97-438.89)	101.25 (84.87-317.07)	89.38 (70.54-313.75)
0.5mg AFE	110.98 (75.83-193.72)	96.93 (70.84-137.92)	111.80 (38.34-128.83)	102.64 (62.40-166.42)	87.53 (72.48-135.72)	93.05 (77.99-239.47)	137.35 (87.14-152.38)	125.94 (67.96-274.51)	97.40 (61.33-198.55)	119.64 (88.10-655.28)	106.67 (66.67-596.90)	121.26 (102.17-751.26)
1.6mg AFE	95.21 (57.26-137.87)	109.10 (72.17-136.94)	152.87 (66.29-209.89)	86.55 (59.40-152.46)	100.34 (74.97-104.77)	104.72 (62.95-134.65)	170.39 (114.29-720.00)	170.81 (70.00-401.72)	147.31 (93.00-238.39)	116.66 (96.49-170.11)	120.41 (68.65-182.05)	133.28 (78.53-203.66)
5.0mg AFE	100.30 (66.10-121.31)	121.35 (84.31-171.56)	121.98 (90.22-192.96)	84.43 (67.00-97.37)	111.97 (105.55-139.37)	124.60 (85.96-192.97)	161.60 (109.30-750.00)	122.69 (0.00-262.79)	116.58 (90.41-279.63)	102.81 (80.84-165.52)	110.00 (82.04-142.82)	108.73 (80.15-177.14)

(b)

	T_E	T_I	$T_I:T_E$	V'_E	V'_{Emax}	V'_{Imax}	Wb_{el}	Wb_{res}	Wb_{Eres}	Wb_{Ires}	Wb_{Itot}
SALINE	101.35 (83.06-219.93)	110.18 (86.29-150.99)	102.19 (58.48-110.54)	104.88 (43.76-117.87)	115.84 (43.28-137.88)	102.03 (56.11-140.35)	127.55 (48.26-231.46)	92.15 (48.17-277.79)	76.47 (41.95-223.61)	108.27 (58.49-298.91)	117.22 (51.88-259.08)
0.5mg AFE	91.94 (57.49-153.43)	96.05 (61.96-129.16)	100.62 (79.65-147.55)	95.12 (45.74-140.09)	97.00 (71.98-138.63)	104.17 (90.82-127.24)	75.83 (35.17-223.30)	100.18 (70.27-159.68)	109.35 (13.94-167.23)	109.34 (68.56-164.81)	87.28 (58.93-197.79)
1.6mg AFE	112.64 (57.16-126.97)	113.30 (92.75-128.97)	101.30 (84.66-171.74)	84.86 (43.97-151.48)	81.10 (75.73-112.24)	87.89 (76.28-117.08)	121.65 (67.46-149.23)	116.46 (67.27-156.83)	101.23 (32.63-177.76)	119.01 (65.55-152.66)	123.13 (66.59-149.65)
5.0mg AFE	116.32 (95.69-156.50)	120.27 (100.79-130.14)	95.44 (73.27-124.52)	102.37 (70.10-112.78)	104.00 (77.37-131.04)	105.12 (87.99-112.75)	135.07 (108.78-181.06)	132.33 (113.55-277.72)	153.20 (93.47-312.24)	126.69 (106.56-253.77)	128.25 (114.34-215.27)

4.5.1.4 BALF cytology

BALF neutrophil counts and ratios following saline and AFE challenges are presented in Appendix 4.5, and summarised in Table 4.2.

Table 4.2: BALF neutrophil counts (x10^5/ml) and ratios (%) (median and range) in heaves (n=6) horses at 6h following inhalation challenge with saline, 0.5, 1.6 and 5.0mg AFE.

	BALF neutrophil count (x10^5/ml)	BALF neutrophil ratio (%)
SALINE	0.07 (0.03-0.20)	2.2 (0.6-4.5)
0.5mg AFE	0.18 (0.07-0.48)	6.8 (2.1-16.2)
1.6mg AFE	0.94 (0.68-2.10)	26.7 (11.3-53.9)
5.0mg AFE	1.08 (0.36-1.83)	24.5 (7.9-44.5)

When compared with saline inhalation, both 1.6mg and 5mg AFE inhalation challenges resulted in a significant ($P<0.05$) increase in BALF neutrophil count (1.6mg - increase in median 0.84 x 10^5/ml, 95% CI 0.65-2.02: 5.0mg - increase in median 0.95 x 10^5/ml, 95% CI 0.31-1.76) and neutrophil ratio (Table 4.2; Figs 4.4 and 4.5). Although inhalation of 0.5mg AFE did not significantly increase the BALF neutrophil count, it did induce a slight, yet significant ($P<0.05$) increase in BALF neutrophil ratio (Table 4.2, Fig. 4.5). When compared with 0.5mg AFE inhalation, both 1.6mg and 5mg AFE inhalation resulted in a significantly ($P<0.05$) greater BALF neutrophil count (1.6mg - difference in median 0.78 x 10^5/ml, 95% CI 0.37-1.89: 5.0mg - difference in median 0.85 x 10^5/ml, 95% CI 0.29-1.35) and neutrophil ratio. A 5h-hay/straw challenge (2.5.2.6) induced a significantly ($P<0.05$) greater BALF neutrophil count and ratio than 0.5mg AFE, and a greater BALF neutrophil

count and ratio than 1.6mg and 5mg which approached significance (P=0.059) (Figs. 4.4 and 4.5).

Fig. 4.4: BALF neutrophil counts ($x10^5$/ml) in heaves (n=6) horses at 6h following inhalation challenge with saline, 0.5, 1.6 and 5.0mg AFE and mouldy hay/straw challenge (H/S).

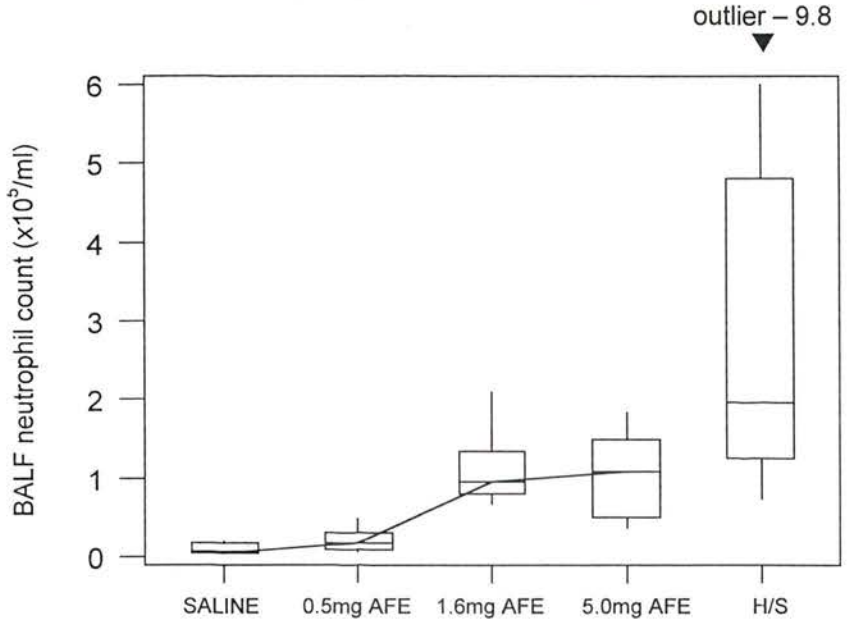
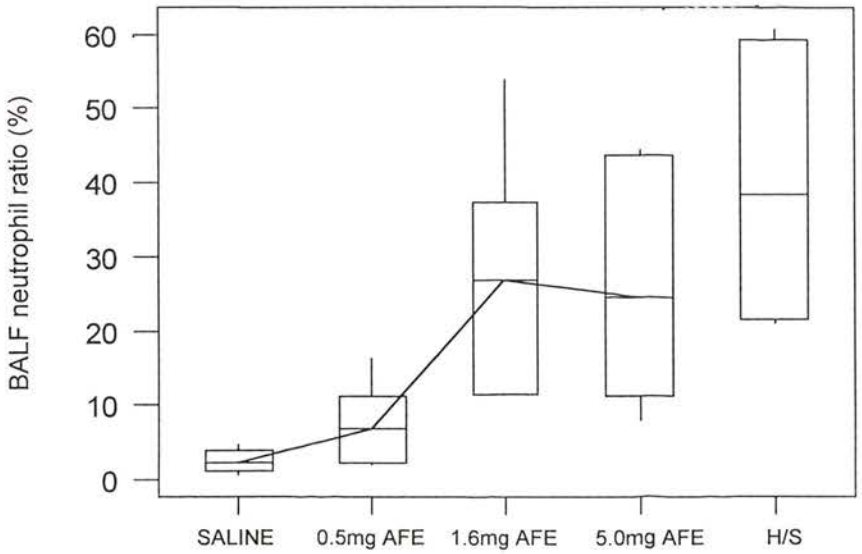
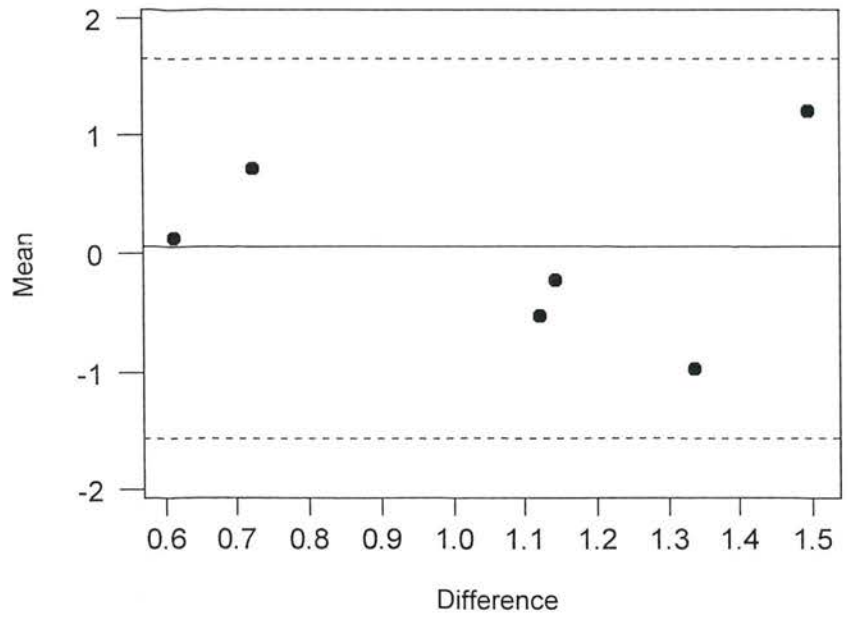


Fig. 4.5: BALF neutrophil ratio (%) in heaves (n=6) horses at 6h following inhalation challenge with saline, 0.5, 1.6 and 5.0mg AFE and mouldy hay/straw challenge (H/S).



There was no significant difference in the neutrophil count or ratio between the 1.6mg and 5mg AFE challenges. In addition, as all of the 6 calculated differences in total BALF neutrophil number values for the 1.6 and 5mg AFE challenges fell within 2 standard deviations of the mean of the differences and the mean of the differences approximated zero, agreement was considered good (Bland and Altman 1986) (Fig. 4.6). Both of these findings were indicative of a plateau in the neutrophilic response at higher doses of AFE.

Fig. 4.6: Difference between BALF neutrophil counts ($x10^5$/ml) plotted against the mean of the BALF neutrophil counts ($x10^5$/ml) in heaves (n=6) horses at 6h following inhalation challenge with 1.6 and 5.0mg AFE.
Solid line = mean of the differences; dotted line = mean of the differences ± 2 standard deviations of the differences.



Due to the influence of a BALF neutrophilia on the ratio of other BALF cell types, the latter cells were considered only as absolute numbers. The absolute number of other BALF cell types 6h following challenge is summarised in Table 4.3.

Table 4.3: Total (TCC) and differential BALF cell counts (x10^5/ml) (median and range) in heaves (n=6) horses at 6h following inhalation challenge with saline, 0.5, 1.6 and 5.0mg AFE.

	TCC	Lymphocytes	Macrophages	Mast cells	Basophiloid cells	Eosinophils
SALINE	4.50 (3.20-5.60)	2.22 (1.54-3.33)	1.77 (1.24-2.96)	0.14 (0.09-0.19)	0.01 (0.01-0.11)	0.00 (0.00-0.01)
0.5mg AFE	3.45 (1.50-5.10)	1.38 (0.36-2.01)	1.72 (0.85-2.56)	0.04 (0.03-0.11)	0.00 (0.00-0.02)	0.00 (0.00-0.02)
1.6mg AFE	3.85 (2.70-9.40)	1.21 (0.56-6.04)	1.57 (0.72-2.78)	0.05 (0.04-0.14)	0.00 (0.00-0.02)	0.00 (0.00-0.01)
5.0mg AFE	4.45 (2.00-6.60)	1.86 (0.74-2.38)	1.62 (0.33-2.69)	0.05 (0.02-0.14)	0.00 (0.00-0.01)	0.01 (0.00-0.02)

When compared with saline inhalation, both 1.6mg and 5mg AFE inhalation resulted in a significant ($P<0.05$) reduction in absolute BALF basophiloid numbers (1.6mg - decrease in median 0.02, 95% CI 0.00-0.11; 5mg – decrease in median 0.02, 95% CI 0.01-0.11) at 6h (Fig. 4.7). In addition, inhalation of 1.6mg AFE resulted in a significant ($P<0.05$) reduction in BALF mast cell count (reduction in median 0.08 x 10^5/ml, 95% CI 0.04-0.12) (Fig. 4.8). A similar reduction albeit non-significant ($P=0.059$) occurred following 0.5mg (reduction in median 0.09 x 10^5/ml, 90% CI 0.04-0.14) and 5mg (reduction in median 0.09 x 10^5/ml, 90% CI 0.03-0.13) AFE inhalation (Fig 4.8). Interestingly, a significant ($P<0.05$) increase in BALF eosinophils was noted following 5mg AFE inhalation (increase in median 0.08 x 10^4/ml, 95% CI 0.01-0.15) (Fig. 4.9).

Fig. 4.7: BALF basophiloid cell counts ($x10^5$/ml) in heaves (n=6) horses at 6h following inhalation challenge with saline, 0.5, 1.6 and 5.0mg AFE.

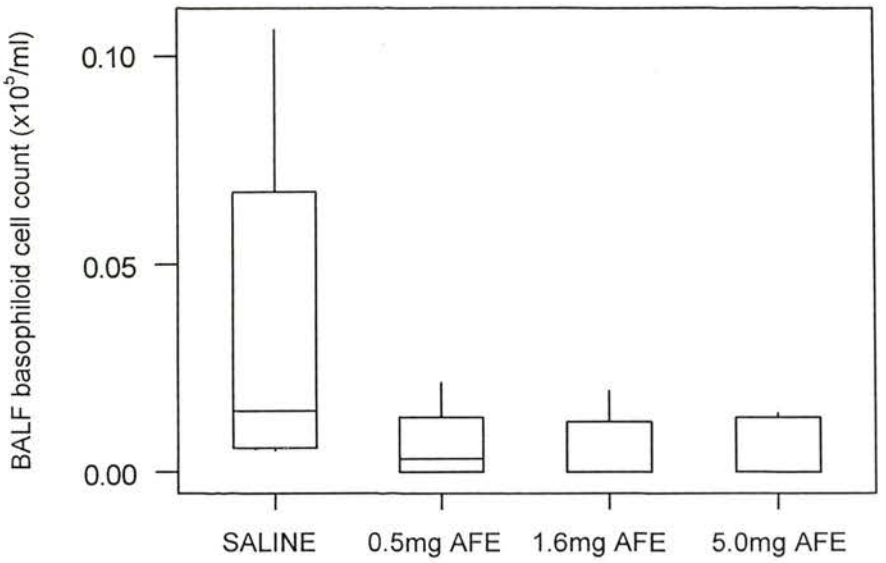


Fig. 4.8: BALF mast cell counts ($x10^5$/ml) in heaves (n=6) horses at 6h following inhalation challenge with saline, 0.5, 1.6 and 5.0mg AFE.

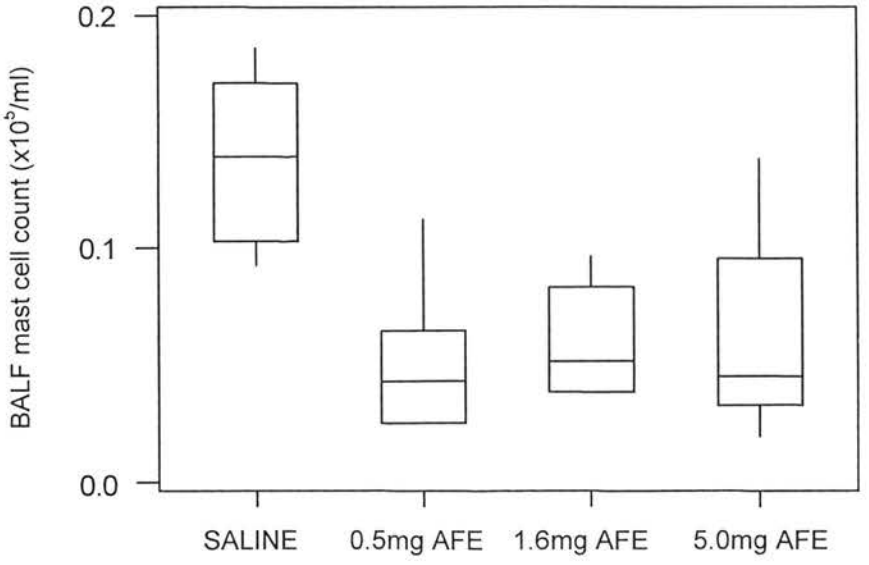
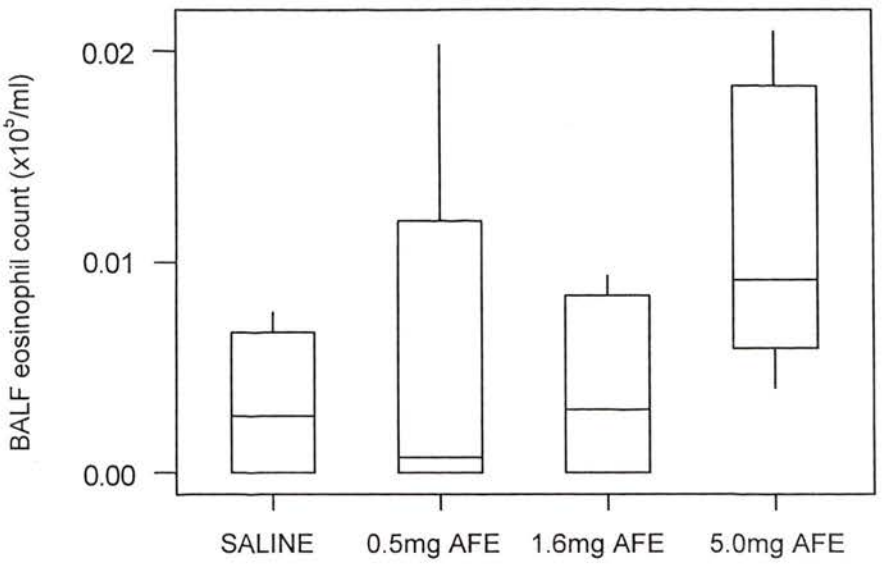


Fig. 4.9: BALF eosinophil counts ($x10^5$/ml) in heaves (n=6) horses at 6h following inhalation challenge with saline, 0.5, 1.6 and 5.0mg AFE.



4.6 Discussion

This study reports the results of a series of dose-response inhalation experiments in heaves-susceptible horses using a soluble aqueous *Aspergillus fumigatus* extract (AFE). In agreement with other studies, AFE inhalation resulted in both pulmonary inflammation and dysfunction (McPherson *et al.*, 1979; McGorum *et al.*, 1993c). However a plateau was demonstrated in both of these measured indices with the 2 higher AFE doses, as indicated by the agreement between the responses to the 1.6 and 5mg doses. This plateau in response could have been further supported if higher challenge doses than used in the study also failed to result in an increasing response, however this work was not possible due to unavailability of sufficient quantity of the

same batch of AFE. It is however likely that the observed plateau was a real phenomenon, considering that it was reached at an exposure (1.6mg AFE) less than 3.15-fold higher than the response threshold for neutrophilic inflammation (i.e. >0.5mg AFE) and was maintained following a further 3.15-fold increase in the AFE exposure.

Although randomisation of the challenge doses would have been more appropriate, it is unlikely that a carry over effect of the 5mg AFE challenge resulted in an exaggerated response to the 1.6mg AFE challenge. Firstly all horses received a 0.5mg AFE challenge following the 5mg AFE challenge, yet only a minor response to this low exposure was detected. Secondly, a lack of carry-over effects was supported by the failure to detect any significant differences between the baseline data for both lung function and arterial blood gas and pH measurements prior to all challenges.

Although the reason for the plateau in the neutrophilic and lung function response is unclear, a similar plateau in skin reactivity has been reported in children with allergic eczema following atopy patch testing with house dust mite and grass pollen allergens (Darsow *et al.,* 1999). This phenomenon has also been demonstrated in a guinea pig model of dust mite antigen-induced asthma whereby the degree of airway eosinophilia induced following inhalation of a low dose of crude dust mite extract did not increase with increasing doses of inhaled extract (Hsiue *et al.,* 1997). As previous studies have proposed the involvement of a type I hypersensitivity response in the pathogenesis of heaves (McPherson *et al.,* 1979; Derksen *et al.,* 1988; McGorum *et al.,* 1993b), with a suggested role for pulmonary mast cells (McGorum *et al.,* 1993b),

it is feasible that the observed plateau in response may reflect a state of "allergen saturation". Under such circumstances the maximal capacity for allergen-specific IgE-mediated mast cell degranulation may have been reached following challenge with the middle dose of AFE. It is possible to attribute the plateau in both the pulmonary neutrophilic and functional response to such a phenomenon (Cairns and Walls, 1996).

It has been suggested that a reduction in BALF mast cells and basophiloid cells may reflect mast cell/basophiloid cell degranulation, with subsequent failure to identify the degranulated cells on BALF cytospin preparations stained using Leishmans' stain. Therefore the hypothesis of "allergen saturation" may be supported by the significant reduction in BALF basophiloid cells following 1.6mg and 5mg AFE challenges and the reduction in BALF mast cell numbers following all 3 challenges, although this only approached significance following 0.5mg and 5mg AFE challenge. However, the data presented in Figs. 4.7 and 4.8 suggest that the magnitude of the reduction in the mast cell and basophiloid numbers did not appear to increase with increasing doses of AFE.

Also of interest was the increase in BALF eosinophil numbers detected following the 5mg AFE challenge. While this was statistically significant, it was only a small increase, which probably has little biological significance. However, despite the eosinophil being classically associated with allergic responses in the lung (Coyle *et al.,* 1996; Ohkawara *et al.,* 1997), following all AFE challenges, the neutrophil was the most abundant polymorphonuclear inflammatory cell detected in BALF,

consistent with previous mould extract inhalation studies (Derksen *et al.,* 1988; McGorum *et al.,* 1993c) and naturally occurring heaves (Derksen, 1993; Fairbairn *et al.,* 1993).

The plateau in response to increasing doses of mould extract may explain the results of previous mould inhalation studies where the severity of pulmonary inflammation and dysfunction associated with the natural disease was not reproduced (McGorum *et al.,* 1993c). Although only the 0.5mg AFE dose resulted in significantly lower BALF neutrophil count than the hay/straw challenge performed in the same horses (Chapter 3), the difference between the BALF neutrophilic response to hay/straw challenge and both the 1.6 and 5.0mg doses approached significance. This failure to achieve statistical significance in both cases resulted from the same horse, which developed a BALF neutrophilia following hay/straw challenge of equivalent magnitude to that following both 1.6mg and 5.0mg AFE challenge (H/S – BALF neutrophil ratio = 27%, 1.6mg AFE – 31%, 5.0mg AFE 28%).

Proposed explanations for this reduced response compared with hay/straw challenge include variations between responses to short-term and long-term inhalation challenge, the involvement of other inhalants in the natural disease and insufficient dosage of extract (McGorum *et al.,* 1993c). However it would appear from the current study that the latter explanation is unlikely. The plateau in response may therefore support the role of inhalants other than those present within AFE in the natural disease. This is perhaps not surprising considering the myriad of likely inhalants present within stable dust (6.4.1), many of which have pro-inflammatory

properties (Clarke, 1987a; Clarke and Madelin, 1987) including endotoxin (Chapters 2 and 3). It is possible that the combined exposure to fungal allergens and endotoxin could result in an increase in disease severity in susceptible horses, even if the underlying susceptibility reflects the individuals' hypersensitivity response to fungal allergen, as opposed to their endotoxin responsiveness. Another explanation for the reduced response to soluble mould extract inhalation compared with hay/straw exposure is that the AFE contains only soluble and not particulate components. Particulates, such as mould spores, have been shown *in vitro* to significantly enhance IgE-mediated histamine release from suspensions of BAL cells (Larsen *et al.,* 1996).

In conclusion, the current study supports the role of other inhalants in addition to soluble mould allergens in the aetiopathogenesis of heaves. Considering the wide variety of inhalants to which stabled horses are exposed, the clinical features of this disease may reflect a hypersensitivity to inhaled mould allergens, which results in a magnification in the host response to other pro-inflammatory components of stable dust, such as endotoxin. Consequently, further work was performed to investigate whether, in heaves horses, inhaled endotoxin could potentiate the pulmonary inflammatory and functional response to AFE (Chapter 5).

CHAPTER 5: EFFECT OF CONTAMINANT LPS ON THE RESPONSE TO INHALED SOLUBLE *ASPERGILLUS FUMIGATUS* EXTRACT

5.1 Summary

To investigate the role of endotoxin contamination of fungal extract in the response of heaves horses following inhalation challenge, the response of 6 heaves horses to inhalation of 1.6mg soluble *A. fumigatus* extract (AFE) was assessed before and after lipopolysaccharide (LPS) depletion. LPS depletion of AFE resulted in a significant reduction in airway (BALF) neutrophil numbers and increase in arterial oxygen tension when compared with AFE. There was no significant difference between saline and the LPS-depleted AFE challenges with respect to BALF neutrophil count and lung function. The reduction in airway neutrophil numbers was greater than predicted by extrapolation from soluble LPS dose-response inhalation experiments. While it was not determined whether the reduction in effect was due *entirely* to removal of LPS and not other AFE components, this study supports the potentiating role of LPS in this AFE-induced model of heaves. This study also supports the role of inhaled endotoxin in the pulmonary inflammation and dysfunction in naturally occurring heaves, given the high concentration of both *A. fumigatus* and endotoxin in stable dust.

5.2 Introduction

The study described in Chapter 4 suggested that insufficient dose delivery was unlikely to explain the reduced pulmonary inflammatory response following fungal

extract inhalation as compared to that following mouldy hay/straw exposure. It appeared more likely that additional components of the airborne dust contributed to this pulmonary inflammation, and this thesis has concentrated on the potential role of endotoxin in this respect for 2 main reasons. Firstly, stable dust contains relatively high quantities of endotoxin (Olenchock *et al.,* 1992; Dutkiewicz *et al.,* 1994; McGorum *et al.,* 1998; Tanner *et al.,* 1998), and secondly, LPS inhalation challenges in horses results in neutrophilic airway inflammation (2.5.2.6) and mild obstructive lung dysfunction (2.5.2.3), both features of naturally occurring heaves. Despite the proposed similarities between heaves and human allergic asthma (Derksen, 1993), unlike asthma, eosinophil infiltration into the airways is not a feature of heaves (Derksen *et al.,* 1985b; Fairbairn *et al.,* 1993). However in asthma, endotoxin contamination of inhaled allergens can alter the predominant cell population recruited to the airways from eosinophils to neutrophils (Hunt *et al.,* 1992; Hunt *et al.,* 1994). Indeed endotoxin contamination of inhaled allergens has been proposed as a cause of the predominantly neutrophilic lung infiltration in sudden-onset fatal asthma (Sur *et al.,* 1993). It is therefore possible that the high concentration of inhaled endotoxin to which horses are exposed may be a major contributor to the neutrophilic inflammation in heaves. This chapter describes the pulmonary inflammatory and functional response to inhalation challenge with LPS depleted fungal extract.

5.3 Materials and methods

5.3.1 Subjects

6 horses (3 geldings, 3 mares; age 17 years, 8-28; weight 434 kg, 323-594) with a history and clinical diagnosis of heaves were used (3.3.1). The disease status of all subjects was confirmed by mouldy hay/straw challenge as previously described (2.3.4.2). All horses were kept in a low dust environment (2.3.2.1) throughout the duration of the study.

5.3.2 Inhalation challenge material

5.3.2.1 Evaluated challenge

Endotoxin-depleted 1.6mg soluble *A. fumigatus* extract (AFE-LPS) was used. The AFE was of the same batch as that previously described (4.3.2).

5.3.2.2 Positive (1.6mg AFE) and negative (saline) control challenges

To relate the AFE-LPS responses with those of a negative and positive challenge, comparisons were made with saline inhalation (Chapter 2) and 1.6mg AFE challenges (Chapter 4) performed in the same horses.

5.3.2.3 Endotoxin analysis of AFE

Endotoxin analysis of 1.6mg AFE and AFE-LPS was performed using an endotoxin-specific *Limulus* amoebocyte assay (2.3.6.2), following appropriate dilution of the extract to reduce the contaminating endotoxin concentration to a level between the standard supplied with the assay and a 1:8 dilution of that standard.

5.3.2.4 Endotoxin depletion of AFE

Polymixin-coated agarose beads suspended in 50% glycerol (polymixin B-agarose, Sigma Aldrich Co. Ltd., Poole, Dorset) were used to achieve endotoxin depletion of AFE (Molig and Baek, 1987). The binding capacity of the polymixin-coated bead suspension is reported as 200-500μg LPS from *Escherichia coli* serotype 0128:B12 per ml. 10ml of the polymixin-coated bead suspension was added to 5ml of the stock solution of AFE (10mg/ml) in a sterile conical tube. The tube was rotated for 30min and the resulting mixture was centrifuged (1600g; 15min) to pellet the beads. The LPS-depleted supernatant (AFE-LPS) was decanted and frozen at -80°C until diluted in saline to a concentration of 1.6mg/ml and used for the inhalation challenges.

5.3.3 Inhalation challenges

For all challenges, 1ml of challenge substance was delivered to the facemask. To facilitate subject cooperation, horses were sedated immediately prior to each inhalation challenge as previously described (2.3.4.1). The aerosol was generated and delivered as previously described (2.3.4.1).

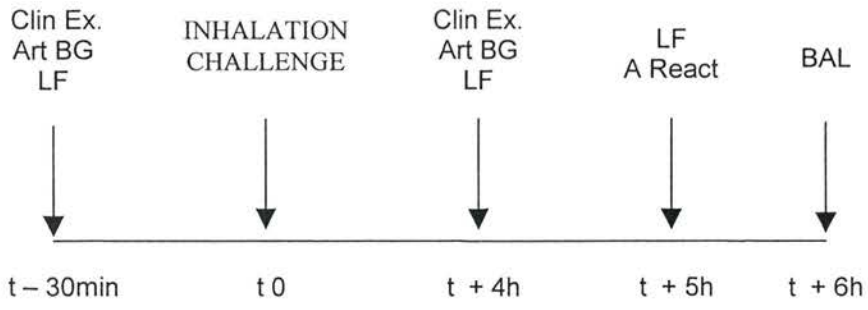
To minimise potential carry-over effects of a preceding on subsequent challenge, inhalation challenges were conducted a minimum of 14 days apart and all horses were shown to have normal clinical findings immediately prior to each inhalation challenge. In order to assess any carry-over effects from previous inhalation challenges in the same horses, baseline lung function and arterial blood gas values prior to AFE and AFE-LPS challenge were compared.

5.3.4 Monitoring the response to challenges

The method and timing of assessment of response to each challenge is summarised in Fig. 5.1. Responses to the AFE-LPS, 1.6mg AFE (Chapter 4) and saline (Chapter 2) challenges were assessed using clinical scoring, lung mechanics, airway reactivity, blood gas analyses and BALF cytology, as previously described (2.3.7).

Fig. 5.1: Study design.

BAL = bronchoalveolar lavage; LF = lung function evaluation; Clin Ex = clinical examination; Art BG = Arterial blood gases and pH analysis; A React = airway reactivity evaluation.



5.4 Statistical analyses

As the data were either not normally distributed and/or the groups of compared data did not have equal variance, non-parametric tests were used. The effects of each challenge were determined by performing within-group analyses.

To check for the presence of any carry-over effects of one challenge on a subsequent challenge, pre-challenge (baseline) measurements of arterial blood gases and pH and lung function were compared for the 3 challenges using a Friedman test, and when significant, a Wilcoxon Rank Sum test was performed on paired data.

To assess the effects of challenge where pre-challenge measurements were made at t-30min (arterial blood gases and pH analyses and lung mechanics), the post-challenge values were expressed as % of baseline value, except for clinical scores, where actual values were used. As saline was the vehicle for AFE and AFE-LPS delivery, the effect of AFE challenge was assessed by pairing and subtracting post-AFE/AFE-LPS (% of baseline value) and post-saline (% of baseline value) data. Where no pre-challenge data was collected (BALF cytology), comparisons were made with saline (placebo) challenge data at an equivalent time point. A Friedman test was performed on sets of paired data to reduce the risk of type 1 errors, and when significant, a Wilcoxon Rank Sum test was performed on paired data.

Significance was assumed if $P<0.05$. Following correction for any effect of saline inhalation, changes from baseline are expressed as increase/decrease or change in median values, with 95% confidence interval for the difference in median, calculated

for non-parametric data as described by Campbell and Gardner (1994). Results in tables are expressed as median and range.

5.5 Results

5.5.1 LPS depletion from AFE

Following mixing of 5ml AFE (10mg/ml) with 10ml the polymixin bead suspension and centrifugation to pellet the beads, the volume of the resulting supernatant was 10ml, as the 5ml glycerol in which the beads were suspended was not separated by centrifugation. Therefore the concentration of AFE in the final stored supernatant was 5mg/ml (1:2 dilution of 10mg/ml) and this was used as the stock solution for subsequent dilution and nebulisation.

The endotoxin content of the original AFE (10mg/ml) was shown to be 13.1μg/ml. The endotoxin content of the LPS depleted AFE (5mg/ml) was 2.1μg/ml. Both the AFE-LPS and the AFE were diluted in physiologic saline, to result in a final challenge concentration equivalent to 1.6mg/ml AFE. Therefore polymixin treatment reduced the endotoxin concentration in the final 1.6mg AFE challenge from 2.1μg/ml (13.1μg/ml x [1.6/10]) to 0.66μg/ml (2.1μg/ml x [1.6/5]), i.e. a reduction of 1.44μg/ml, equating to a 69% reduction in endotoxin activity.

5.5.2 Response to inhalation challenge with saline, AFE and AFE-LPS

The responses to inhalation challenge with saline and 1.6mg AFE are previously described (2.5.2 and 4.5.1, respectively).

5.5.2.1 Clinical examination

All horses had a clinical score of zero prior to all challenges, and when compared with baseline values, no significant increase in clinical scores was detected at 4h following AFE-LPS challenge (Appendix 5.1).

5.5.2.2 Arterial blood gases and pH analyses

Raw data for arterial blood gas and pH measurements are presented in Appendix 5.2. There was no significant difference in the baseline blood gases or pH when AFE-LPS and AFE challenges were compared, indicating a lack of detectable carry-over effects. When compared with baseline values, no significant change in blood gases or pH values were detected at 4h following AFE-LPS challenge. AFE challenge induced a significantly ($P<0.05$) greater decrease in median arterial oxygen tension at 4h than AFE-LPS (difference in median - 11%, 95% CI 4-27).

5.5.2.3 Lung mechanics and airway reactivity

Raw data for lung mechanics measurements are presented in Appendix 5.3. With the exception of $RL_{E50\%}$, $RL_{E75\%}$ and Wb_{Eres}, there was no significant difference in the baseline lung mechanics measurements prior to AFE and AFE-LPS challenges, indicating a lack of detectable carry-over effects. The percentage change in lung

function measurements from baseline is presented in Table 5.1. PCCdyn70 values following challenge are presented in Appendix 5.4.

Following correction for the effects of saline inhalation, the significant ($P<0.05$) increase in $R_{LE25\%}$ at 5h following 1.6mg AFE challenge (4.5.1.3) was not detected following AFE-LPS challenge. AFE-LPS challenge did not induce a significant alteration in airway reactivity at 5h. There was no significant difference in PCCdyn70 values when the AFE-LPS and 1.6mg AFE challenges were compared.

Table 5.1 (a and b): Percent (%) of baseline lung mechanics measurements (median and range) in heaves (n=6) horses at 5h following inhalation challenge with saline, 1.6mg AFE and 1.6mg AFE-LPS.

(a)

	Cdyn	dPpl	RL_{iso}	RR	VT	Wb	$RL_{E25\%}$	$RL_{E50\%}$	$RL_{E75\%}$	$RL_{I25\%}$	$RL_{I50\%}$	$RL_{I75\%}$
SALINE	96.85 (75.80-104.88)	108.13 (59.98-188.49)	88.70 (76.88-197.35)	93.02 (62.35-110.58)	111.33 (67.19-165.78)	95.82 (28.95-146.50)	62.14 (35.80-172.00)	64.98 (0.00-125.25)	70.33 (50.66-114.96)	124.76 (76.97-438.89)	101.25 (84.87-317.07)	89.38 (70.54-313.75)
1.6mg AFE	95.21 (57.26-137.87)	109.10 (72.17-136.94)	152.87 (66.29-209.89)	86.55 (59.40-152.46)	100.34 (74.97-104.77)	104.72 (62.95-134.65)	170.39 (114.29-720.00)	170.81 (70.00-401.72)	147.31 (93.00-238.39)	116.66 (96.49-170.11)	120.41 (68.65-182.05)	133.28 (78.53-203.66)
1.6mg AFE-LPS	113.31 (33.86-160.16)	103.46 (93.39-191.76)	110.77 (91.64-224.04)	99.47 (64.89-117.86)	106.30 (88.41-127.47)	107.53 (77.87-157.77)	135.98 (71.43-378.95)	99.12 (51.11-164.89)	99.03 (86.55-198.43	119.61 (81.56-270.11)	138.59 (65.93-235.00)	136.67 (69.49-217.76)

(b)

	T_E	T_I	$T_I:T_E$	V'_E	V'_{Emax}	V'_{Imax}	Wb_{el}	Wb_{res}	Wb_{Eres}	Wb_{Ires}	Wb_{Itot}
SALINE	101.35 (83.06-219.93)	110.18 (86.29-150.99)	102.19 (58.48-110.54)	104.88 (43.76-117.87)	115.84 (43.28-137.88)	102.03 (56.11-140.35)	127.55 (48.26-231.46)	92.15 (48.17-277.79)	76.47 (41.95-223.61)	108.27 (58.49-298.91)	117.22 (51.88-259.08)
1.6mg AFE	112.64 (57.16-126.97)	113.30 (92.75-128.97)	101.30 (84.66-171.74)	84.86 (43.97-151.48)	81.10 (75.73-112.24)	87.89 (76.28-117.08)	121.65 (67.46-149.23)	116.46 (67.27-156.83)	101.23 (32.63-177.76)	119.01 (65.55-152.66)	123.13 (66.59-149.65)
1.6mg AFE-LPS	102.45 (85.52-148.70)	100.57 (86.55-148.70)	83.83 (56.36-107.47)	100.94 (78.30-117.02)	98.60 (76.88-110.67)	103.22 (79.88-121.60)	102.40 (65.94-403.97)	105.58 (88.99-230.42)	101.28 (73.47-241.93)	116.21 (92.37-217.20)	112.18 (88.31-268.66)

5.5.2.4 BALF cytology

BALF neutrophil counts and ratios following saline, 1.6mg AFE and AFE-LPS challenges are fully presented in Appendix 5.5, and summarised in Table 5.2.

Table 5.2: BALF neutrophil counts ($x10^5$/ml) and ratios (%) (median and range) in heaves (n=6) horses at 6h following inhalation challenge with saline, 1.6mg AFE and 1.6mg AFE-LPS.

	BALF neutrophil count ($x10^5$/ml)	BALF neutrophil ratio (%)
SALINE	0.07 (0.03-0.20)	2.2 (0.6-4.5)
1.6mg AFE	0.94 (0.68-2.10)	26.7 (11.3-53.9)
1.6mg AFE-LPS	0.44 (0.16-1.90)	12.8 (5.0-47.5)

Inhalation challenge with AFE-LPS resulted in a significant ($P<0.05$) reduction in BALF neutrophil count (decrease in median 0.43 x 10^5/ml, 95%CI 0.10-0.65) and ratio at 6h when compared with AFE challenge (Table 5.2; Figs. 5.2 and 5.3). In addition, the BALF neutrophil count following AFE-LPS challenge was not significantly different from that following saline challenge (Table 5.2; Fig. 5.2). For comparison, previous inhalation challenges in the same 6 horses with 20μg soluble LPS resulted in an increase in BALF neutrophil numbers (increase in median 0.20, 95% CI 0.06-0.48), compared with saline at 6h (calculated from data of study in Chapter 2).

Fig. 5.2: BALF neutrophil counts (x10^5/ml) in heaves (n=6) horses at 6h following inhalation challenge with saline, 1.6mg AFE and 1.6mg AFE-LPS.

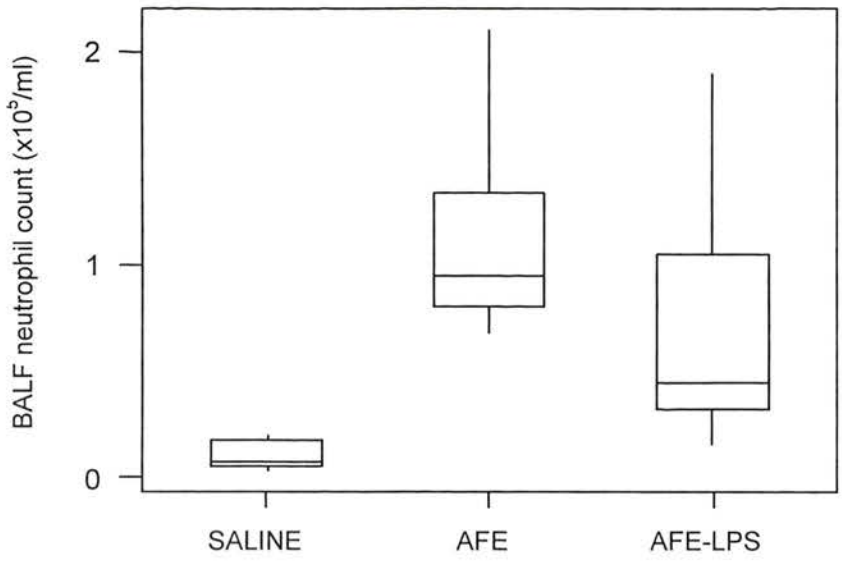
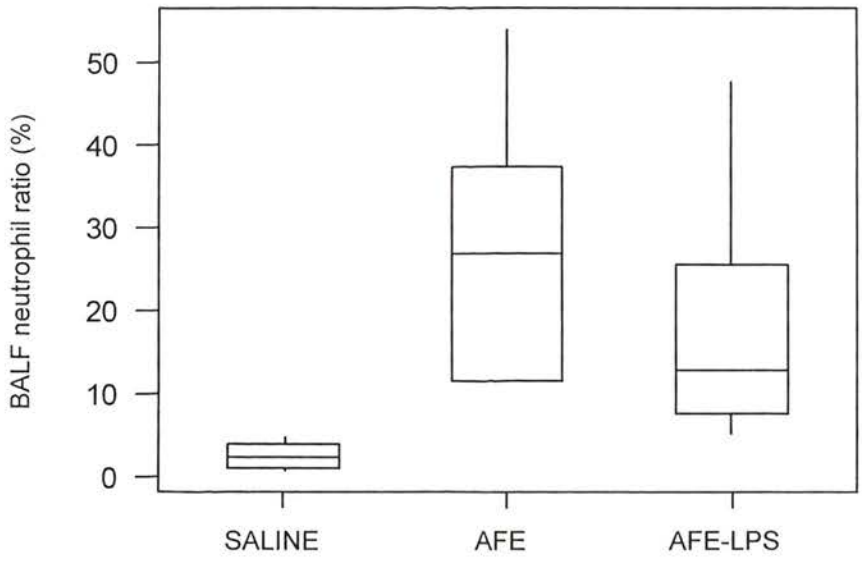


Fig. 5.3: BALF neutrophil ratio (%) in heaves (n=6) horses at 6h following inhalation challenge with saline, 1.6mg AFE and 1.6mg AFE-LPS.



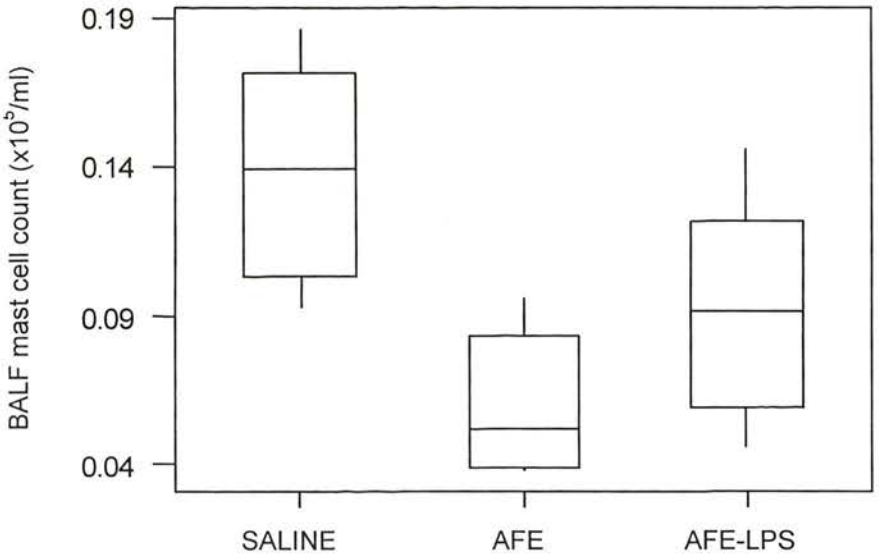
Due to the influence of a BALF neutrophilia on the ratio of other BALF cell types, they were considered only as absolute numbers. The absolute number of other BALF cell types at 6h following challenge are summarised in Table 5.3.

Table 5.3: Total and differential BALF cell counts (x10^5/ml) (median and range) in heaves (n=6) horses at 6h following inhalation challenge with saline, 1.6mg AFE and 1.6mg AFE-LPS.
TCC = total BALF cell count.

	TCC	Lymphocytes	Macrophages	Mast cells	Basophiloid cells	Eosinophils
SALINE	4.50 (3.20-5.60)	2.22 (1.54-3.33)	1.77 (1.24-2.96)	0.14 (0.09-0.19)	0.01 (0.01-0.11)	0.00 (0.00-0.01)
1.6mg AFE	3.85 (2.70-9.40)	1.21 (0.56-6.04)	1.57 (0.72-2.78)	0.05 (0.04-0.14)	0.00 (0.00-0.02)	0.00 (0.00-0.01)
1.6mg AFE -LPS	3.85 (2.70-5.60)	1.30 (1.09-2.37)	1.58 (0.76-2.61)	0.09 (0.05-0.15)	0.01 (0.00-0.06)	0.00 (0.00-0.01)

LPS depletion also resulted in a significant ($P<0.05$) increase in the BALF mast cell count (increase in median 0.03 x 10^5/ml, 0.01-0.06) at 6h, compared with 1.6mg AFE challenge (Fig 5.4). BALF mast cell and basophiloid cell numbers at 6h following AFE-LPS challenge did not significantly differ from those following saline challenge. Compared with saline inhalation, AFE-LPS challenge did not result in a significant alteration in total BALF cell numbers or absolute lymphocyte, macrophage, eosinophil or epithelial cell numbers (Table 5.3).

Fig. 5.4: BALF mast cell counts (x10^5/ml) in heaves (n=6) horses at 6h following inhalation challenge with saline, 1.6mg AFE and 1.6mg AFE-LPS.



5.6 Discussion

In addition to supporting the role of inhaled fungi in the aetiopathogenesis of heaves, the results presented in Chapter 4 also supported a role for other inhalants in determining the severity of the pulmonary inflammatory response. Comparing the inhalation responses to standard doses of AFE and endotoxin, with the response to inhalation of a combination of these components would have provided a valid method of determining the relative role of each component. However the finding that the AFE, used in the dose-response inhalation challenges reported in Chapter 4, was contaminated with LPS offered a suitable alternative to further investigate the combined effects of these 2 components. Considering the relatively high airborne concentrations of both moulds and endotoxin in stable dust (Clarke, 1987a; Clarke,

1987b; Clarke and Madelin, 1987; Clarke *et al.,* 1987; Webster *et al.,* 1987; Olenchock *et al.,* 1992; Dutkiewicz *et al.,* 1994; Raymond *et al.,* 1994; McGorum *et al.,* 1998; Tanner *et al.,* 1998), this system also potentially offered an insight into the role of inhaled endotoxin in the aetiopathogenesis of naturally occurring heaves. The successful depletion of LPS (estimated 69% reduction) from inhalation challenge material using polymixin-coated agarose beads was previously employed to investigate the role of inhaled endotoxin in a mouse model of organic dust-induced lung disease (Jagielo *et al.,* 1996a). The failure to achieve complete depletion in this study may have resulted from the LPS molecules forming micelles in the AFE, thus preventing exposure and consequently binding of the polymixin to cations on the lipid A component of the LPS molecules (Makela and Stocker, 1984).

The reduction in the neutrophilic response and improvement in lung function following LPS depletion of AFE was greater than would have been predicted by extrapolation of previous LPS dose-response inhalation experiments (Chapter 2). Polymixin treatment of AFE resulted in a reduction in delivery to the facemask equivalent to only 1.44μg LPS. However the difference in the median BALF neutrophil count when the AFE and AFE-LPS challenges were compared was greater than that induced following 20μg LPS inhalation (data presented in 5.5.2.4). This finding suggests that the LPS content of the inhaled extract contributed to the pulmonary inflammatory response to a greater degree than predicted if the contribution was solely additive to that of AFE, or that polymixin treatment also removed other AFE components which had a greater effect than LPS.

Despite the fact that the AFE-LPS challenge consistently resulted in a BALF neutrophil count not significantly different from saline challenge, it is unlikely that the neutrophilic response to AFE (4.5.1.4) could entirely be attributed to the activity of LPS. Firstly, previous LPS dose-response experiments have demonstrated that a significantly higher dose of LPS than that present within both the 1.6mg and 5mg AFE (2.1 and 6.6μg, respectively) is required to induce an equivalent airway neutrophilia and lung dysfunction (2.5.2). Secondly, the dose response curves following AFE inhalation and LPS inhalation in heaves horses are quite different, with the inflammatory response to LPS continuing to increase at exposures 100-fold greater than the response threshold (<20μg) (2.5.2.6; Pirie *et al.*, 2001b). This compares with a plateau in the neutrophilic inflammatory response to increasing doses of AFE only at a dose 3.2-fold greater than the response threshold (4.5.1.4).

It could be argued that the endotoxins present in AFE may be of a different LPS type to those present within the *Salmonella* R60 mutant used in the LPS challenges. However the *Salmonella* R60 mutant LPS represents a structure shared by many of the Enterobacteriaceae and is responsible for a major part of the biological activity of LPS (Prof. IR Poxton, personal communication). It would have been interesting to determine the effect of challenge with AFE-LPS following the addition of LPS from the *Salmonella* R60 mutant at a dose equivalent to that removed during the original depletion. Re-establishment of the neutrophilic response to a degree similar to that following challenge with AFE would have supported the theory that the depleted LPS and the *Salmonella* R60 mutant had similar biological activities. This would also have provided confirmation that the reduction in response resulted entirely from LPS

depletion, and was not due to any alteration in the activity of other agents present within AFE, nor due to contamination of the AFE-LPS with glycerol.

This study therefore demonstrates that the LPS contamination contributes markedly to the response to AFE challenge in horses. The phenomenon of disease severity being related to exposure to other inhalants, in addition to allergen, has been documented in human asthma, whereby endotoxin exposure can be a greater determinant of disease severity than allergen exposure (Michel *et al.,* 1991; Michel, 1996; Michel *et al.,* 1996; Rizzo *et al.,* 1997). In addition, inhalation challenge studies in a murine model of asthma have shown that co-exposure of mice to LPS and allergen results in a greater degree of airway neutrophilia when compared with allergen challenge alone (Goldsmith *et al.,* 1999).

It is possible that the presence of LPS contamination in the current AFE model resulted in a magnification in the response to mould allergens present within the extract, consistent with previous studies which have demonstrated an augmentation of the immunoglobulin responses to allergen by LPS (Rylander and Holt, 1998; Slater *et al.,* 1998; Tulic *et al.,* 2000). Alternatively, the response to inhaled LPS may have be magnified by the co-presence of allergen, perhaps via an increase in the concentration of lipopolysaccharide binding protein and soluble CD14 receptors in the bronchoalveolar compartment after allergen challenge, as has been demonstrated in human asthma (Martin *et al.,* 1992; Dubin *et al.,* 1996).

Interestingly, an alteration of the type of cellular response to inhaled allergen has been demonstrated in man following endotoxin contamination of allergen, whereby neutrophils instead of eosinophils were the predominant cell type detected within the airways (Hunt *et al.,* 1992; Hunt *et al.,* 1994). It is possible therefore that endotoxin contamination of mould extracts used in previous investigations of heaves contributed significantly to the reported neutrophil influx in the airways (Derksen *et al.,* 1988; McGorum *et al.,* 1993c). Although reduction in the LPS content of AFE in the current study did not alter the type of inflammatory cell recruited to the airways from neutrophils to eosinophils, complete LPS depletion was not achieved. Consequently, the level of endotoxin contamination in the AFE-LPS challenge, albeit reduced, may have contributed to the neutrophilic influx.

Also of interest was the increased BALF mast cell count following AFE-LPS challenge when compared with AFE challenge. It has been hypothesised that the reduction in the BALF mast cell count following AFE challenge when compared with saline inhalation resulted from a failure to identify degranulated mast cells on cytospin preparations (Derksen *et al.,* 1988). Consequently, the higher BALF mast cell count following challenge with AFE-LPS compared with AFE may support a role for LPS in mast cell degranulation, as has been demonstrated previously in studies on rat skin and colon (Brown *et al.,* 1998; Iuvone *et al.,* 1999). In addition, endotoxin has been shown to enhance histamine release from human pulmonary mast cells *in vitro* by both immunological and non-immunological mechanisms (Norn *et al.,* 1994).

In conclusion, this work has further supported a potentiating role of endotoxin in the pulmonary inflammatory and functional response to inhaled AFE. Ideally an add-back experiment would have been done to confirm if the difference in response to AFE and AFE-LPS was due solely to LPS.

CHAPTER 6: PRODUCTION AND CHARATERISATION OF A HAY DUST SUSPENSION (HDS), FOR USE IN INHALATION CHALLENGES IN HEAVES-SUSCEPTIBLE SUBJECTS

6.1 Summary

Currently, heaves is investigated by exposing susceptible horses to dusty hay. Consequently, the response will be dependent on the organic dust content and composition of the hay, as well as other factors including stable ventilation. It was hypothesised that the use of a nebulised hay dust suspension (HDS) would reduce the variability of these challenges and thus standardise experimental protocols. Furthermore, analysis of HDS would also permit further investigation of the organic dust components responsible for the pulmonary inflammatory and functional response.

Three hay dust suspensions (HDS-1, 2 and 3) were prepared from 3 batches of dusty hay. HDS-1 and 3 were analysed for endotoxin, β-D-glucan and protein concentrations, general protease activity, and enumeration and size distribution of particulates. Protease activity was mainly attributable to a 28kDa serine protease and to 85kDa and 160kDa metalloproteases. The particulate and soluble components of HDS could be aerosolised by jet nebulisation. It was therefore concluded that detailed analysis of HDS is possible, that such a challenge system provides a method of standardising experimental protocols within and among laboratories, and that all components of HDS (both soluble and particulate) can be delivered to the lung using standard nebulisation techniques.

6.2 Introduction

The diagnosis of heaves may be problematic, especially in horses with mild disease (Clarke, 1987c). In such cases, the diagnosis is frequently based upon the induction of pulmonary inflammation and dysfunction by exposure to mouldy hay/straw, with subsequent resolution of signs when this exposure ceases (Robinson *et al.,* 1996). However, no standardised hay/straw challenge protocol exists, and the variable and undefined composition of airborne dust in a conventional hay/straw challenge system (Clarke, 1993), and variation in stable ventilation can result in poor reproducibility of the responses to such challenges (Chapter 3). There is thus a requirement for a standardised and repeatable inhalation challenge that induces the functional and inflammatory responses of heaves in susceptible horses, but not in controls.

Theoretically, a standardised, prolonged duration, dry dust challenge, as used in other species (Rylander, 1988; Jolie *et al.,* 1999), may best reproduce the dust challenge encountered naturally in a dusty stable. However, the potential benefits of such a technique may be outweighed by practical problems associated with standardised and prolonged delivery of dry dust to horses. In addition, manipulation of the individual components of dry dust would be problematic. Soluble aqueous extracts of various organic dusts have been used extensively to investigate dust related respiratory disease in man and other species (Gao *et al.,* 1993; Schwartz *et al.,* 1994; Blaski *et al.,* 1996; Jagielo *et al.,* 1996a; Deetz *et al.,* 1997; Jagielo *et al.,* 1997; Jagielo *et al.,* 1998; Trapp *et al.,* 1998). However, these extracts do not contain dust particulates (*eg.* spores, mite debris, inorganic dust, plant fragments) which may directly contribute to pulmonary inflammation (Kurup *et al.,* 1997), and/or affect the

pulmonary distribution of soluble components. Therefore only the response to soluble dust components (*eg.* proteases and soluble fungal antigens, endotoxins and glucans) can be assessed. Surprisingly, there are apparently no reports of the use of aqueous hay dust suspensions (HDS), which contain both soluble and particulate dust components, in the investigation of dust related disease in any species. This chapter describes the production and analysis of HDS, to be used in the diagnosis and investigation of heaves.

6.3 Materials and methods

6.3.1 Production of HDS

6.3.1.1 Collection of dust particles

Three different HDS (HDS-1, 2 and 3) were produced from 3 visibly mouldy batches of hay. Only HDS-1 was produced from a batch of hay *known* to induce heaves in susceptible horses (2.3.2.2). To harvest hay dust, hay was agitated manually onto a clean surface and the large stems were manually removed. The resultant dust was sieved (grid size 3x2mm) twice to remove larger plant debris. The remaining dust was spread evenly on a flat surface in an unventilated area (Fig. 6.1) and left for 1h to allow airborne dust to settle. The dust was then aspirated using a dual vortex household vacuum (DC01, Dyson Appliances Ltd., Malmesbury, Wiltshire, UK) (Fig. 6.2), which collected coarse dust in the outer dust collection drum (Fig. 6.3) and fine dust (Fig. 6.4) in a separate central compartment. The fine dust was transferred to sterile containers and stored at -20°C until required.

Fig 6.1: Following sieving, hay dust was spread on a flat clean surface in an unventilated room

Fig. 6.2: A dual vortex household vacuum was used to aspirate the settled dust

Fig. 6.3: Coarse dust was separated from fine dust. The coarse dust, collected in the outer dust collection drum, was discarded.

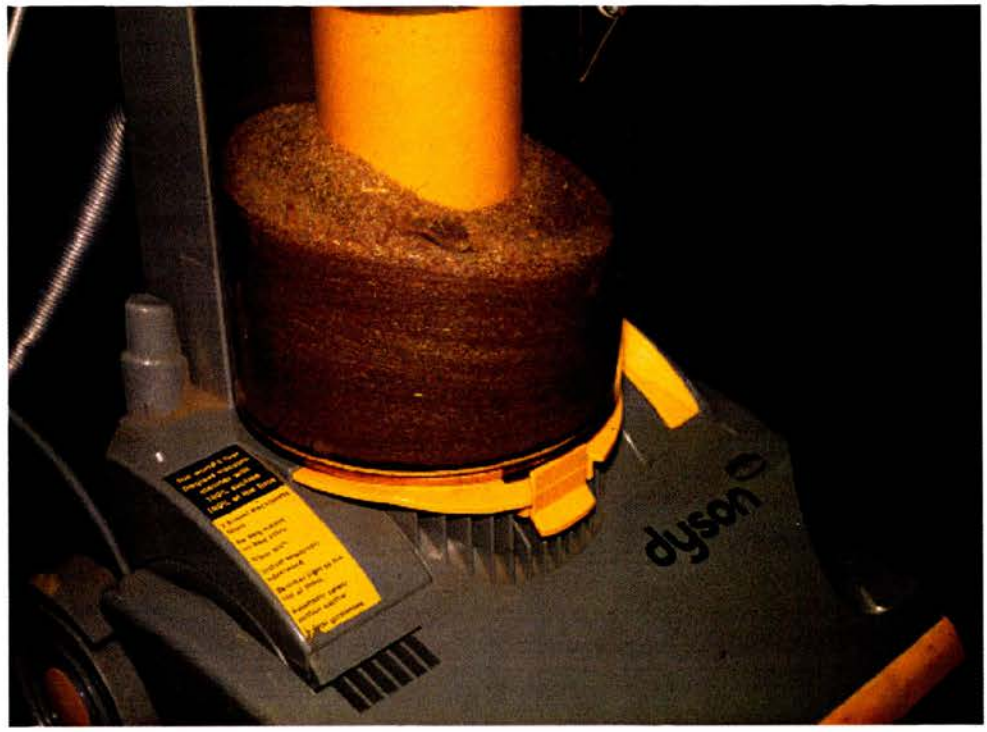


Fig. 6.4: Fine dust particles collected from the separate central compartment were used to prepare HDS.

6.3.1.2 Production of suspension

To prepare the HDS, 10ml sterile physiologic saline (Vetivex, 0.9% w/v Sodium Chloride, Ivex Pharmaceuticals, Larne, UK) was added to each gram of dust. The suspension was then vortexed for 30s, shaken for 30min and rolled for 30min. It was then filtered through 60μm pore size nylon gauze mesh (Nytex gauze, Nytex, UK), aliquoted into 1.8ml eppendorfs and stored at -80°C.

6.3.2 Characterisation of HDS

6.3.2.1 Microscopic analysis

All three suspensions were examined microscopically (Leica Microsystems UK Ltd., Milton Keynes) under 400x magnification (Fig. 6.5) and the particulates sized using an eyepiece graticule and compared with their microscopic appearance prior to filtration (Fig. 6.6).

Fig. 6.5: Photomicrograph of HDS following filtration through 60μm pore size nylon gauze showing mainly small fungal spores (2-4μm diameter) in addition to occasional "fair weather air spora" (e.g. Alternaria spore [A], rust uredospore [r]), mite faeces [m] and small fragments of plant/vegetable debris. x 400 magnification.

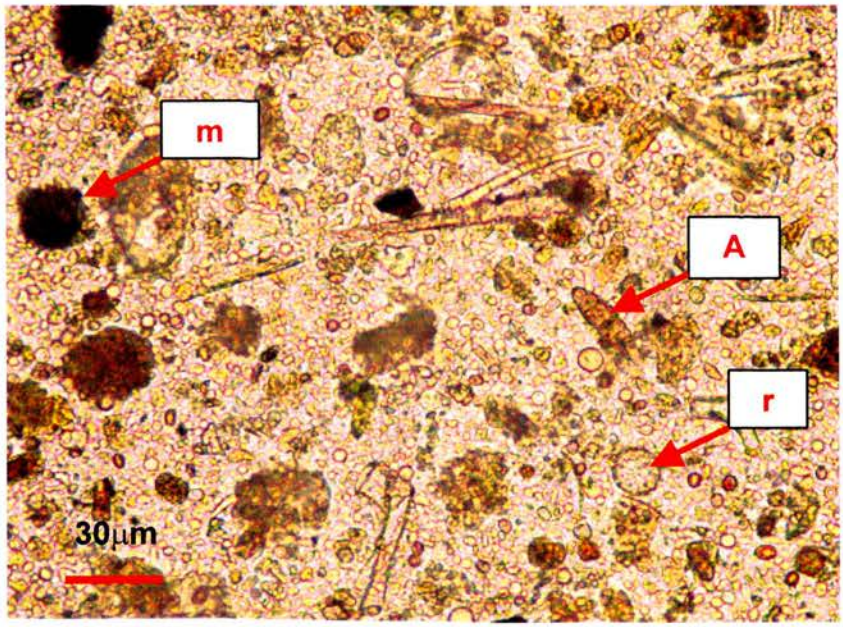
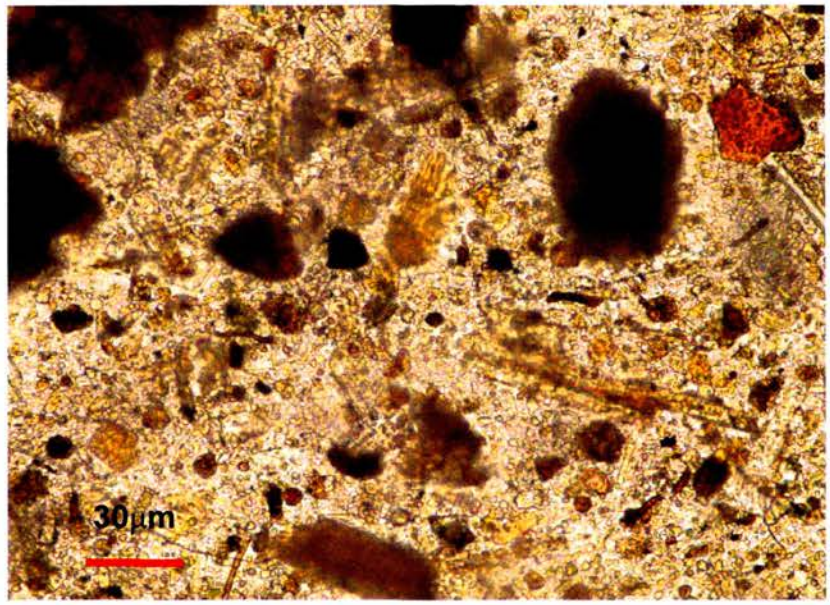


Fig. 6.6: Photomicrograph of HDS prior to filtration through 60μm pore size nylon gauze showing larger plant and non-defined particles. x 400 magnification.



6.3.2.2 Particulate loss during filtration

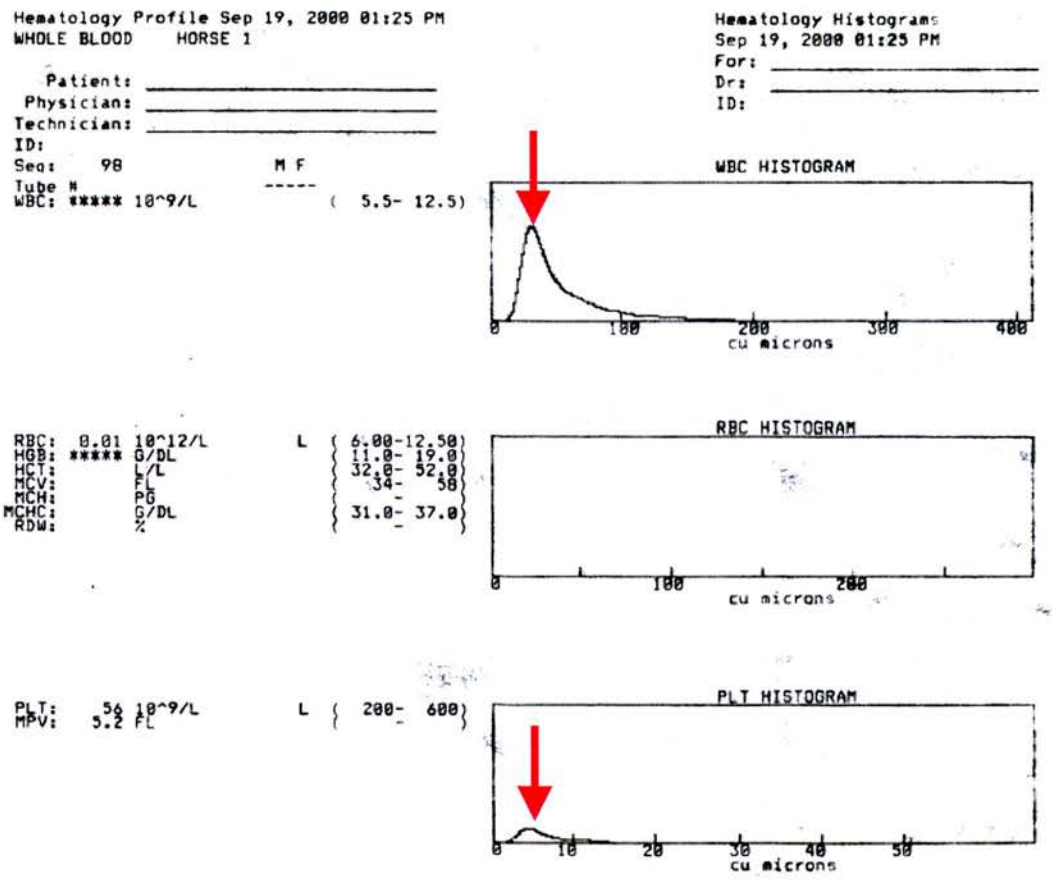
To determine the mass of particulates in the final suspensions, and consequently particulate loss during filtration, a sample of HDS-1 was weighed before and after evaporation of solution (including a correction for the mass of the sodium chloride).

6.3.2.3 Particulate count

Particulate counts for HDS-1 and HDS-3 were determined both manually using a haemocytometer (Neubauer haemocytometer, Fischer Scientific UK Ltd., Loughborough, Leics., UK) and by the impedence principle using an electronic cell counter (Baker System 9120 plus CP, Biochem Immunosystems, Allentown, PA, USA). This equipment also calculated the frequency distribution of the different sized particles. Counts were obtained for particulates in the 0-40, and the 40-300 μm^3

ranges (Fig. 6.7), which, if particles were spherical, corresponded to particulate diameters of 0-4.2 and 4.2-8.3µm, respectively. The particulate count as assessed using the haemocytometer was calculated as a mean of 10 separate counts.

Fig. 6.7: Printout of electronic cell counter data indicating frequency distribution of different sized particles. Two distinct peaks can be seen at the 2 most abundant particle volumes (arrows).



6.3.2.4 Analysis of endotoxin and glucan content

Endotoxin and glucan concentrations in HDS-1 and HDS-3 were determined using endotoxin-specific and glucan-specific assays, respectively. HDS samples were diluted in sterile water prior to analysis, and the particulates were not removed by centrifugation. Endotoxin analysis was then performed as previously described (2.3.6.2). For glucan analysis, an equal volume of 3M NaOH was added to the diluted sample prior to analysis to unwind the triple helical structure of the β-D-glucan as recommended for particulate samples by Thorn (1999). Otherwise β-D-glucan analysis was performed as previously described (3.3.4.3).

6.3.2.5 Analysis of protein content

The protein concentrations of HDS-1 and HDS-3 were measured using a commercial kit (Urinary Protein, Randox Laboratories Ltd., Co. Antrim, UK) by the method of Pesce and Kaplan (1987).

6.3.2.6 Analysis for general protease activity

General proteolytic activity was measured in HDS-1 and HDS-3 using a commercial assay (Universal protease substrate, Roche Diagnostics GmbH, Mannheim, Germany), employing the method of Twining (1984), using resorufin-coupled casein as a general protease substrate. For this assay, 1mg casein from cow's milk was coupled with 9μg activated resorufin (N-[resorufin-4-carbonyl]piperidine-4-carbonic acid N'-hydroxysuccinimide ester) and purified by gel chromatography. Protease

activity releases resorufin-labelled peptides from casein which are not precipitated by trichloroacetic acid. Following treatment with trichloroacetic acid and centrifugation to pellet precipitates, the concentration of these resorufin-labelled peptides in the supernatant is used as a quantitative assessment of the proteolytic activity present.

For the assay, 75µl substrate solution (0.4% resorufin labelled casein in distilled water) or 75µl distilled water (assay blank) was added to 75µl incubation buffer (0.2M Tris-HCl pH 7.8, 0.02M $CaCl_2$) and 150µl sample (1:10 dilution of HDS) or 150µl distilled water (sample blank). Following incubation at 37°C for 15min, 50µl mixture was transferred to a sterile eppendorf and the remainder returned to the incubator. 120µl of stop reagent (5% w/v trichloroacetic acid in distilled water) was added to the 50µl aliquot, this mixture was incubated for a further 10min, then centrifuged for 5min, and 100µl supernatant pipetted into a well of a 96 well microtitre plate. 150µl assay buffer (0.5M Tris-HCl, pH 8.8) was added and the absorbance read at 570nm. This procedure was repeated at 15min intervals whereby a further 50µl aliquot was transferred from the original mixture. A 1:10 dilution of the HDS was used as this provided a linear curve for absorbance over time during the first 60min period, prior to the development of a plateau in absorbance units due to substrate depletion. As the assay measured general protease activity, results could not be expressed in actual units of protease activity, however comparisons could be made between HDS samples.

6.3.2.7 Analysis for specific protease activity

To identify specific protease mechanistic classes, the assay was repeated on HDS-1 following the addition of, and co-incubation with, a variety of broad spectrum (Complete Protease Inhibitor Cocktail Tablets [EDTA-free], Roche Diagnostics GmbH) and group-specific (ethylenediaminetetraacetic acid [EDTA], Sigma-Aldrich Co. Ltd., Dorset, UK; Aprotinin, Roche Diagnostics GmbH; Pepstatin, Roche Diagnostics GmbH; E-64, Roche Diagnostics GmbH) protease inhibitors. Each inhibitor was pre-incubated with the sample at 37°C for 1h prior to analysis as described above. A variety of concentrations of inhibitors were used: EDTA 1mg/ml and 10mg/ml; Aprotinin 1mg/ml and 10mg/ml; Pepstatin 0.2mg/ml and 2mg/ml; E-64 0.5mg/ml and 5mg/ml. For the Complete Protease Inhibitor Cocktail Tablets [EDTA-free], 1 tablet per 10ml extraction solution is recommended for protease inhibition. They were added to the HDS (1:10) from a stock solution of 1 tablet per 1.5ml to give final concentrations in the HDS equivalent to 1 tablet per 40ml, 20ml, 10ml, 5ml and 2.5ml.

6.3.2.8 Identification of protease activity by modified SDS-page electrophoresis

Proteolytic activities of HDS-1 and HDS-3 were also characterised using sodium dodecyl sulfate (SDS)-page electrophoresis (Lundy et al 1995), using 1mg/ml azocasein incorporated into a 12% agarose gel as the general protease substrate. Undiluted, diluted (1:10) and 10x concentrated HDS samples were analysed. Concentration of the samples was achieved by centrifugation through filters with a 10kDa cut-off (Centron-10, Amicon Ltd., Stonehouse, Gloucestershire, UK). Prior to

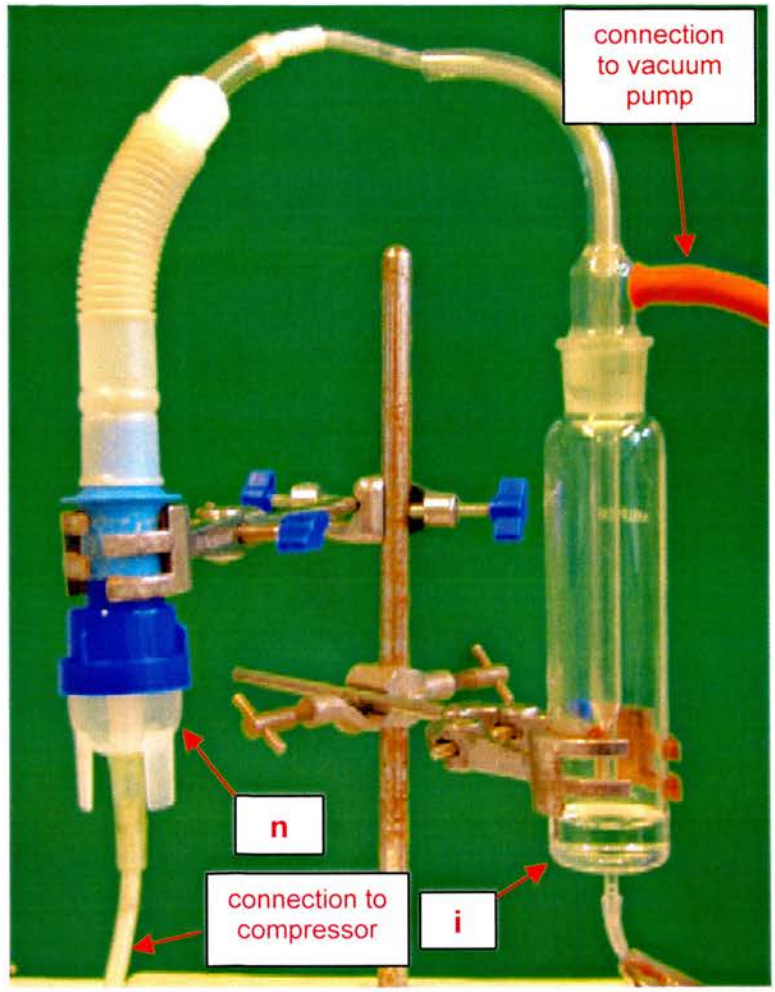
loading of the wells in the gel, HDS samples were diluted 1:2 with sample buffer (distilled water, 0.5M Tris-HCl [pH 6], glycerol, 10% SDS, 0.05% bromophenol blue). Following electrophoresis, gels were washed twice for 45min with 2.5% Triton-X 100 solution and incubated overnight at 37°C in phosphate buffered saline. To ensure preservation of protease activity, samples were non-denatured prior to electrophoresis. Following staining with Coomassie blue, specific bands of protease activity were identified as clear areas against the blue background of the stained azocasein substrate. These bands were compared with broad-range SDS-page molecular weight markers (Molecular weight standards, Bio-Rad, Hercules, California, USA).

This procedure was then repeated, following 1h pre-incubation at 37°C of HDS with broad spectrum (Complete Protease Inhibitor Cocktail Tablets [EDTA-free]) and 2 group-specific (EDTA and Pefabloc [Pentapharm AG, Basel, Switzerland]) protease inhibitors. For pre-incubation, EDTA was added to the HDS at a concentration of 10mg/ml, Complete Protease Inhibitor Cocktail Tablets [EDTA-free] were added at a concentration equivalent to 1 tablet per ml, and Pefabloc was added to the HDS at a concentration of 25mg/ml. With the exception of Pefabloc, which is an irreversible inhibitor, all other inhibitors were also incorporated into the 2.5% Triton-X 100 washing solution and PBS incubation stage. EDTA was incorporated into both steps at a concentration of 5mg/ml, and Complete Protease Inhibitor Cocktail Tablets [EDTA-free] were incorporated into the 2.5% Triton X 100 washing stage at a concentration of 1tablet per ml, and into the PBS incubation stage at a concentration of 0.5 tablets per ml.

6.3.3 Efficiency of nebulisation of HDS particulates

In order to assess the efficiency of particulate nebulisation, HDS-1 was nebulised as previously described (2.3.4.1) into an impinger chamber (All glass impinger, Millipore, Hertfordshire, UK) containing 5ml saline (Fig. 6.8). 1ml HDS was nebulised from a total volume of 2ml, as assessed by a 1g reduction in mass of the nebuliser cup. The particulate concentration of the suspension retained within the impinger was measured as previously described (6.3.2.3.). This concentration was multiplied by the final volume of suspension within the impinger, which gave the total number of particulates aerosolised from 1ml HDS. Comparison of the total particulate count of the nebulised suspension and the suspension collected within the impinger permitted the calculation of the efficiency of particle nebulisation. In addition, measurement of the frequency distribution of different sized particulates in both aliquots permitted calculation of the relative efficiency of nebulisation of particulates within the 0-40, and the 40-300 μm^3 ranges.

Fig 6.8: Apparatus for the assessment of the efficiency of particulate nebulisation. 1ml of HDS within the nebuliser cup (n) was nebulised into an all glass impinger (i) containing 5ml saline.



6.3.4 Fractionation of HDS

HDS-1 was centrifuged at 1600g for 15 min to yield HDS supernatant (SUP) and particulate debris. The particulates, which comprised mainly mould spores, were washed 3 times with separate aliquots of sterile physiologic saline, by repeated re-suspension and centrifugation (15 min, 1600g). The washed particulates (WP) were then re-suspended in saline, to the volume of the original HDS. This washing

procedure was repeated on a further sample of HDS-1, but the same aliquot of saline (a volume equivalent to the decanted HDS supernatant) was used for all 3 washes. Following the 3 washes, the resultant supernatant, which contained saline and soluble components washed from the surface of the particulates, was collected and termed the "wash fraction" (WF). All 3 fractions were aliquoted into 1.8ml ependorfs and stored at –80°C.

6.3.5 Characterisation of HDS fractions

6.3.5.1 Analysis for endotoxin and glucan content

The endotoxin and glucan concentrations of the 3 fractions (SUP, WP and WF) were determined using endotoxin-specific and glucan-specific assays, respectively, as previously described (6.3.2.4).

6.3.5.2 Analysis for general protease content

General proteolytic activity was measured in the 3 fractions using a commercial assay employing resorufin-coupled casein as a general protease substrate, as previously described (6.3.2.6).

6.4 Results

6.4.1 Analysis of HDS

6.4.1.1 Microscopic analysis

All 3 HDS samples contained particulates which comprised predominantly fungal spores 2-4μm in diameter (Fig. 6.5). Less abundant particulate constituents included mite exoskeleton fragments, mite faeces, larger mould spores (e.g. *Alternaria*), plant and other unidentifiable debris. It was not possible to differentiate the 3 HDS samples by microscopic examination.

6.4.1.2 Particulate loss during filtration

4ml of HDS weighed 3.9654g (1ml=0.99g). Following evaporation of solution from 4ml HDS, the resulting weight of particulates (including NaCl crystals) was 0.1234g. Following subtraction of the weight of NaCl in 4ml HDS (0.036g), the weight of dust within 4ml HDS was calculated as 0.0874g. Hence the weight of dust particles within 1ml HDS was calculated as 0.0219g (21.9mg), indicating that approximately 78% of the original dust (100mg/ml) was removed by filtration through the 60μm pore size nylon gauze.

6.4.1.3 Particulate count, endotoxin, glucan and protein concentrations of HDS

The particulate, endotoxin, glucan and protein concentrations of HDS are summarised in Table 6.1. Unfortunately, only limited assay of HDS-2 was possible due to

insufficient sample quantity. The co-efficient of variance of the 10 counts using the haemocytometer was 9.3%.

Table 6.1: Particulate, protein, endotoxin and β-D-glucan concentrations of HDS. NP = not performed.

	HDS-1	HDS-2	HDS-3
Particulate concentration ($x10^6$/ml) (0-40μm^3 range)	553	NP	929
Particulate concentration ($x10^6$/ml) (40-300μm^3 range)	181	NP	187
Mean total particulate concentration ($x10^6$/ml) (manual count using haemocytometer)	710	NP	NP
Protein concentration (mg/ml)	0.47	NP	0.46
Endotoxin concentration (μg/ml)	21.6	18.2	15.2
β-D-glucan concentration (μg/ml)	184	NP	596

6.4.1.4 Protease activities of HDS

HDS-1 and HDS-3 had similar general proteolytic activities (Fig. 6.9). A dose-dependant reduction in protease activity was observed when HDS-1 was co-incubated with the Complete Protease Inhibitor Cocktail Tablets [EDTA-free], however significant protease activity remained even following co-incubation with a high concentration of inhibitor (Fig 6.10). Protease activity was reduced when HDS-1 was co-incubated with the metalloprotease inhibitor EDTA or the serine protease inhibitor aprotinin (Fig. 6.11), but not with the aspartate protease inhibitor pepstatin, or the cysteine protease inhibitor E-64.

Fig. 6.9: Casein-resorufin hydrolysis (A570nm) by HDS-1 (1:10 and 1:100 dilution) and HDS-3 (1:10 and 1:100 dilution) plotted against incubation time (min).

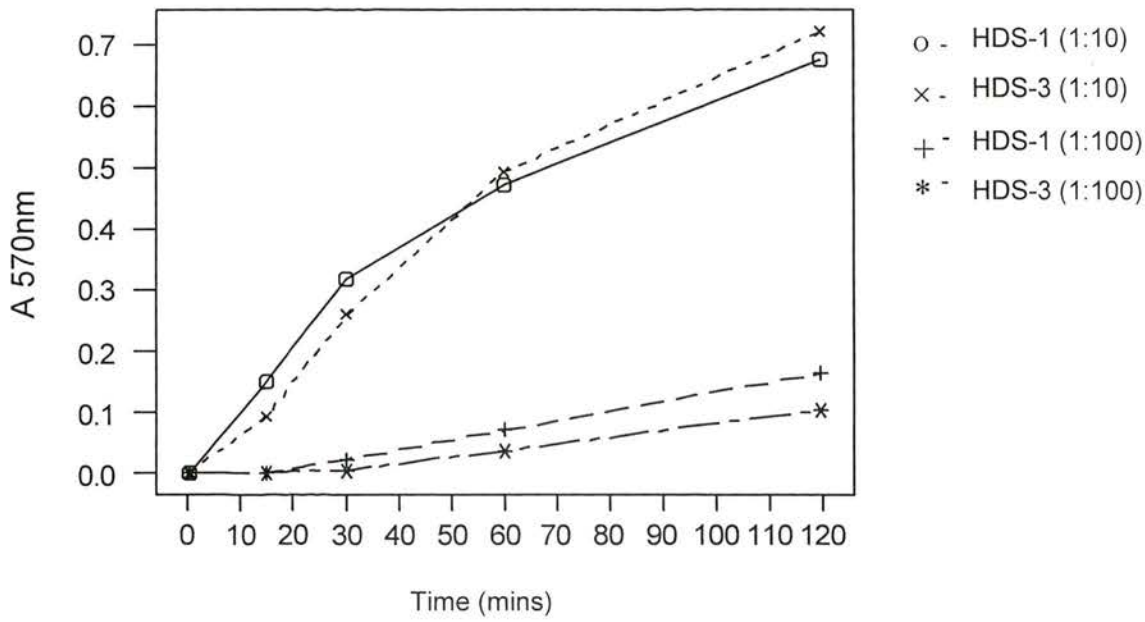


Fig 6.10: Casein-resorufin hydrolysis (A570nm) by HDS-1 (1:10 dilution) co-incubated with doubling Complete Protease Inhibitor Cocktail Tablets [EDTA-free] plotted against incubation time (min).

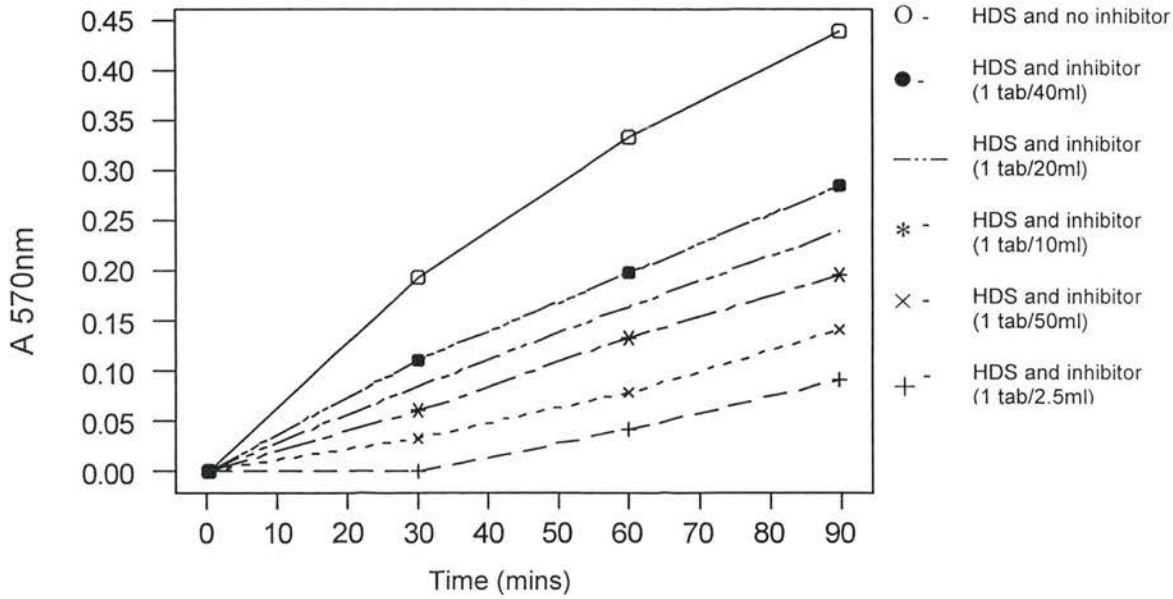
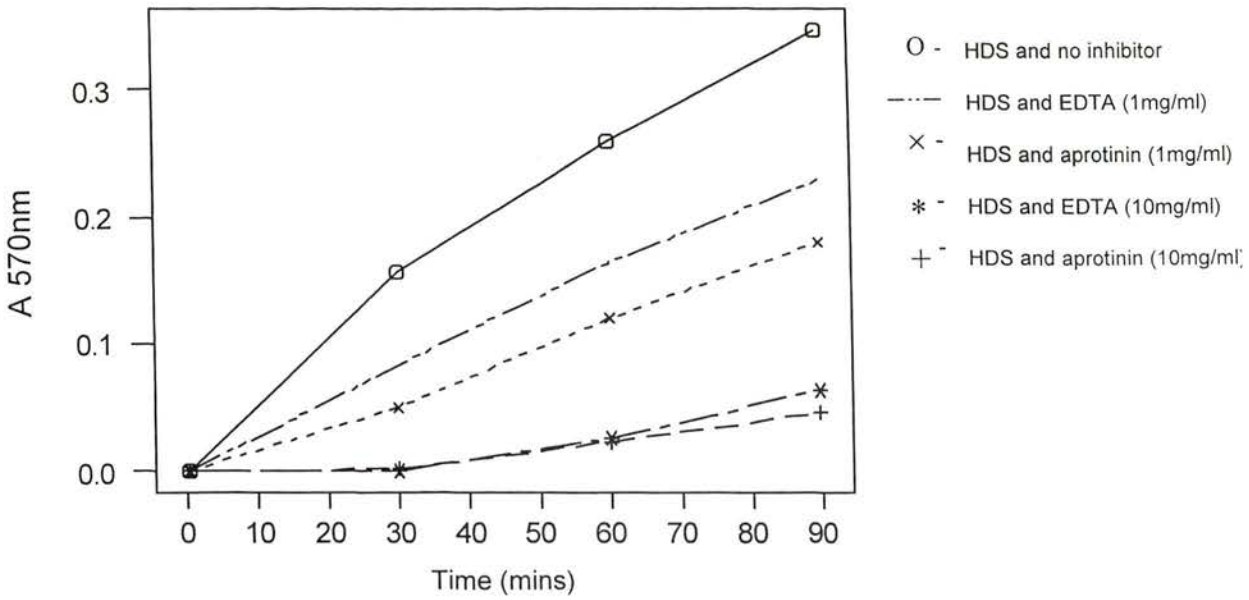


Fig. 6.11: Casein-resorufin hydrolysis (A570nm) by HDS-1 (1:10 dilution) co-incubated with aprotinin (10mg/ml and 1mg/ml) and EDTA (10mg/ml and 1mg/ml) plotted against incubation time (min).



6.4.1.5 SDS-page electrophoresis

Following electrophoresis of 1:10 diluted samples of HDS, faint bands of protease activity were visible. Following electrophoresis of 10x concentrated samples, HDS-1 had distinct bands of protease activity at 160 and 85kDa and HDS-3 had a broad area of protease activity extending from 200 to 85kDa (Figs. 6.12 and 6.13), which following sample dilution, revealed 2 distinct bands at approximately 160 and 85kDa. A band of protease activity in the 28kDa region was present in both HDS, but was more marked in HDS-3 (Figs. 6.12 and 6.13).

Thirty minute pre-incubation at 37°C of both 10x concentrated HDS samples with Pefabloc®, reduced the intensity of the 28kDa band, revealed a faint band of activity in the 21kDa region, but did not affect the protease activity in the higher molecular weight regions (Figs. 6.12 and 6.13). Pre-incubation with EDTA, and its subsequent incorporation in the wash and incubation steps, removed the 160 and 85kDa bands of activity in HDS-1, and markedly reduced the intensity of the broad area of activity in the 200 to 85kDa range in HDS-3 (Figs. 6.12 and 6.13). Interestingly, EDTA markedly enhanced the 28kDa band and revealed a faint band of activity at 21kDa (Figs. 6.12 and 6.13). The modified gel electrophoresis data supported the results of the general protease activity assay, indicating that protease activity of HDS-1 was mainly due to 28kDa serine protease, and 85 and 160kDa metalloproteases.

Fig. 6.12: Modified SDS-PAGE electrophoresis of 10 x concentrated samples of HDS-1 and HDS-3 with and without pre- and co-incubation with pefabloc (HDS-1/P; HDS-3/P) and EDTA (HDS-1/E; HDS-3/E).

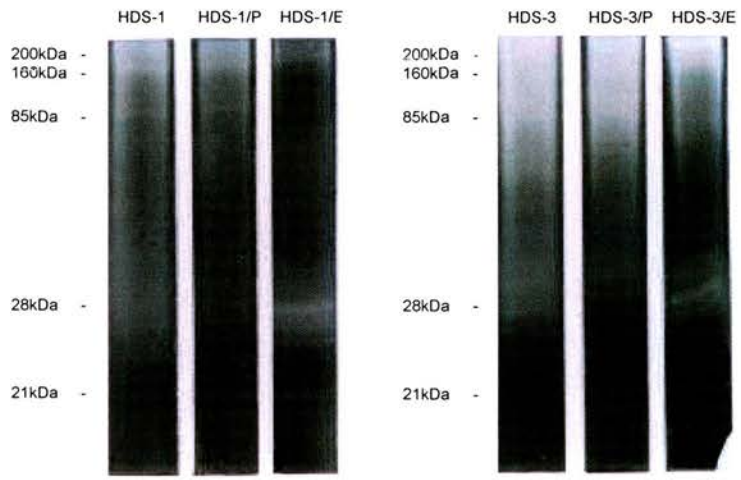
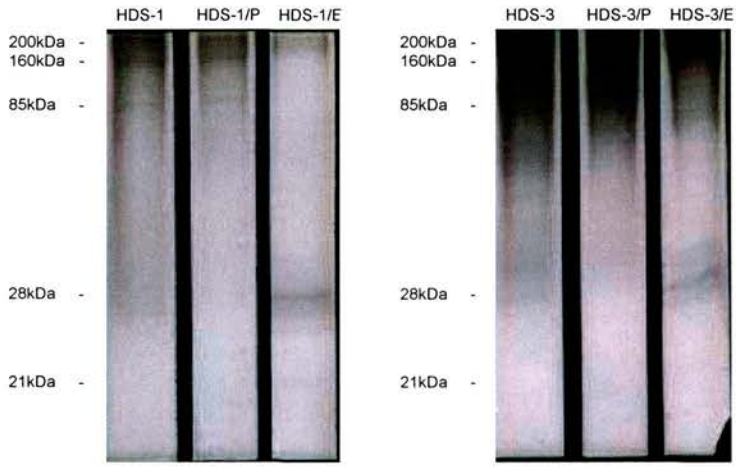


Fig. 6.13: Negative image of modified SDS-PAGE electrophoresis of 10 x concentrated samples of HDS-1 and HDS-3 with and without pre- and co-incubation with pefabloc (HDS-1/P; HDS-3/P) and EDTA (HDS-1/E; HDS-3/E).



6.4.2 Efficiency of nebulisation of HDS particulates

Following nebulisation of 1ml HDS, the final volume in the impinger was 5.9ml (original volume 5ml). Following comparison of the total number of particulates in the impinger with those in the 1ml of nebulised HDS, it was calculated that 26% of particulates in the 0-40μm^3 range, and 8% of particulates in the 40-300μm^3 range, were successfully aerosolised.

6.4.3 Endotoxin and β-D-glucan content, and protease activity of HDS fractions

6.4.3.1 Endotoxin and β-D-glucan content

The endotoxin and β-D-glucan concentrations of the 3 HDS fractions (SUP, WP and WF) are summarised in Table 6.2. The endotoxin concentration of SUP and HDS were comparable, and markedly greater than that of WP and WF. The glucan concentration of WP and HDS were comparable and markedly greater than that of SUP and WF.

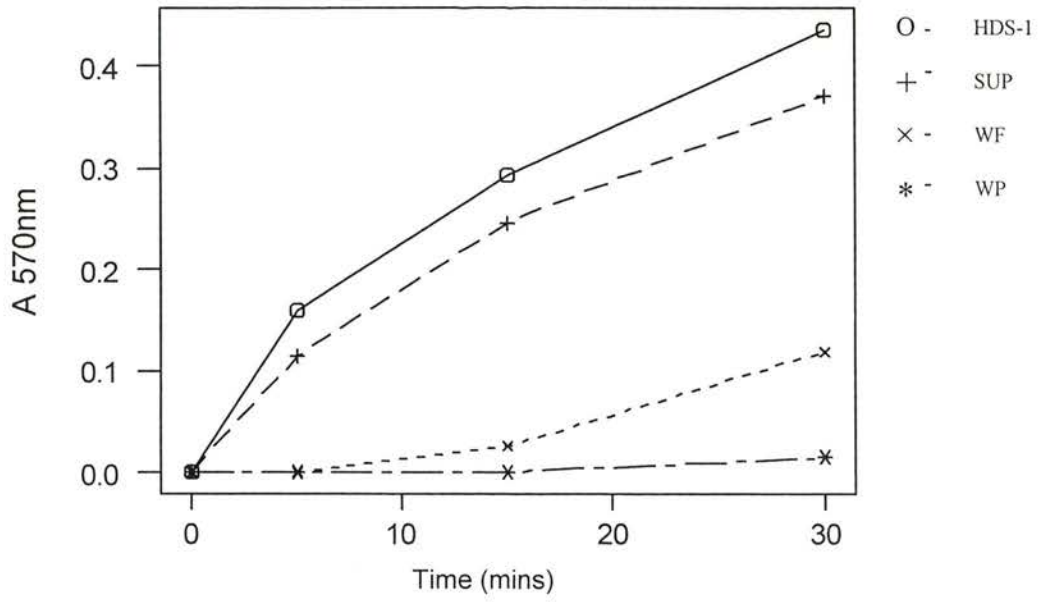
Table 6.2: Endotoxin and β-D-glucan concentrations of HDS, SUP, WP and WF.

	HDS	SUP	WP	WF
Endotoxin concentration (μg/ml)	21.6	17.9	0.8	2.3
β-D-glucan concentration (μg/ml)	183.7	3.0	166.8	1.4

6.4.3.2 Protease activity of HDS fractions

The protease activities of the HDS fractions (SUP, WP and WF) are summarised in Fig. 6.12. SUP contained most of the general protease activity of the original HDS.

Fig. 6.12: Casein-resorufin hydrolysis (A570nm) by HDS-1, SUP, WP and WF plotted against incubation time (min).



6.5 Discussion

This chapter describes the production and characterisation of saline suspensions of hay dust (HDS), which contain both soluble and particulate dust components, and which could be used as a tool for the diagnosis and investigation of heaves. There are apparently no previous reports of organic dust suspensions being used for this purpose in any species.

When preparing the HDS, a dual-cyclone vacuum was specifically used to collect large quantities of dust containing a high proportion of respirable dust particles. This was considered important since respirable particles are more likely to deposit in the

lower airways and induce pulmonary disease (Jacobs, 1997b). In addition, the collection of large dust particles would have resulted in an appreciable overrepresentation of certain soluble dust components in the final suspensions following filtration. Although it is possible to collect particles *entirely* within the respirable range (<5μm), such a practice would not have been feasible on such a large scale. Despite this, the method used in this report to ensure the collection of very fine dust in the final yield did employ the principle of separating particles according to their inertial properties under centrifugal forces. This principle is well recognised and is utilised by cyclone personal air samplers, which are in common use as a preclassifier for sampling respirable dust fractions, as previously described (2.3.5).

Microscopic examination of the resultant HDS indicated that the majority of particles were mould spores, as described by Clarke and Madelin (1987), with the two most abundant spore types being approximately 2 and 4 μm in diameter. An electronic counter employing the impedence principle was used to further evaluate the concentration and size range of the particles. Although this equipment is designed for haematological analysis, it proved accurate in determining the particulate concentration when compared with a haemocytometer count. In addition, the electronic counter provided particle size frequency distributions, indicating the presence of 2 peaks of particle volumes, which corresponded with the diameters of the two most abundant spore sizes as determined microscopically, assuming that spores were spherical. Given that these spores were predominantly in the 2-4μm diameter size range, they were likely to be respirable (Clarke, 1987a).

The HDS glucan concentration (an indicator of total fungal content [Douwes *et al.,* 1998; Dillon *et al.,* 1999; Douwes *et al.,* 1999]), was approximately 3-fold greater in HDS-3 than in HDS-1. While this likely reflected the higher concentration of mould spores in HDS-3, this difference may alternatively reflect a difference in mould species, since different fungi may contain varying amounts of glucan (Fogelmark and Rylander, 1997). Previous mould inhalation studies have supported a role for inhaled moulds in heaves (McPherson *et al.,* 1979; Derksen *et al.,* 1988; McGorum *et al.,* 1993c). All three HDS preparations contained similar endotoxin concentrations, however it is possible that the levels are higher than the determined values since the *Limulus* amoebocyte lysate assay employed mainly detects soluble endotoxin and underestimates particulate endotoxin (Rylander *et al.,* 1989). Interestingly, the endotoxin concentrations detected exceeded those of soluble grain dust extracts (Schwartz *et al.,* 1994; Jagielo *et al.,* 1996b), which were also produced by mixing 1g dust per ml of diluent. The high endotoxin content of HDS is consistent with reports that equine stable dust contains relatively high endotoxin concentrations (Dutkiewicz *et al.,* 1994; McGorum *et al.,* 1998), and supports the need for further investigation into the contribution of inhaled endotoxin in the aetiopathogenesis of heaves.

The detection of proteases in HDS-1 and HDS-3 is of interest, since inhaled proteases can induce respiratory epithelial damage, inflammatory cell recruitment and mucus hypersecretion, ultimately resulting in airway inflammation and bronchoconstriction (Suzuki *et al.,* 1996). The source of the proteases in the HDS is unclear, however possibilities include fungi (Chow *et al.,* 2000), pollens (Tomee *et al.,* 1998; Widmer *et al.,* 2000), bacteria (Milanowski *et al.,* 1995b) and mites (Stewart *et al.*, 1998). Of

particular interest is the detection of serine proteases in HDS-1 and HDS-3, since *Aspergillus fumigatus* is a common component of dust from mouldy hay (Clarke and Madelin, 1987) and serine proteases of *Aspergillus fumigatus* origin can degrade pulmonary epithelium (Iadarola *et al.,* 1998). Furthermore, the serine proteases trypsin and chymotrypsin contribute to the allergenicity of house dust mites (Stewart *et al.,* 1991; Stewart *et al.,* 1994), an important cause of human allergic asthma (PlattsMills *et al.,* 1997). Storage mites, which were identified microscopically in the collected hay dust, also produce a similar protease profile (Stewart *et al.,* 1998), and thus may be a source of proteases in the HDS. While the importance of inhaled storage mite products in equine pulmonary inflammation is unclear (Robinson *et al.,* 1996), they have been implicated in acute airway obstruction in farmers exposed to organic dusts (Vanhagehamsten and Johansson, 1998).

As expected, the soluble components of HDS were more effectively aerosolised by the jet nebuliser than the particulates. However, as 26% and 8% of particles in the 0-40μm^3 and 40-300μm^3 size range, respectively, were aerosolised, this means that HDS can be used to investigate the potential important role of particulates in heaves. The importance of inhaled particulates was highlighted by Kurup et al (1997), who demonstrated enhancement of the murine pulmonary inflammatory response to inhaled soluble *Aspergillus fumigatus* by co-exposure with inert polystyrene beads, resulting in a magnitude of response similar to that following exposure to whole *Aspergillus fumigatus* spores.

In conclusion, the production and analysis of HDS provides a potentially useful standardised and characterised tool for the diagnosis and investigation of heaves. As both soluble and particulate components of HDS may be successfully nebulised, this enables assessment of the role of stable dust particulates in heaves.

CHAPTER 7: RESPONSE TO INHALED HAY DUST SUSPENSION IN ASYMPTOMATIC HEAVES HORSES AND CONTROLS

7.1 Summary

To evaluate inhaled hay dust suspensions (HDS) as a tool for the diagnosis and investigation of heaves, the pulmonary inflammatory and functional consequences of inhalation challenge with 3 different HDS were determined in 6 control and 7 asymptomatic heaves horses. Heaves horses given HDS challenge developed the characteristic features of heaves, including airway neutrophilia, obstructive airway dysfunction and airway mucus hyper-secretion. While HDS challenge induced mild airway neutrophilia in controls, the no-response threshold for controls was greater than that of heaves horses, and there was no overlap in BALF neutrophil counts of controls and heaves horses post challenge. Furthermore, HDS challenge did not induce pulmonary dysfunction or mucus hyper-secretion in controls. Thus HDS challenges enabled differentiation of control and heaves horses. Interestingly, in both groups, the airway neutrophilia was a dose dependent, rather than an "all or nothing", response. This study suggests that inhalation challenge with HDS is of value in the diagnosis and investigation of heaves.

7.2 Introduction

The traditional method of inducing heaves, for diagnostic and research purposes, involves housing horses in a poorly ventilated stable containing mouldy hay or straw (McGorum *et al.,* 1993c). However there is no standardised challenge protocol, and

the variable and undefined composition of airborne dust in this challenge system (Clarke, 1993) and differences in ventilation rates can result in poor reproducibility of the responses to such challenges (Chapter 3). In order to develop a more standardised, repeatable and defined challenge system, hay dust suspensions (HDS) were produced and characterised (Chapter 6), with a view to their use as a model of disease induction in susceptible horses. If successful, such a model would serve to improve the diagnosis of heaves. Additionally, a HDS-induced model would also broaden research potential by permitting selective manipulation of its constituents, thus allowing some assessment of their individual contribution to the clinicopathological features of heaves, a process which is not possible using the current methods of disease induction. This chapter describes the pulmonary inflammatory and functional response of control and heaves horses to inhalation challenge with 3 different HDS.

7.3 Materials and methods

7.3.1 Subjects

Six previously described healthy, control horses with no detectable respiratory tract disorders (2.3.1.2) and 7 previously described horses with a history and clinical diagnosis of heaves (2.3.1.1) were used, as previously described. Throughout the study all horses were kept in a low dust environment, as previously described (2.3.2.1).

7.3.2 Inhalation challenge material

The origin, production and characterisation of the 3 HDS (HDS-1, HDS-2 and HDS-3) have been previously described (6.3.1 and 6.3.2).

7.3.2.1 Dose-response relationship to HDS-1 challenge

To determine the dose-response relationship, four doses of HDS-1 were used in the inhalation challenges, namely; HDS-1 [31], HDS-1 [57], HDS-1 [100] and HDS-1 [316] (Table 7.1). The number in parenthesis relates to the original weight (mg) of dust used to produce 1ml of HDS prior to filtration through nylon gauze (60μm pore size) (6.3.1.2). As the stock solution was HDS-1 [100], the HDS-1 [31] and HDS-1 [57] doses were prepared by appropriate dilution with saline immediately prior to inhalation challenge, thus ensuring that the volume of the final challenge substance was constant. HDS-1 [316] equated to 3.16 x volume of HDS-1 [100] challenge.

7.3.2.2 Effect of challenge with HDS from different dust sources

To investigate whether the response to HDS inhalation was dependent on the batch of hay dust used, the response to inhalation challenge with HDS-2 [100] and HDS-3 [100] was also determined.

7.3.3 HDS inhalation challenges

7.3.3.1 Inhalation challenge protocol

The challenges given to each group are summarised in Table 7.1. Each group received 3 separate dose of HDS-1. The heaves group received inhalation challenge with HDS-1 [31], HDS-1 [57] and HDS-1 [100]. The control group received inhalation challenge with HDS-1 [57], HDS-1 [100] and HDS-1 [316]. Both groups also received inhalation challenge with HDS-2 [100], and the heaves group also received inhalation challenge with HDS-3 [100].

For all challenges, 1ml of challenge substance was delivered to the facemask, except for HDS-1 [316], when 3.16ml was used (7.3.2.1). To facilitate subject cooperation, horses were intravenously sedated with 20μg/kg romifidine and 10μg/kg butorphanol immediately prior to each inhalation challenge. The aerosol was generated and delivered as previously described (2.3.4.1). The challenges were not randomised, with the exception of the HDS-1 [31] and HDS-1 [57] challenges in the heaves group. The order in which the challenges were given is summarised in Table 7.1.

To minimise potential carry-over effects of a preceding challenge on subsequent challenges, inhalation challenges were conducted a minimum of 14 days apart and all horses were shown to have normal clinical findings immediately prior to each challenge. In order to assess any carry-over effects, all measured baseline lung function and arterial blood gases and pH values were compared. In addition, following completion of the other challenges, heaves horses received a repeat

inhalation challenge of HDS-2 [100] (termed HDS-2R [100]) which, as well as confirming the absence of any carry over effects, allowed an assessment of repeatability of the response to challenge.

Table 7.1: Summary of the various HDS challenges given to heaves (n=7) and control (n=6) horses, including in brackets the order of challenges.

	HDS-1 [31]	HDS-1 [57]	HDS-1 [100]	HDS-1 [316]	HDS-2 [100]	HDS-2R [100]	HDS-3 [100]
CONTROLS	✗	✓ (2)	✓ (1)	✓ (4)	✓ (3)	✗	✗
HEAVES	✓ (2 or 3)	✓ (2 or 3)	✓ (1)	✗	✓ (4)	✓ (6)	✓ (5)

7.3.3.2 Positive (hay/straw exposure) and negative (saline) control challenges

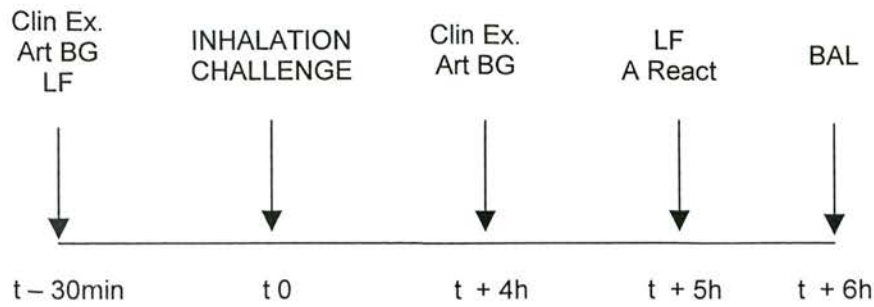
To relate the HDS responses with those of placebo and a conventional hay/straw challenge, comparisons were made with saline inhalation (negative control) and a 5h-duration conventional hay/straw challenge (positive control) in the same horses, as previously described (2.3.4.2).

7.3.4 Monitoring the response to challenges

The timing and method of assessment of response to each challenge is summarised in Fig. 7.1. Responses to the HDS-1 [100], HDS-2 [100] and HDS-3 [100] inhalation challenges as well as the hay/straw (Chapter 2) and saline (Chapter 2) challenges were assessed using clinical scoring, lung mechanics, airway reactivity, blood gases and pH analyses and BALF cytology, as previously described (2.3.7). The quantity of tracheal mucus was also blindly scored following HDS-1 [100] and hay/straw challenges as previously described (2.3.7.4). Responses to all other challenges were assessed solely by clinical scoring and BALF cytology.

Fig. 7.1: Study design.

BAL = bronchoalveolar lavage (all challenges); LF = lung function evaluation (HDS-1 [100], HDS-2 [100]; HDS-2R [100], HDS-3 [100]); Clin. Ex. = clinical examination (all challenges); Art BG = Arterial blood gases and pH analysis (HDS-1 [100], HDS-2 [100]; HDS-2R [100], HDS-3 [100]); A React = airway reactivity evaluation (HDS-1 [100], HDS-2 [100]; HDS-2R [100], HDS-3 [100]).



7.4 Statistical analysis

As the data were either not normally distributed and/or the groups of compared data did not have equal variance, non-parametric tests were used. The effects of each challenge were determined mostly by performing within-group analyses.

To check for the presence of any carry-over effects of one challenge on a subsequent challenge, pre-challenge (baseline) measurements of arterial blood gases and pH and lung mechanics were compared using a Friedman test, and when significant, a Wilcoxon Rank Sum test was performed on paired data.

To check for any effects of challenge where pre-challenge measurements were made at t-30min (arterial blood gas and pH analyses and lung mechanics), the post-challenge values were expressed as % of baseline value, except for clinical scores

where actual values were used. As saline was the vehicle for HDS delivery, the effect of HDS challenge was assessed by pairing and subtracting post-HDS (% of baseline value) and post-saline (% of baseline value) data. Where no pre-challenge data was collected (BALF cytology), comparisons were made with saline (placebo) challenge data at equivalent time points. A Friedman test was performed on sets of paired data to reduce the risk of type 1 errors, and when significant, a Wilcoxon Rank Sum test was performed on paired data.

Between group (heaves *vs* controls) analyses were performed for BALF neutrophil numbers, using the Mann Whitney test.

Significance was assumed if $P<0.05$. Following correction for any effect of saline inhalation, changes from baseline are expressed as increase/decrease or change in median values, with 95% confidence interval for the difference in median, calculated for non-parametric data (Campbell and Gardner 1994).

The two separate HDS-2 [100] inhalation challenges were compared using a Wilcoxon Rank Sum test, and as an indication of repeatability, the differences in paired values were plotted against their mean (Bland and Altman 1986). Good repeatability was assumed if the calculated differences in paired values fell within 2 standard deviations of the mean of the differences (British Standards Institution, 1979). Results in tables are expressed as median and range.

7.5 Results

7.5.1 Response to inhalation challenge with 3 separate HDS doses

7.5.1.1 Clinical examination

All horses had a clinical score of zero prior to all challenges, and when compared with baseline values, no significant increase in clinical scores was detected in either group following any of the challenges (Appendix 7.1).

7.5.1.2 Tracheal secretion score

Only the heaves group had significantly ($P<0.05$) increased tracheal secretion scores after HDS-1 [100] (Table 7.2) when compared with saline (2.5.2.4). This response was comparable to the response to hay/straw challenges (2.5.2.4).

Table 7.2 Tracheal secretion scores (median and range) in heaves (n=7) and control (n=6) horses, 6h following inhalation challenge with HDS-1 [100] and hay/straw challenge.

	SALINE	HDS-1 [100]	HAY/STRAW
CONTROLS	0 (0-0)	0 (0-0)	0 (0-0)
HEAVES	0 (0-0)	2 (1-4)	2 (1-3)

7.5.1.4 BALF cytology

The BALF neutrophil counts and ratios following challenge with saline and all HDS-1 doses are fully presented in Appendix 7.2, and summarised in Table 7.3.

Table 7.3: BALF neutrophil counts ($x10^5$/ml) and ratios (%) (median and range) in control (n=6) and heaves (n=7) horses at 6h following inhalation challenge with saline, HDS-1 [31], HDS-1 [57], HDS-1 [100] and HDS-1 [316].
NP = not performed.

	BALF neutrophil count ($x10^5$/ml)		BALF neutrophil ratio (%)	
	CONTROLS	HEAVES	CONTROLS	HEAVES
SALINE	0.06 (0.01-0.17)	0.06 (0.03-0.20)	1.3 (0.2-3.2)	2.3 (0.6-4.5)
HDS-1 [31]	NP	0.14 (0.06-2.30)	NP	3.2 (1.9-30.9)
HDS-1 [57]	0.05 (0.01-0.24)	1.01 (0.43-3.32)	1.3 (0.3-6.2)	25.2 (5.6-42.3)
HDS-1 [100]	0.28 (0.08-0.67)	2.17 (0.54-3.81)	6.3 (2.8-13.2)	50.7 (19.4-70.4)
HDS-1 [316]	1.72 (0.90-4.28)	NP	40.7 (34.6-71.0)	NP

When compared with saline, absolute BALF neutrophil counts and ratios were significantly ($P<0.05$) increased in heaves horses following inhalation challenge with HDS-1 [57] and HDS-1 [100] and in controls following inhalation challenge with HDS-1 [100] and HDS-1 [316] (Table 7.3). The BALF neutrophilia induced by HDS-1 in both groups was dose-dependent (Figs. 7.2 to 7.5). When compared with saline inhalation, the BALF neutrophilia following HDS-1 [100] inhalation challenge was significantly greater ($P<0.01$) in heaves horses (34-fold increase in median count) than controls (5-fold increase in median count) (Figs. 7.6 and 7.7). There was no overlap in BALF neutrophil ratios between the 2 groups following HDS-1 [100] challenge (Fig. 7.7).

Fig. 7.2: BALF neutrophil counts (x10^5/ml) in heaves (n=7) horses at 6h following inhalation challenge with saline, HDS-1 [31], HDS-1 [57], HDS-1 [100] and mouldy hay/straw challenge (H/S). NP = not performed.

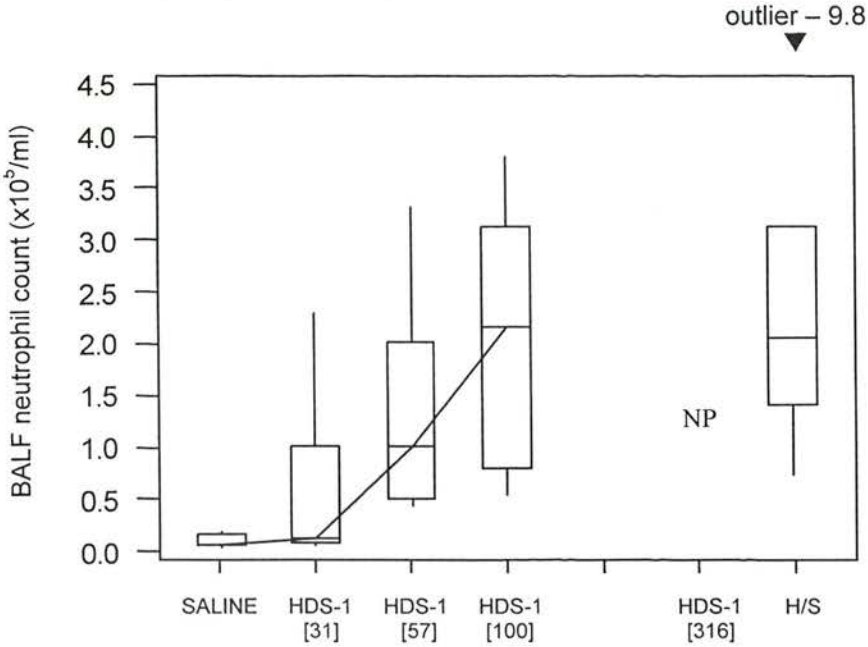


Fig. 7.3: BALF neutrophil counts (x10^5/ml) in control (n=6) horses at 6h following inhalation challenge with saline, HDS-1 [57], HDS-1 [100], HDS-1 [316] and mouldy hay/straw challenge (H/S). * = outlier. NP = not performed.

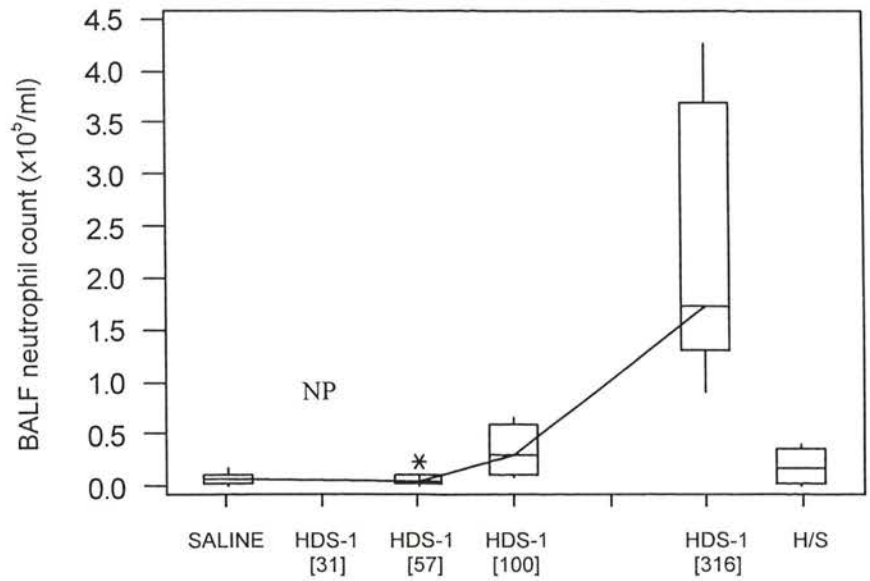


Fig. 7.4: BALF neutrophil ratio (%) in heaves (n=7) horses at 6h following inhalation challenge with saline, HDS-1 [31], HDS-1 [57], HDS-1 [100] and mouldy hay/straw challenge (H/S). NP = not performed.

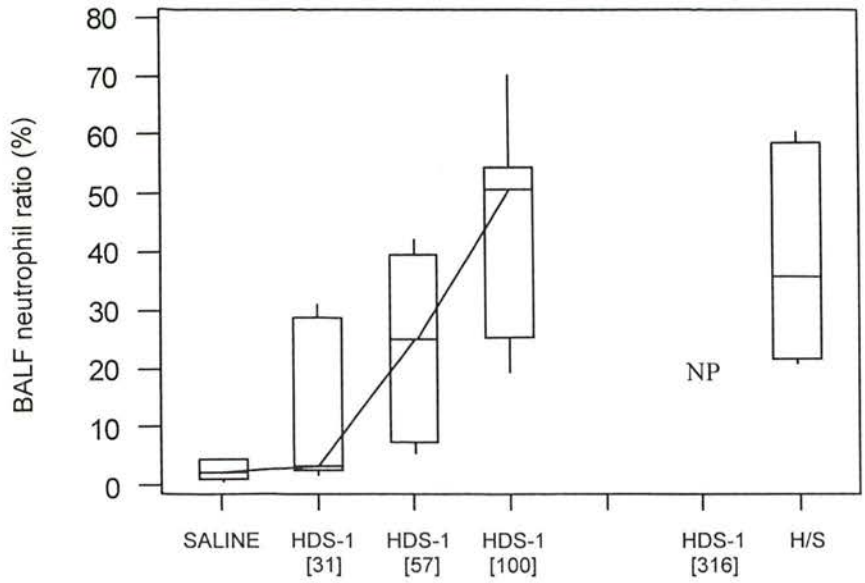


Fig. 7.5: BALF neutrophil ratio (%) in control (n=6) horses at 6h following inhalation challenge with saline, HDS-1 [57], HDS-1 [100], HDS-1 [316] and mouldy hay/straw challenge (H/S). NP = not performed.

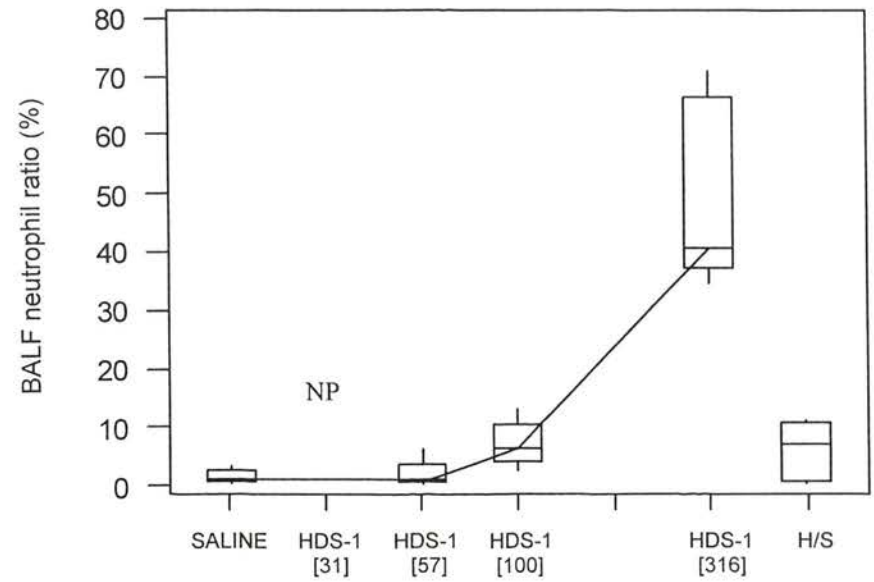


Fig. 7.6: BALF neutrophil counts (x10^5/ml) in heaves (n=7) and control (n=6) horses at 6h following inhalation challenge with HDS-1 [100].

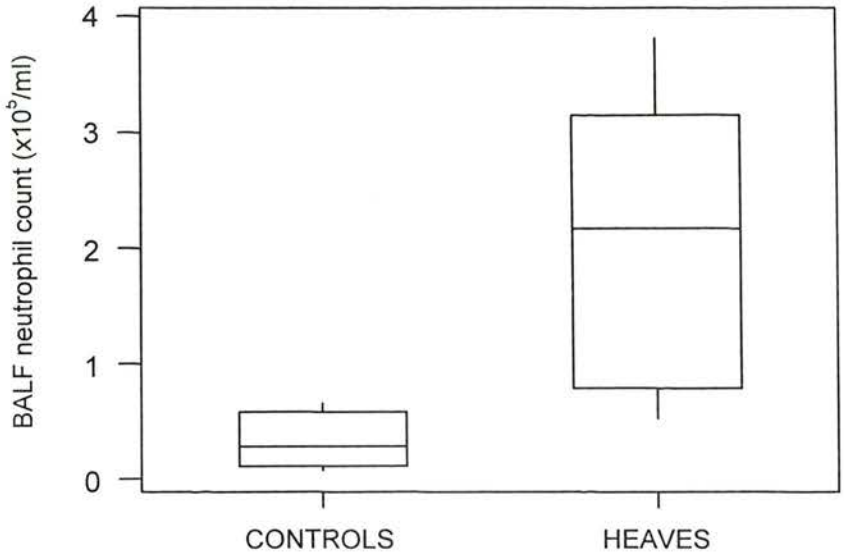
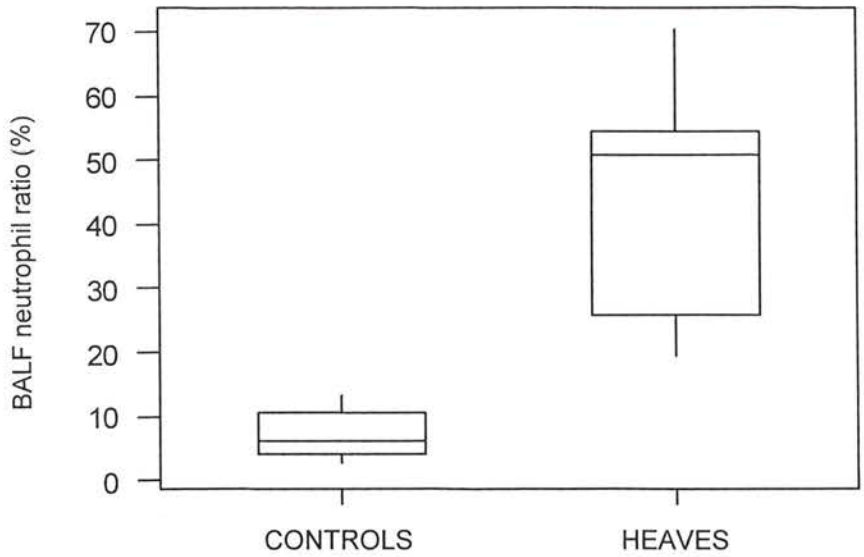


Fig. 7.7: BALF neutrophil ratio (%) in heaves (n=7) and control (n=6) horses at 6h following inhalation challenge with HDS-1 [100].



Due to the influence of a BALF neutrophilia on the ratio of other BALF cell types, the latter were considered only as absolute numbers. The absolute numbers of other BALF cell types were not significantly affected by any of the challenges and are summarised in Table 7.4.

Table 7.4: Total and differential BALF cell counts ($x10^5$/ml) (median and range) in heaves (H; n=7) and control (C; n=6) horses at 6h following inhalation challenge with saline, HDS-1 [31], HDS-1 [57], HDS-1 [100] and HDS-1 [316].
NP = not performed, TCC = total cell count.

		TCC	Lymphocytes	Macrophages	Mast cells	Basophiloid cells	Eosinophils
SALINE	C	3.85 (2.00-9.60)	1.59 (0.99-6.22)	1.98 (0.73-2.80)	0.25 (0.06-0.39)	0.02 (0.00-0.22)	0.03 (0.00-0.06)
	H	3.80 (1.30-5.60)	2.09 (0.80-3.33)	1.58 (0.34-2.96)	0.13 (0.09-0.19)	0.01 (0.00-0.11)	0.01 (0.00-0.01)
HDS-1 [31]	C	NP	NP	NP	NP	NP	NP
	H	3.40 (3.00-8.00)	1.80 (1.03-2.52)	1.63 (0.90-3.06)	0.07 (0.04-0.16)	0.01 (0.00-0.02)	0.00 (0.00-0.10)
HDS-1 [57]	C	3.40 (1.80-4.10)	1.10 (0.61-2.06)	1.78 (0.77-2.55)	0.14 (0.05-0.19)	0.01 (0.00-0.03)	0.01 (0.00-0.29)
	H	5.10 (2.00-8.80)	2.12 (0.95-4.23)	1.68 (0.33-2.80)	0.08 (0.02-0.30)	0.01 (0.00-0.02)	0.02 (0.00-0.57)
HDS-1 [100]	C	4.65 (1.60-8.00)	1.49 (0.66-2.29)	2.18 (0.51-4.19)	0.14 (0.11-0.24)	0.02 (0.00-1.04)	0.01 (0.00-0.23)
	H	4.10 2.10-7.00)	1.19 (0..61-1.86)	1.16 (0.41-1.75)	0.07 (0.02-0.14)	0.00 (0.00-0.10)	0.01 (0.00-0.13)
HDS-1 [316]	C	4.40 (2.20-6.90)	1.32 (0.62-1.79)	0.76 (0.37-1.43)	0.08 (0.03-0.18)	0.00 (0.00-0.24)	0.00 (0.00-0.14)
	H	NP	NP	NP	NP	NP	NP

7.5.2 Response to inhalation with HDS from separate dust sources

7.5.2.1 Clinical examination

All horses had a clinical score of zero prior to all challenges, and when compared with baseline values, no significant increase in clinical scores was detected in either group following any of the challenges (Appendix 7.3).

7.5.2.2 Arterial blood gases and pH analyses

Arterial blood gas measurements raw data are presented in Appendix 7.4. There was no significant difference in the baseline blood gas indices prior to each of the challenges, indicating a lack of any carry-over effects. Following correction for saline inhalation, none of the challenges significantly altered arterial PaO_2, $PaCO_2$ or pH when compared with baseline values in either group.

7.5.2.3 Lung mechanics and airway reactivity

Raw data for lung mechanics measurements are presented in Appendix 7.5. With the exception of respiratory rate in both groups, there was no significant difference in the baseline lung function measurements prior to each of the challenges, indicating a lack of any carry-over effects. The percent of baseline lung function measurements following challenge is presented in Table 7.5. PCCdyn70 values following challenge are presented in Table 7.6.

Heaves group: Following correction for any effects of saline inhalation, HDS-1 [100] inhalation significantly increased $RL_{E50\%}$ (median increase 39%, 95% confidence

interval 4-75; $P<0.05$) when compared with baseline (Fig. 7.8). Following correction for any effects of saline inhalation, HDS-2 [100] inhalation significantly increased $RL_{E50\%}$ (increase in median 88%, 95% CI 25-303; $P<0.05$) (Fig. 7.8) and $RL_{E75\%}$ (increase in median 58%, 95% CI 49-347; $P<0.05$) (Fig. 7.9) when compared with baseline. Following correction for any effects of saline inhalation, HDS-3 [100] inhalation increased $RL_{E50\%}$ (increase in median 119%, 95% CI 21-355; $P<0.05$) (Fig. 7.8), $RL_{E75\%}$ (increase in median 150%, 95% CI 48-252; $P<0.05$) (Fig. 7.9) and Rliso (increase in median 93%, 95% CI 17-189; $P<0.05$) (Fig. 7.10) when compared with baseline. HDS-3 [100] induced a significantly ($P<0.05$) greater increase in Rliso than HDS-1 [100] (Fig. 7.10). Hay/straw challenge had no significant effect on lung function (Chapter 2).

Control group: Lung mechanics of controls were unaffected by HDS-1 [100] and HDS-2 [100] challenges. Hay/straw challenge had no significant effect on lung function in controls (Chapter 2).

Only HDS-2R [100] challenge in the heaves group significantly increased airway reactivity, as indicated by a reduced PCCdyn70 when compared with saline (3.0mg/ml, 0.4-4.1 vs 5.6mg/ml, 2.5-10.5; $P<0.05$) (Table 7.6). Airway reactivity did not significantly differ between HDS-2 [100] (8.0mg/ml, 1.4-32.8) and HDS-2R [100] (3.0mg/ml, 0.4-4.1) challenges. Although all the calculated differences in these paired values for airway reactivity fell within 2 standard deviations of the mean of the differences, indicative of good agreement, the mean of the differences was sufficiently different from zero to render the assessment of repeatability invalid.

Table 7.5(a): Percent (%) of baseline lung function measurements (median and range) in heaves (n=7) and control (n=6) horses at 5h following inhalation challenge with saline, HDS-1[100], HDS-2 [100], HDS-2R [100] and HDS-3 [100].
NP = not performed, C = control group, H = heaves group.

		Cdyn	dPpl	RL_{iso}	RR	VT	Wb	$RL_{E25\%}$	$RL_{E50\%}$	$RL_{E75\%}$	$RL_{I25\%}$	$RL_{I50\%}$	$RL_{I75\%}$
SALINE	C	68.4 (47.1-157.7)	134.3 (104.2-162.2)	137.0 (85.3-180.3)	91.5 (54.4-106.5)	110.3 (97.7-165.4)	127.9 (78.9-1345.7)	104.2 (26.9-2441.7)	105.9 (43.8-1689.6)	164.0 (132.0-217.6)	148.6 (115.9-190.5)	125.3 (102.8-152.9)	120.1 (106.2-141.9)
	H	96.4 (57.9-104.9)	111.6 (60.0-260.6)	95.9 (76.9-197.3	90.1 (48.6-110.6)	113.2 (67.2-200.7)	70.8 (35.8-172.0)	66.1 (0.00-125.3)	69.3 (42.7-115.0)	129.4 (77.0-593.2)	102.5 (84.9-590.5)	89.4 (70.5-446.9)	99.2 (29.0-232.9)
HDS-1 [100]	C	92.7 (53.2-138.8)	140.8 (100.5-157.6)	110.9 (70.5-170.2)	93.8 (59.8-102.4)	120.6 (90.0-152.6)	136.3 (30.9-212.5)	155.0 (7.1-279.7)	103.9 (38.0-182.5)	140.2 (111.0-275.8)	136.2 (107.3-257.4)	116.5 (79.8-157.0)	118.3 (103.9-140.1)
	H	105.4 (70.1-139.8)	123.0 (97.0-172.6)	118.2 (85.8-193.2)	87.6 (66.8-110.6)	111.0 (98.0-159.9)	108.9 (8.2-274.1)	120.2 20.8-148.7)	132.9 (75.0-175.2)	128.0 (69.7-180.8)	126.4 (83.2-181.2)	142.5 (70.1-159.4)	115.3 (79.3-196.1)
HDS-2 [100]	C	93.3 (53.5-137.8)	113.3 (84.2-148.3)	141.3 (77.7-250.0)	73.7 (61.7-111.2)	112.4 (94.9-141.8)	193.6 (75.5-336.6)		143.3 (55.0-705.3)	138.5 (71.0-235.8)	135.4 (50.6-243.2)	112.3 (84.4-181.5)	97.1 (89.2-163.1)
	H	74.4 (27.5-97.0)	123.9 (95.9-432.9)	186.9 (89.3-565.0)	98.3 (67.8-133.2)	94.5 (79.4-129.2)	146.0 (102.2-1883.3)	118.5 (94.0-550.0)	123.2 (102.6-445.5)	184.9 (72.7-533.9)	148.0 (71.1-608.6)	116.7 (77.0-463.4)	112.2 (93.7-705.3)
HDS-2R [100]	C	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP
	H	76.0 (18.9-135.0)	116.8 (95.3-375.5)	122.1 (83.6-406.2)	95.2 (74.1-113.8)	101.8 (69.1-137.5)	152.0 (70.1-676.7)		145.7 (109.9-356.3)	134.3 (87.2-448.9)	118.0 (80.2-416.7)	134.7 (87.6-333.3)	130.8 (57.2-430.0)
HDS-3 [100]	C	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP
	H	73.7 (25.0-110.4)	142.9 (96.9-306.8)	190.0 (112.2-365.7)	113.6 (62.3-173.1)	79.2 (70.3-129.4)	182.9 (126.0-262.4)	173.0 (88.4-515.0)	173.0 (125.0-361.7)	153.7 (100.0-351.2)	188.4 (104.5-404.7)	158.6 (122.5-283.8)	156.5 (114.1-389.8)

Table 7.5(b): Percent (%) of baseline lung function measurements (median and range) in heaves (n=7) and control (n=6) horses at 5h following inhalation challenge with saline, HDS-1[100], HDS-2 [100], HDS-2R [100] and HDS-3 [100].
NP = not performed, C = control group, H = heaves group.

		T_E	T_I	T_I:T_E	V'_E	V'_{Emax}	V'_{Imax}	Wb_{el}	Wb_{res}	Wb_{Eres}	Wb_{Ires}	Wb_{Itot}
SALINE	C	105.3 (90.7-175.9)	107.5 (92.0-179.8)	100.3 (40.1-253.8)	100.7 (89.8-103.3)	93.5 (79.5-116.5)	173.5 (119.5-194.8)	137.8 (110.3-204.7)	98.3 (6.7-222.4)	160.5 (134.3-199.4)	174.2 (130.6-183.7)	95.4 (89.1-116.8)
	H	103.4 (83.1-219.9)	111.8 (86.3-263.8)	101.5 (41.3-110.5)	104.7 (43.8-117.9)	116.0 (43.3-137.9)	131.6 (48.3-646.1)	95.3 (48.2-505.7)	86.2 (42.0-439.0)	112.0 (58.5-545.3)	118.1 (51.9-597.2)	101.2 (56.1-140.4)
HDS-1 [100]	C	107.5 (91.4-170.7)	104.1 (92.7-154.3)	89.2 (87.8-117.6)	101.9 (78.2-121.7)	99.8 (79.8-124.7)	155.7 (102.0-260.3)	123.0 (97.7-210.5)	156.5 (19.5-209.4)	154.3 (99.9-211.0)	149.8 (104.9-235.0)	106.0 (88.8-170.0)
	H	122.1 (82.3-217.1)	108.6 (90.0-162.6)	92.8 (37.4-123.8)	95.8 (76.1-139.5)	100.1 (61.6-144.6)	125.9 (100.7-183.6)	137.2 (94.7-230.2)	131.2 (86.6-381.0)	136.0 (92.3-176.7)	138.1 (97.4-180.5)	100.8 (83.2-129.8)
HDS-2 [100]	C	134.9 (79.9-162.8)	133.0 (98.4-171.1)	98.8 (88.3-127.8)	79.5 (68.3-113.2)	93.9 (58.7-123.2)	138.8 (94.7-172.5)	138.6 (93.8-164.6)	171.7 (83.1-1588.7)	115.4 (89.0-162.1)	130.4 (98.8-151.7)	91.4 (66.0-129.9)
	H	106.7 (81.0-146.9)	95.9 (67.3-136.3)	93.4 (62.1-100.4)	88.7 (76.4-118.8)	91.9 (62.6-127.8)	119.0 (80.3-255.9)	123.8 (99.7-528.7)	113.9 (104.3-418.2)	133.9 (94.4-618.9)	112.6 (89.6-499.7)	95.0 (62.6-127.8)
HDS-2R [100]	C	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP
	H	107.2 (87.6-152.1)	96.5 (81.2-119.5)	84.1 (81.7-103.6)	100.0 (62.8-124.3)	94.9 (55.5-111.9)	139.2 (55.4-423.1)	147.1 (65.4-419.6)	158.1 (100.1-384.8)	143.6 (50.2-449.1)	141.5 (53.2-441.7)	110.7 (57.5-119.6)
HDS-3 [100]	C	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP
	H	89.5 (66.1-163.7)	82.1 (52.9-151.4)	93.7 (80.2-107.9)	99.6 (79.6-128.7)	88.1 (58.0-114.3)	132.5 (41.3-377.0)	140.0 (89.5-346.6)	168.3 (91.5-254.4)	142.2 (87.4-440.8)	127.6 (66.5-419.4)	83.8 (72.8-125.2)

Fig. 7.8: Percent of baseline $RL_{E50\%}$ in heaves horses (n=7) at 5h following inhalation challenge with saline, HDS-1[100], HDS-2 [100] and HDS-3 [100] minus percent of baseline $RL_{E50\%}$ at 5h following inhalation challenge with saline. * = outlier.

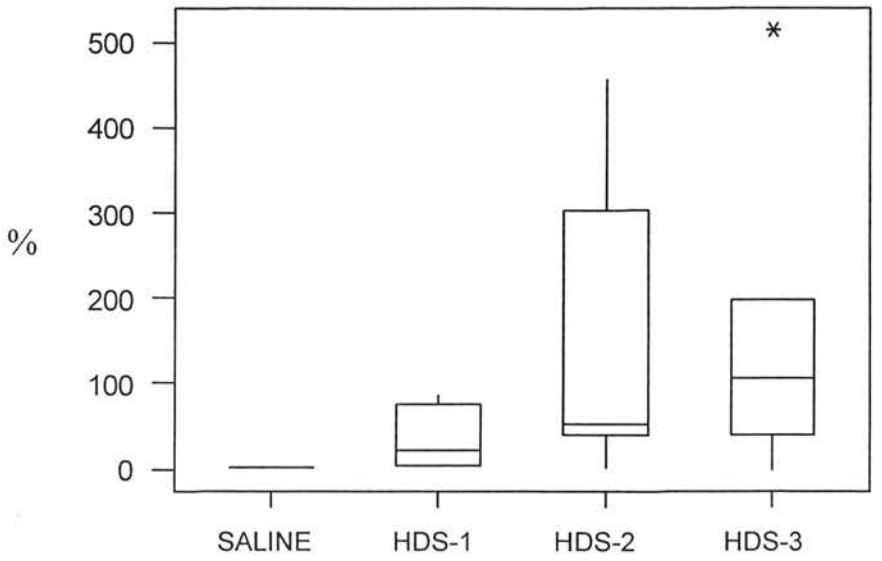


Fig. 7.9: Percent of baseline $RL_{E75\%}$ in heaves horses (n=7) at 5h following inhalation challenge with saline, HDS-1[100], HDS-2 [100] and HDS-3 [100] minus percent of baseline $RL_{E75\%}$ at 5h following inhalation challenge with saline. * = outlier.

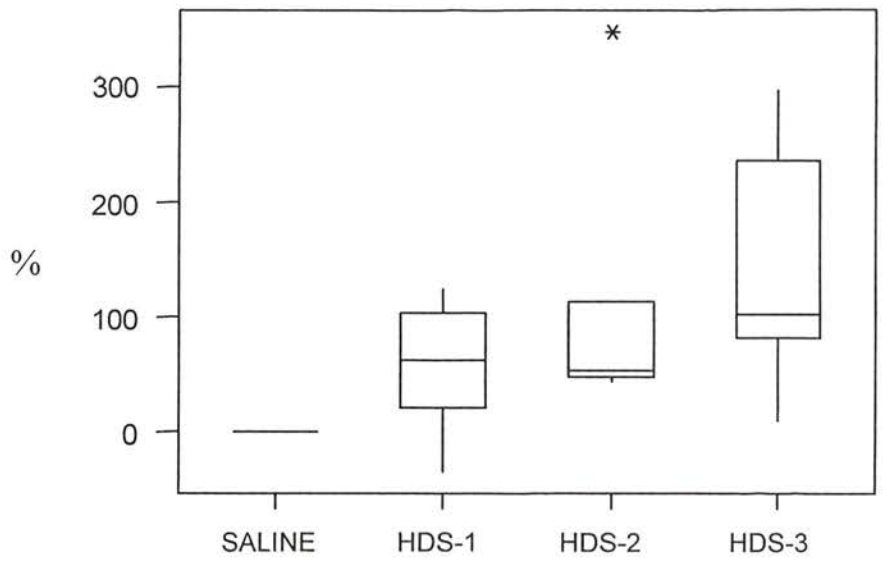


Fig. 7.10: Percent of baseline RL_{ISO} in heaves horses (n=7) at 5h following inhalation challenge with saline, HDS-1[100], HDS-2 [100] and HDS-3 [100] minus percent of baseline RL_{ISO} at 5h following inhalation challenge with saline. * = outlier.

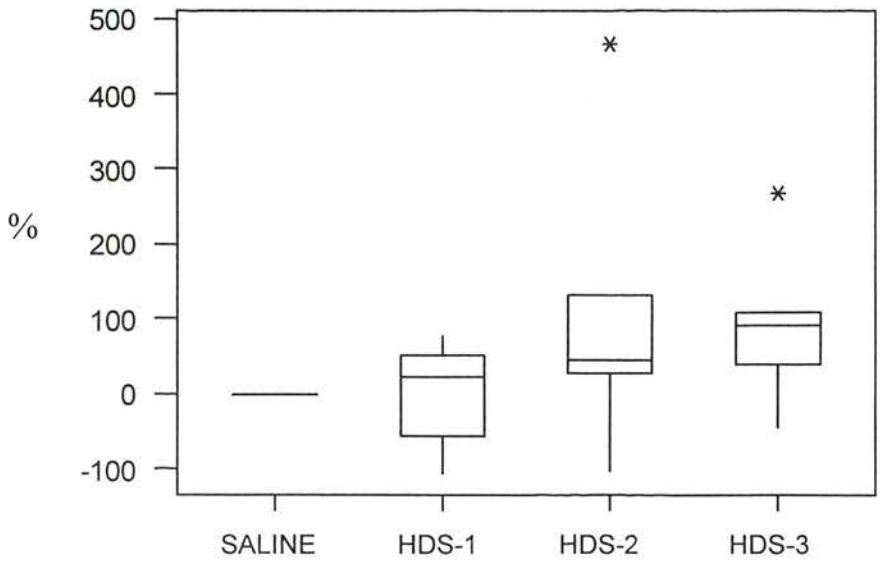


Table 7.6 PCCdyn70 (mg/ml methacholine chloride) (median and range) in control (n=6) and heaves (n=7) horses at approximately 5h following inhalation challenge with saline, HDS-1[100], HDS-2[100], HDS-2R [100] and HDS-3[100]. NP = not performed.

	CONTROLS	HEAVES
SALINE	3.64 (2.29-9.91)	5.64 (2.46-10.53)
HDS-1[100]	4.69 (2.07-7.08)	6.91 (4.26-11.43)
HDS-2	9.02 (1.47-18.22)	7.96 (1.43-32.84)
HDS-2R	NP	2.99 (0.42-4.09)
HDS-3	NP	3.36 (1.22-8.50)

7.5.1.5 BALF cytology

The BALF neutrophil counts and ratios following challenge with saline, HDS-2 [100], HDS-2R [100] and HDS-3 [100] are fully presented in Appendix 7.4, and summarised in Table 7.7.

Table 7.7: BALF neutrophil counts ($x10^5$/ml) and ratios (%) (median and range) in control (n=6) and heaves (n=7) horses at 6h following inhalation challenge with saline, HDS-1[100], HDS-2 [100], HDS-2R [100] and HDS-3 [100].
NP = not performed.

	BALF neutrophil count ($x10^5$/ml)		BALF neutrophil ratio (%)	
	CONTROLS	HEAVES	CONTROLS	HEAVES
SALINE	0.06 (0.01-0.17)	0.06 (0.03-0.20)	1.3 (0.2-3.2)	2.3 (0.6-4.5)
HDS-1[100]	0.28 (0.08-0.67)	2.17 (0.54-3.81)	6.3 (2.8-13.2)	50.7 (19.4-70.4)
HDS-2 [100]	0.16 (0.02-0.41)	4.95 (1.68-6.61)	6.4 (0.7-11.0)	65.7 (39.0-81.9)
HDS-2R [100]	NP	5.73 (1.13-17.29)	NP	68.5 (28.3-83.1)
HDS-3 [100]	NP	8.55 (6.92-16.30)	NP	84.7 (71.3-86.0)

When compared with saline, absolute BALF neutrophil counts and ratios were significantly ($P<0.05$) increased in heaves horses following inhalation challenge with HDS-2 [100], HDS-2R [100] and HDS-3 [100] (Figs. 7.11 and 7.12). When compared with saline, absolute BALF neutrophil counts and ratios were significantly ($P<0.05$) increased in controls following inhalation challenge with HDS-2 [100] (Figs. 7.13 and 7.14). When compared with saline, the BALF neutrophilia following HDS-2 [100] challenge was significantly greater ($P<0.01$) in heaves horses (77-fold increase in median count) than controls (3-fold increase in median count). There was no overlap in BALF neutrophil ratio and count between the groups following HDS-2 [100] challenge (Figs. 7.15 and 7.16).

In comparison to all other inhalation challenges, HDS-3 [100] challenge in the heaves group induced a markedly greater BALF neutrophil count (134-fold increase in median count) and ratio ($P<0.05$) (Figs. 7.11 and 7.12).

Fig. 7.11: BALF neutrophil count (x10^5/ml) in heaves (n=7) horses at 6h following inhalation challenge with saline, HDS-1[100], HDS-2 [100] and HDS-3 [100] and in heaves (n=6) horses at 6h following inhalation challenge with HDS-2R [100]. * = outlier.

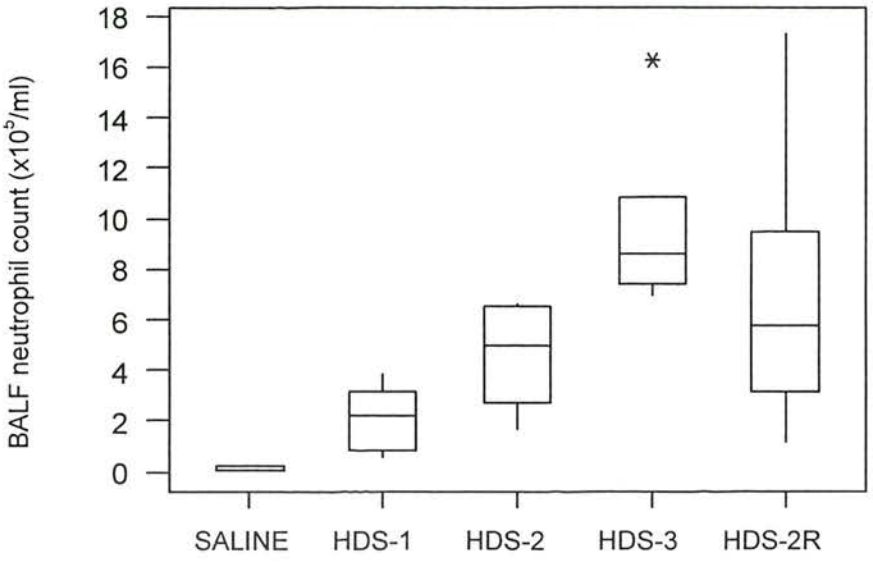


Fig. 7.12: BALF neutrophil ratio (%) in heaves (n=7) horses at 6h following inhalation challenge with saline, HDS-1[100], HDS-2 [100] and HDS-3 [100] and in heaves (n=6) horses at 6h following inhalation challenge with HDS-2R [100]. * = outlier.

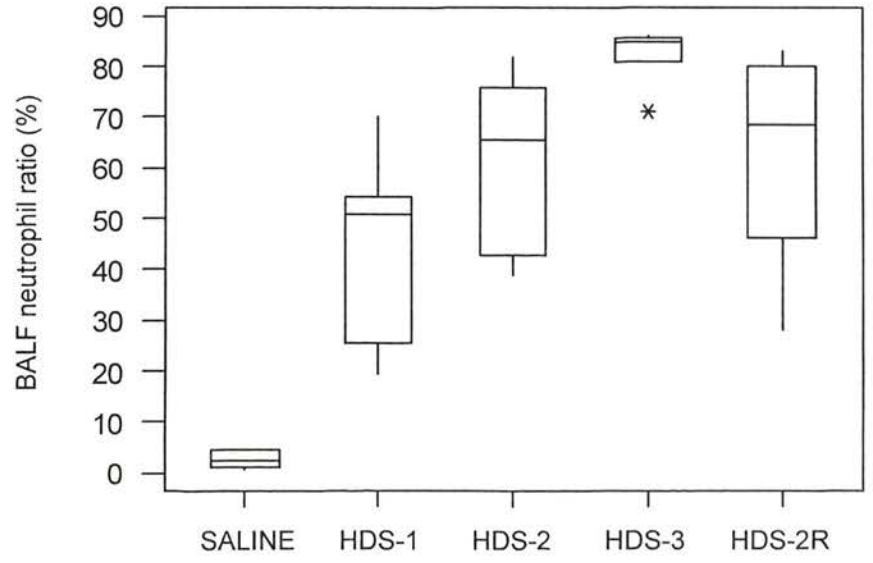


Fig. 7.13: BALF neutrophil count (x10^5/ml) in control (n=6) horses at 6h following inhalation challenge with saline, HDS-1[100] and HDS-2 [100].

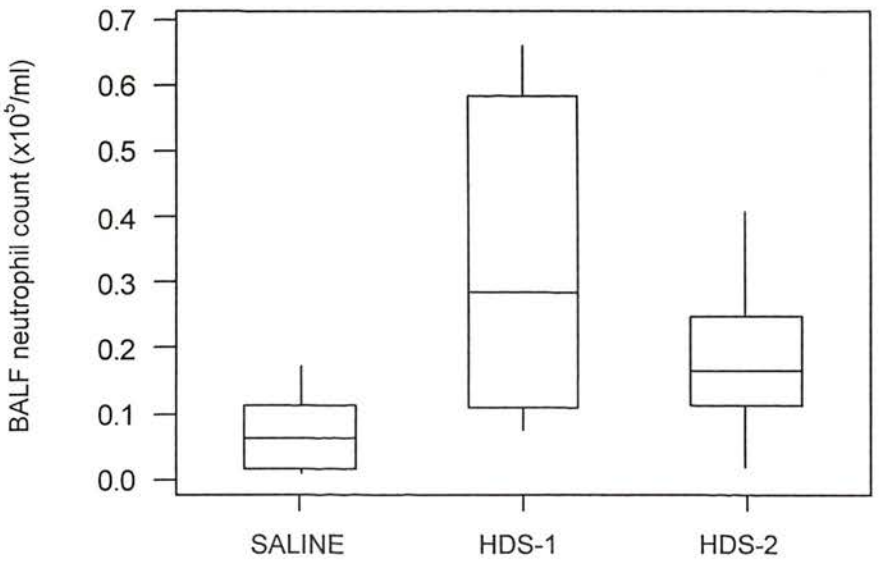


Fig. 7.14: BALF neutrophil ratio (%) in control (n=6) horses at 6h following inhalation challenge with saline, HDS-1[100] and HDS-2 [100].

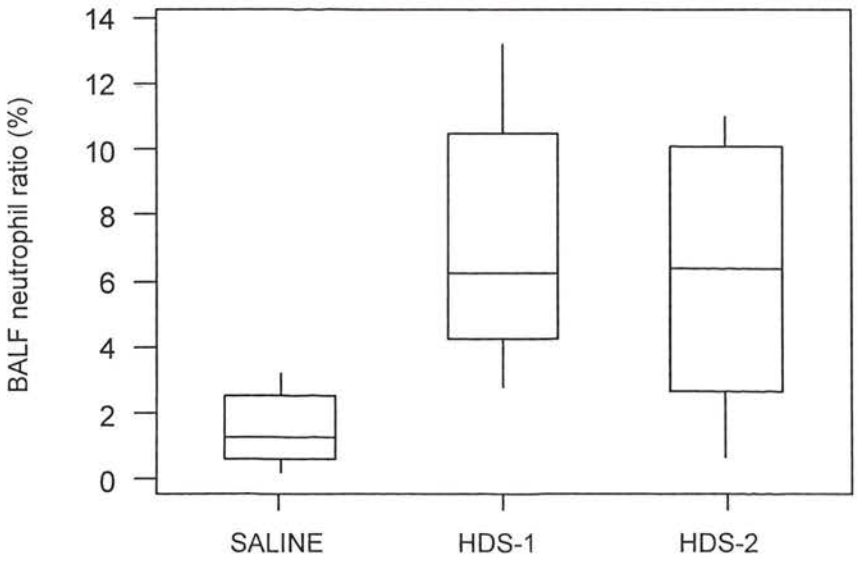


Fig. 7.15: BALF neutrophil count (x10^5/ml) in heaves (n=7) and control (n=6) horses at 6h following inhalation challenge with HDS-2 [100].

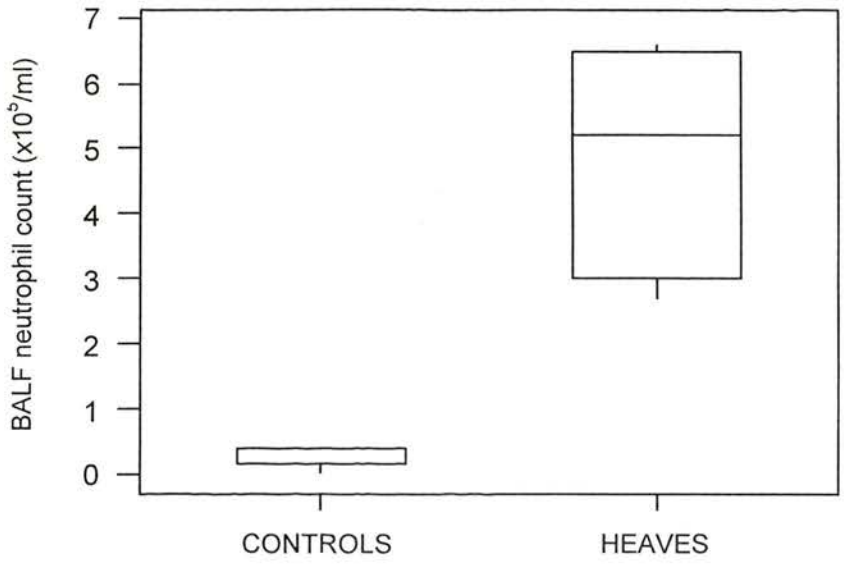
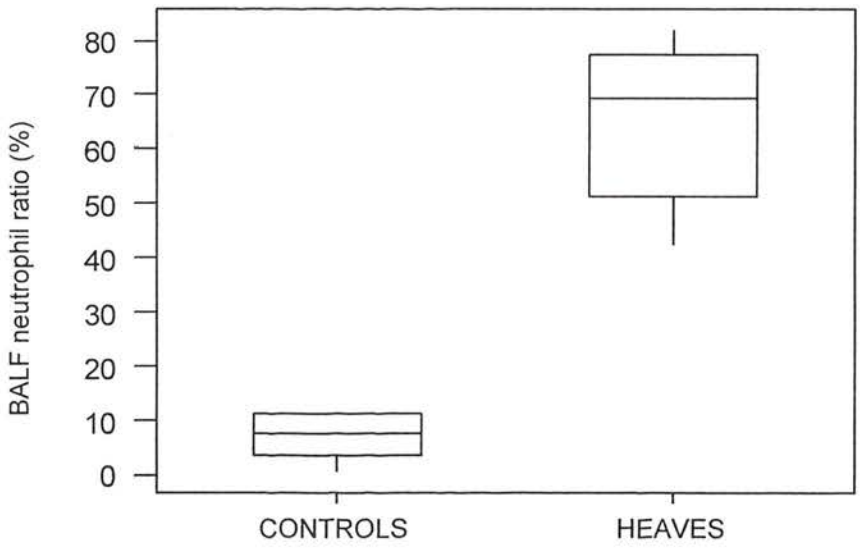


Fig. 7.16: BALF neutrophil ratio (%) in heaves (n=7) and control (n=6) horses at 6h following inhalation challenge with HDS-2 [100].



Due to the influence of a BALF neutrophilia on the ratio of other BALF cell types, the latter were considered only as absolute numbers. The absolute numbers of other BALF cell types are summarised in Table 7.8.

Table 7.8: Total and differential BALF cell counts ($x10^5$/ml) (median and range) in heaves (n=7) and control (n=6) horses at 6h following inhalation challenge with saline, HDS-1[100], HDS-2 [100] and HDS-3 [100] and in heaves (n=6) horses at 6h following inhalation challenge with HDS-2R [100].
NP = not performed, C = control group, H = heaves group, TCC = total cell count.

		TCC	Lymphocytes	Macrophages	Mast cells	Basophiloid cells	Eosinophils
SALINE	C	3.85 (2.00-9.60)	1.59 (0.99-6.22)	1.98 (0.73-2.80)	0.25 (0.06-0.39)	0.02 (0.00-0.22)	0.03 (0.00-0.06)
	H	3.80 (1.30-5.60)	2.09 (0.80-3.33)	1.58 (0.34-2.96)	0.13 (0.09-0.19)	0.01 (0.00-0.11)	0.01 (0.00-0.01)
HDS-1 [100]	C	4.65 (1.60-8.00)	1.49 (0.66-2.29)	2.18 (0.51-4.19)	0.14 (0.11-0.24)	0.02 (0.00-1.04)	0.01 (0.00-0.23)
	H	4.10 2.10-7.00)	1.19 (0.61-1.86)	1.16 (0.41-1.75)	0.07 (0.02-0.14)	0.00 (0.00-0.10)	0.01 (0.00-0.13)
HDS-2 [100]	C	2.90 (1.70-4.30)	1.10 (0.92-2.37)	1.41 (0.30-1.62)	0.14 (0.06-0.33)	0.04 (0.00-0.09)	0.04 (0.00-0.15)
	H	7.90 (3.70-9.10)	1.53 (0.51-2.34)	1.19 (0.39-1.78)	0.08 (0.04-0.29)	0.02 (0.00-0.06)	0.00 (0.00-0.25)
HDS-2R [100]	C	NP	NP	NP	NP	NP	NP
	H	7.70 (4.00-21.80)	1.28 (0.49-2.24)	1.68 (0.83-2.27)	0.08 (0.00-0.12)	0.01 (0.00-0.07)	0.03 (0.00-0.15)
HDS-3 [100]	C	NP	NP	NP	NP	NP	NP
	H	10.10 (8.10-19.00)	1.17 (0.36-2.14)	0.72 (0.24-1.93)	0.06 (0.00-0.09)	0.00 (0.00-0.03)	0.01 (0.00-0.21)

Heaves horses had significantly ($P<0.05$) increased total BALF cell counts after HDS-2 [100] and HDS-3 [100], when compared with saline (Fig. 7.17). HDS-3 [100] also significantly ($P<0.05$) reduced macrophage and mast cell numbers in heaves horses when compared with saline (Figs. 7.18 and 7.19). Absolute numbers of the other BALF cell types were not significantly affected by any of the challenges.

Fig. 7.17: BALF total cell count (x10^5/ml) in heaves (n=7) horses at 6h following inhalation challenge with saline, HDS-1[100], HDS-2 [100] and HDS-3 [100].

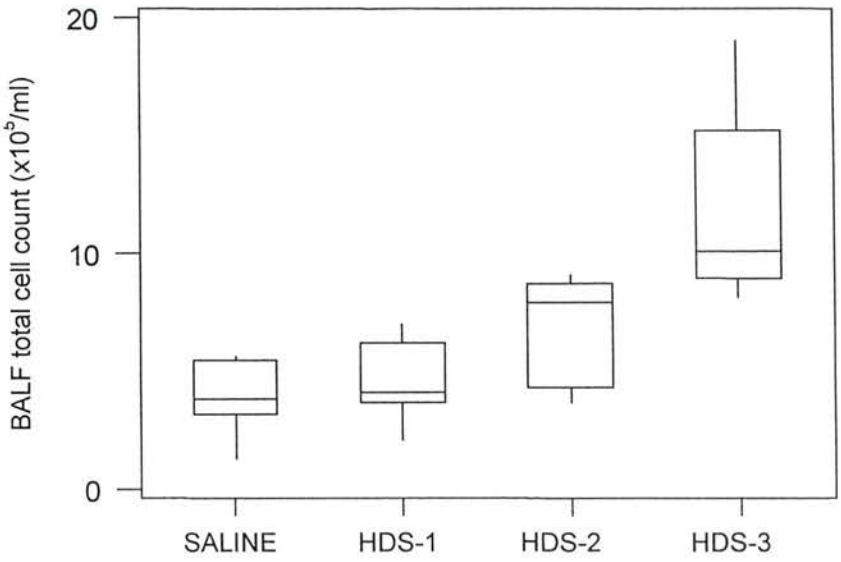


Fig. 7.18: BALF macrophage count (x10^5/ml) in heaves (n=7) horses at 6h following inhalation challenge with saline, HDS-1[100], HDS-2 [100] and HDS-3 [100]. * = outlier.

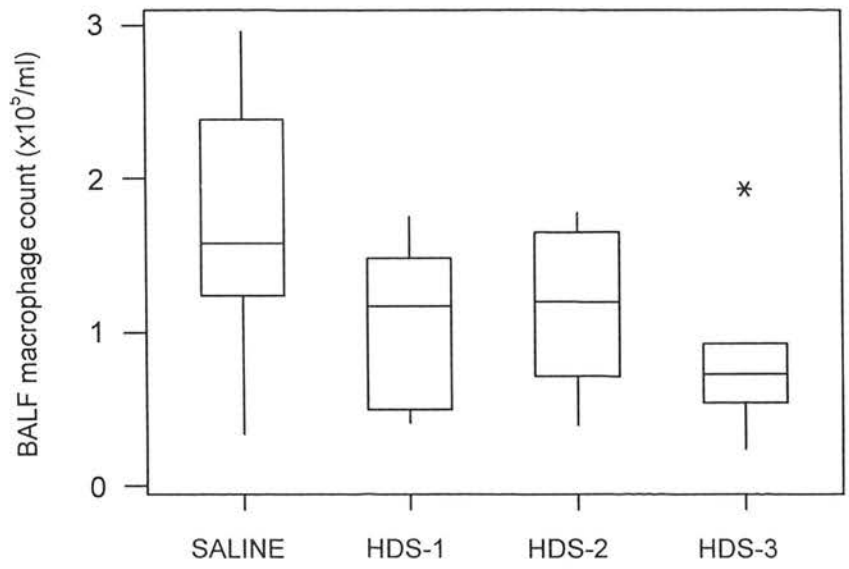
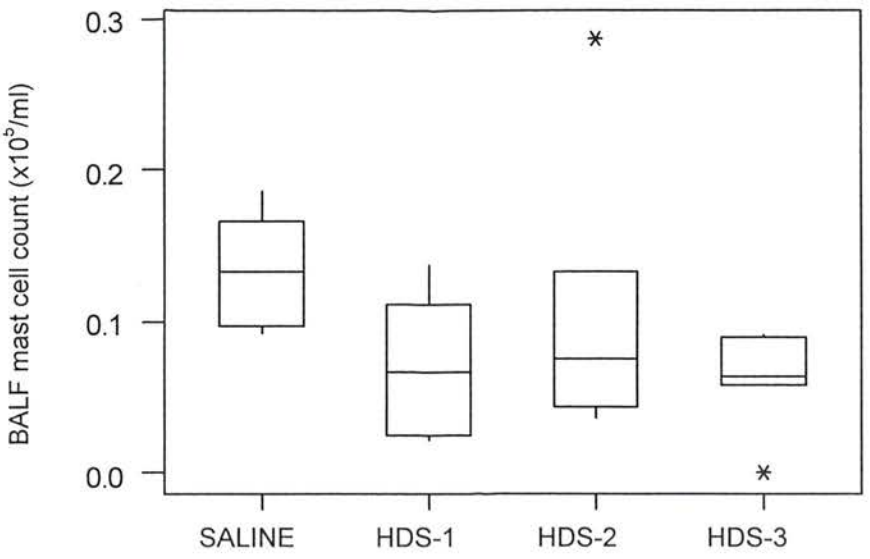
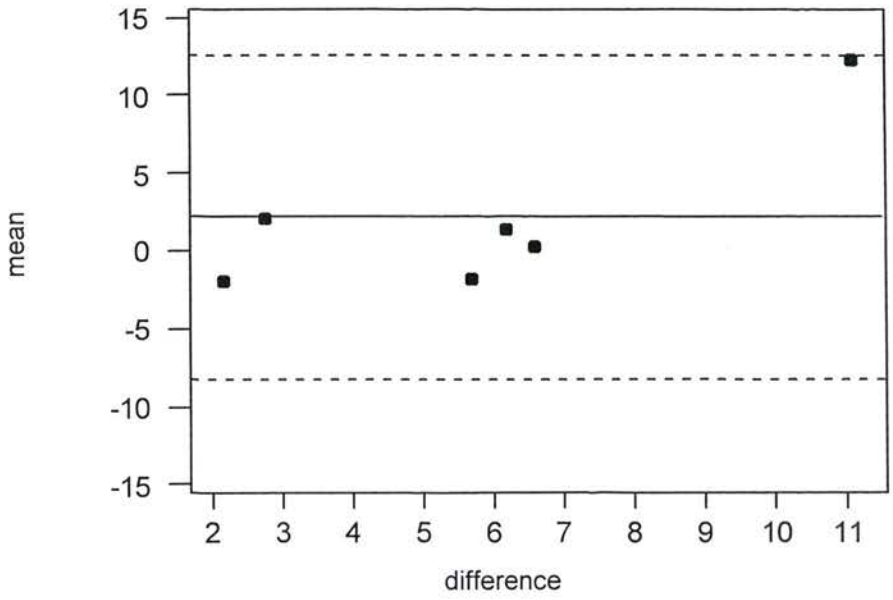


Fig. 7.19: BALF mast cell count (x10^5/ml) in heaves (n=7) horses at 6h following inhalation challenge with saline, HDS-1[100], HDS-2 [100] and HDS-3 [100]. * = outlier.



There was no significant difference in the counts of any BALF cell type in heaves horses between the HDS-2 [100] and HDS-2R [100] challenge. Furthermore, the mean of the differences in paired values for BALF neutrophil counts following these 2 challenges approximated to zero, and all of the calculated differences in paired values fell within 2 standard deviations of the mean of the differences (Fig. 7.20). Consequently, the agreement between the neutrophilic response to both challenges was considered to be good.

Fig. 7.20: Difference between BALF neutrophil counts ($x10^5$/ml) plotted against the mean of the BALF neutrophil counts ($x10^5$/ml) in heaves (n=6) horses at 6h following both HDS-2 inhalation challenges. Solid line = mean of the differences; dotted line = mean of the differences $\pm$ 2 standard deviations of the differences.



7.6 Discussion

In this study, asymptomatic heaves horses given inhaled hay dust suspension (HDS) developed the neutrophilic airway inflammation, obstructive airway dysfunction and mucus hypersecretion that characterise heaves. While high doses of HDS also induced BALF neutrophilia in controls, the magnitude of the neutrophilia was markedly lower than that of heaves horses, and there was no overlap in the neutrophil ratios for the two groups. Furthermore, HDS did not induce lung dysfunction or increase tracheal mucus volume in controls. These findings suggest that HDS is a

valuable tool for the diagnosis and investigation of heaves. Indeed the HDS challenges were more effective in reproducing the airway dysfunction than a 5h duration conventional hay/straw challenge, which induced significant airway neutrophilia but no significant alteration in pulmonary mechanics (Chapter 2). Previous experiments (2.5.1) showed a standard hay/straw challenge protocol resulted in a mean total airborne dust exposure of 2.8mg/m^3, and a mean hourly ventilation rate in the challenged horses of 3.1m^3/h. Therefore, as each HDS [100] challenge originated from 100mg of dust, this equates approximately to the dust exposure encountered during an 11.5h conventional hay/straw challenge. When the repeatability was assessed, there was good reproducibility with respect to airway neutrophilia.

Interestingly, in both groups, the neutrophilic response to HDS-1 inhalation was dose-dependent, rather than being an all or nothing response. This feature has been previously noted in endotoxin-sensitive mice following inhalation of corn dust extract, whereby the dose-dependent inflammatory response was related to the endotoxin concentration of the extract (Schwartz *et al.,* 1994). Interestingly, a significant inflammatory response was not observed in endotoxin resistant mice until high concentrations of extract were administered (Schwartz *et al.,* 1994). In this study, the no-response threshold dose for inducing airway neutrophilia was lower for the heaves group (between HDS-1 [57] and HDS-1 [100]), than for the control group (between HDS-1 [31] and HDS [57]). However, the finding that an extremely high dose of HDS (HDS-1 [316]) induced a marked BALF neutrophilia in controls suggests that even horses without pulmonary disease may develop significant

pulmonary inflammation when exposed to very high airborne dust levels, as may be encountered when they are housed in stables with particularly poor air hygiene. This further emphasises the benefits of a standard, defined challenge protocol for use in the diagnosis of heaves.

The magnitude of the airway neutrophilia and dysfunction in the heaves group was shown to be dependent on the source of dust used to prepare the HDS, with the order of potency being HDS-3 > HDS-2 > HDS-1. Although the order of inhalation challenges was not randomised, it is unlikely that this variable potency resulted from carry-over effects. Firstly, there were no significant differences in baseline lung function prior to the 3 HDS inhalations. Secondly, there was good repeatability of airway neutrophilia between the two separate HDS-2 inhalation challenges. Lastly, the slight, yet significant BALF neutrophilia noted in the control group following HDS-1 [100] challenge was greater than following HDS-2 challenge, despite the HDS-1 [100] challenge being performed first.

It is probable that the potencies of the 3 different HDS reflect their content of pro-inflammatory agents. The greater potency of HDS-3 compared with HDS-1 may be due to its approximately 3 fold higher β-D-glucan content and/or higher level of particulates (mostly mould spores) in the 0-40μm^3 range (6.4.1.3). As these two features reflect a higher fungal content, this finding supports the role of inhaled moulds in the aetiopathogenesis of heaves (McPherson *et al.,* 1979; Derksen *et al.,* 1988; McGorum *et al.,* 1993c). In contrast, the potency of the 3 different HDS preparations did not appear to relate to their endotoxin content (HDS-1 – 21.6μg/ml;

HDS-2 – 18.2μg/ml; HDS-3 – 15.2μg/ml) (6.4.1.3). However, it is possible that the greater glucan and/or particulate content in HDS-3 acted synergistically with, and so magnified the response to, endotoxin and other inflammatory agents in the HDS (Fogelmark *et al.,* 1992; Rylander, 1994; Brown and Donaldson, 1996; Kurup *et al.,* 1997). The differing potencies of HDS-1 and HDS-3 did not appear to relate to their protease content, because they exhibited similar general protease activity (6.4.1.4).

In conclusion, this study demonstrated the successful use of HDS inhalation challenges in reproducing the neutrophilic airway inflammation and dysfunction and mucus hyper-secretion that characterise heaves, and in differentiating heaves-susceptible horses from controls.

CHAPTER 8: RESPONSE TO INHALATION CHALLENGE WITH SEPARATE FRACTIONS OF HAY DUST SUSPENSION IN ASYMPTOMATIC HEAVES HORSES AND CONTROLS

8.1 Summary

To investigate the relative importance of inhaled particulate and soluble components in the pulmonary response to inhaled hay dust suspension (HDS), 6 control and 7 asymptomatic heaves horses were given inhalation challenges with fractionated HDS. The HDS fractions included supernatant (SUP), washed particulates (WP) and wash fraction (WF). Inhalation of SUP induced a significant airway neutrophilia in both groups, with a significantly greater response occurring in heaves horses. SUP induced significantly less airway neutrophilia than HDS in both groups, despite the endotoxin and protease content of HDS and SUP being comparable. WP and WF induced a slight airway neutrophilia in heaves horses. These findings suggest that endotoxins and proteases are not the sole determinants of the magnitude of response. A combined challenge with SUP and WP induced a neutrophilic response approaching the magnitude of that following HDS challenge in the heaves group, indicating that dust particulates contribute to the pulmonary recruitment of neutrophils in heaves. Consequently, inhalation challenge with HDS, which contains both particulates and soluble dust components, may be a more useful tool for the diagnosis and investigation of heaves than aqueous dust extracts, which only contain soluble components.

8.2 Introduction

Although aqueous fungal extract inhalation challenges have established a role for inhaled moulds in the aetiopathogenesis of heaves (McPherson *et al.,* 1979; Derksen *et al.,* 1982; Derksen *et al.,* 1988; McGorum *et al.,* 1993c), these challenges have failed to fully reproduce the naturally occurring disease (McGorum *et al.,* 1993c), and some have resulted in a significant airway neutrophilia in control animals (Derksen *et al.,* 1988). This has led to speculation that other inhaled dust components, including inhaled endotoxins, contribute to the aetiopathogenesis of this disease (McGorum *et al.,* 1993c). Unfortunately, the traditional method of disease induction, namely conventional hay/straw challenge, does not permit the investigation of the potential role of the individual inhalants in this disease. Although the use of aqueous soluble extracts of organic dusts has greatly improved the understanding of human organic dust-related respiratory disease (Schwartz *et al.,* 1994; Blaski *et al.,* 1996; Jagielo *et al.,* 1996a; Jagielo *et al.,* 1996b; Jagielo *et al.,* 1997), it has only permitted evaluation of the role of soluble dust components. The successful use of an inhaled HDS (Chapter 7), which contains both soluble and particulate dust components (Chapter 6), to induce the clinical, inflammatory and functional features of heaves, has enabled the investigation of the role of different dust fractions in the aetiopathogenesis of this disease. This chapter describes the response of control and heaves horses to inhalation challenge with different HDS fractions.

8.3 Materials and methods

8.3.1 Subjects

Six previously described healthy control horses (2.3.1.2) with no detectable respiratory tract disorders and 7 previously described horses with a history and clinical diagnosis of heaves (2.3.1.1) were used. The disease status of all subjects was confirmed by mouldy hay/straw challenge as previously described (2.3.4.2). Throughout the study all horses were kept in a low dust environment (2.3.2.1).

8.3.2 Inhalation challenge material

8.3.2.1 HDS fractions in isolation

The production and characterisation of the 3 fractions of HDS-1, namely SUP, WP and WF has been previously described (6.3.4 and 6.3.5). A 1ml volume of challenge substances was delivered to the facemask for all inhalation challenges unless stated otherwise.

8.3.2.2 HDS fractions given in combination

To determine the importance of inhaled dust particulates in the aetiopathogenesis of heaves, heaves horses received 2 combined WP and SUP challenges. The first combined challenge consisted of a mixture of WP and SUP, prepared by re-suspending pelleted WP in SUP, thus the final proportion of WP to SUP was comparable to that in the non-fractionated HDS. This challenge was termed mixed WP/SUP challenge (WP/SUP[m]), with a 1ml volume used in each challenge. The

second combined challenge consisted of a WP challenge and SUP challenge given consecutively. The WP challenge was given first (taking approximately 10min) and was followed immediately by the SUP challenge (also taking approximately 10min). This challenge was termed the separate WP/SUP challenge (WP/SUP[s]). The volume of each individual challenge (WP and SUP) was 1ml, therefore the total volume of the WP/SUP[s] challenge was 2ml.

8.3.3 Inhalation challenges

8.3.3.1 Inhalation challenge protocol

The challenges given to each group are summarised in Table 8.1. The heaves group received separate inhalation challenges with SUP, WP and WF, and both combined challenges (WP/SUP[m] and WP/SUP[s]). The control group received inhalation challenge with SUP only.

To facilitate subject cooperation, horses were sedated with intravenous 20μg/kg romifidine and 10μg/kg butorphanol immediately prior to each inhalation challenge. The aerosol challenge was generated and delivered as previously described (2.3.4.1). The order in which the individual challenges were given is summarised in Table 8.1. The SUP, WP and WF challenges in the heaves group were not randomised. Following completion of the individual challenges, the combined challenges were given. The order of the combined challenges was randomised.

To minimise potential carry-over effects of a preceding challenge on subsequent challenges, inhalation challenges were conducted a minimum of 14 days apart and all horses were shown to have normal clinical findings immediately prior to each challenge. In order to assess any carry-over effects, all measured baseline lung function and arterial blood gases and pH values were compared. In addition, prior to the combined challenges, 6 heaves horses received a repeat inhalation challenge with WP (termed WP[R]) which, as well as confirming the absence of any carry over effects, allowed an assessment of repeatability of the response to WP challenge.

Table 8.1 Summary of the order in which heaves (n=7) and control (n=6) horses received inhalation challenges with the individual and combined HDS fractions.

	WP	WP[R]	SUP	WF	WP/SUP[m]	WP/SUP[s]
CONTROLS	✗	✗	✓	✗	✗	✗
HEAVES	✓ (1)	✓ (4)	✓ (2)	✓ (3)	✓ (5 or 6)	✓ (5 or 6)

7.3.3.2 Positive (hay/straw exposure) and negative (saline) control challenges

To compare the responses with those of placebo and non-fractionated HDS, comparisons were made with saline (negative control) and HDS-1[100] (positive control) challenge in the same horses, as previously described in Chapters 2 and 7.

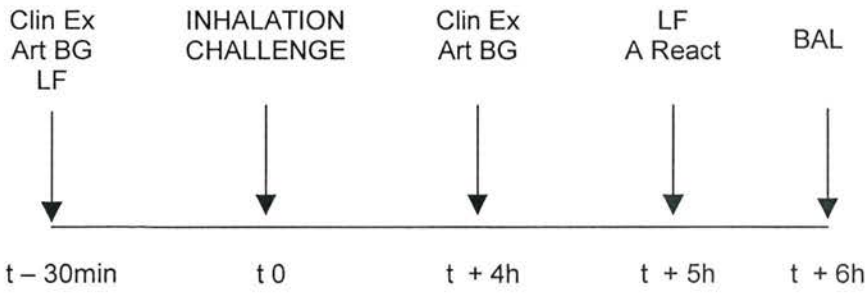
8.3.4 Monitoring the response to challenges

The method and timing of assessment of response to each challenge is summarised in Fig. 8.1. Responses to SUP and WP were assessed using clinical scoring, lung mechanics, airway reactivity, blood gases and pH analyses and BALF cytology as

previously described (2.3.7), while the response to the other single (WF, WP[R]) and combined (WP/SUP[m] and WP/SUP[s]) challenges were assessed solely by BALF cytology.

Fig. 8.1 Study design.

BAL = bronchoalveolar lavage (all challenges); LF = lung function evaluation (SUP, WP); Clin Ex = clinical examination (SUP, WP); Art BG = Arterial blood gases and pH analysis (SUP, WP); A React = airway reactivity evaluation (SUP, WP).



8.4 Statistical analysis

As the data were either not normally distributed and/or the groups of compared data did not have equal variance, non-parametric tests were used. The effects of each challenge were determined mostly by performing within-group analyses.

To check for the presence of any carry-over effects of one challenge on a subsequent challenge, pre-challenge (baseline) measurements of arterial blood gases and pH and lung mechanics were compared using a Friedman test, and when significant, a Wilcoxon Rank Sum test was performed on paired data.

To check for any effects of challenge, where pre-challenge measurements were made at t-30min (arterial blood gases and pH analyses and lung mechanics), the post-challenge values were expressed as % of baseline value, except for clinical scores where actual values were used. As saline was the vehicle for delivery of all HDS fractions, the effect of challenge was assessed by pairing and subtracting post-HDS fraction (% of baseline value) and post-saline (% of baseline value) data. Where no pre-challenge data was collected (BALF cytology), comparisons were made with saline (placebo) challenge data at equivalent time points. A Friedman test was performed on sets of paired data to reduce the risk of type 1 errors, and when significant, a Wilcoxon Rank Sum test was performed on paired data.

Analyses, for response to SUP challenge, between groups (heaves *vs* controls), were performed for BALF neutrophil numbers, using the Mann Whitney test.

Significance was assumed if $P<0.05$. Following correction for any effect of saline inhalation, changes from baseline are expressed as increase/decrease or change in median percent values, with 95% confidence interval for the difference in median, calculated for non-parametric data (Campbell and Gardner 1994).

The two separate WP inhalation challenges were compared using a Wilcoxon Rank Sum test, and as an indication of repeatability, the differences in paired values were plotted against their mean (Bland and Altman 1986). Good repeatability was assumed if the calculated differences in paired values, fell within 2 standard deviations of the

mean of the differences (British Standards Institution, 1979). Results are expressed as median and range.

8.5 Results

8.5.1 Response to inhalation challenge with SUP, WP and WF given in isolation

8.5.1.1 Clinical examination

All horses had a clinical score of zero prior to all challenges. When compared with baseline values, no significant increase in clinical scores was detected in the heaves group following SUP or WP challenge, or in the control group following SUP challenge (Appendix 8.1).

8.5.1.2 Arterial blood gases and pH analyses

Raw data for arterial blood gases and pH measurements are presented in Appendix 8.2. There was no significant difference in the baseline blood gas or pH values prior to the SUP and WP challenges in the heaves group, indicating a lack of any carry-over effects. The percent of baseline arterial blood gases and pH measurements is presented in Table 8.2. Following correction for saline inhalation, SUP challenge did not significantly alter PaO_2, $PaCO_2$ or arterial pH when compared with baseline values in both groups. WP challenge did not significantly alter arterial PaO_2, $PaCO_2$ or pH when compared with baseline values in the heaves group.

8.5.1.3 Lung mechanics and airway reactivity

Raw data for lung mechanics measurements are presented in Appendix 8.3. There was no significant difference in the baseline lung function measurements prior to the WP or SUP challenges in the heaves group, indicating a lack of any carry-over effects. The percent of baseline lung function measurements are presented in Table 8.3. PCCdyn70 values following challenge are presented in Appendix 8.4.

Following correction for any effects of saline inhalation, no alteration in lung mechanics was detected following WP or SUP challenge in the heaves group, or following SUP challenge in the controls. For comparison, following correction for any effects of saline inhalation, HDS-1 [100] inhalation significantly increased $RL_{E50\%}$ (median increase 39%, 95% confidence interval 4-75; $P<0.05$) in the heaves group when compared with baseline (7.5.2.3).

Table 8.3(a): Percent (%) of baseline lung mechanics measurements (median and range) in heaves (n=7) and control (n=6) horses at 5h following inhalation challenge with saline, SUP, WP and HDS-1[100]. NP = not performed, C = control group, H = heaves group.

		Cdyn	dPpl	RL_{iso}	RR	VT	Wb	$RL_{E25\%}$	$RL_{E50\%}$	$RL_{E75\%}$	$RL_{I25\%}$	$RL_{I50\%}$	$RL_{I75\%}$
S	C	68.4 (47.1-157.7)	134.3 (104.2-162.2)	137.0 (85.3-180.3)	91.5 (54.4-106.5)	110.3 (97.7-165.4)	127.9 (78.9-1345.7)	104.2 (26.9-2441.7)	105.9 (43.8-1689.6)	164.0 (132.0-217.6)	148.6 (115.9-190.5)	125.3 (102.8-152.9)	120.1 (106.2-141.9)
	H	96.4 (57.9-104.9)	111.6 (60.0-260.6)	95.9 (76.9-197.3)	90.1 (48.6-110.6)	113.2 (67.2-200.7)	70.8 (35.8-172.0)	66.1 (0.00-125.3)	69.3 (42.7-115.0)	129.4 (77.0-593.2)	102.5 (84.9-590.5)	89.4 (70.5-446.9)	99.2 (29.0-232.9)
SUP	C	94.0 (33.4-204.3)	108.9 (76.7-143.5)	110.6 (76.1-392.5)	106.6 (90.5-118.4)	96.4 (47.5-113.1)	149.0 (58.4-1035.0)	354.3 (86.5-2300)	206.4 (84.4-1450)	99.4 (74.2-416.1)	95.6 (67.1-152.1)	107.5 (82.4-187.7)	126.3 (89.9-139.8)
	H	87.1 (71.2-116.7)	112.0 (94.5-158.0)	97.3 (65.9-112.1)	94.7 (85.1-112.6)	113.3 (91.0-169.1)	86.6 (13.5-3656.3)	84.4 (55.9-1189.1)	103.4 (20.5-749.7)	114.0 (92.2-161.6)	119.0 (105.0-183.2)	99.8 (86.6-115.7)	140.1 (63.8-243.6)
WP	C	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP
	H	83.7 (63.6-121.8)	96.9 (78.3-159.3)	111.5 (47.4-165.8)	97.0 (51.9-133.3)	98.9 (93.0-131.9)	57.8 (9.8-117.6)	72.7 (11.6-141.5)	93.7 (42.1-176.8)	117.3 (63.7-210.0)	119.1 (51.0-293.8)	100.7 (38.9-145.3)	83.4 (41.1-171.5)
HDS-1 [100]	C	92.7 (53.2-138.8)	140.8 (100.5-157.6)	110.9 (70.5-170.2)	93.8 (59.8-102.4)	120.6 (90.0-152.6)	136.3 (30.9-212.5)	155.0 (7.1-279.7)	103.9 (38.0-182.5)	140.2 (111.0-275.8)	136.2 (107.3-257.4)	116.5 (79.8-157.0)	118.3 (103.9-140.1)
	H	105.4 (70.1-139.8)	123.0 (97.0-172.6)	118.2 (85.8-193.2)	87.6 (66.8-110.6)	111.0 (98.0-159.9)	108.9 (8.2-274.1)	120.2 (20.8-148.7)	132.9 (75.0-175.2)	128.0 (69.7-180.8)	126.4 (83.2-181.2)	142.5 (70.1-159.4)	115.3 (79.3-196.1)

Table 8.3(b): The percent (%) of baseline lung mechanics measurements (median and range) in heaves (n=7) and control (n=6) horses at 5h following inhalation challenge with saline, SUP, WP and HDS-1[100]. NP = not performed, C = control group, H = heaves group.

		T_E	T_I	$T_I.T_E$	V'_E	V'_{Emax}	V'_{Imax}	Wb_{el}	Wb_{res}	Wb_{Eres}	Wb_{Ires}	Wb_{Itot}
S	C	105.3 (90.7-175.9)	107.5 (92.0-179.8)	100.3 (40.1-253.8)	100.7 (89.8-103.3)	93.5 (79.5-116.5)	173.5 (119.5-194.8)	137.8 (110.3-204.7)	98.3 (6.7-222.4)	160.5 (134.3-199.4)	174.2 (130.6-183.7)	95.4 (89.1-116.8)
	H	103.4 (83.1-219.9)	111.8 (86.3-263.8)	101.5 (41.3-110.5)	104.7 (43.8-117.9)	116.0 (43.3-137.9)	131.6 (48.3-646.1)	95.3 (48.2-505.7)	86.2 (42.0-439.0)	112.0 (58.5-545.3)	118.1 (51.9-597.2)	101.2 (56.1-140.4)
SUP	C	89.3 (76.2-110.5)	97.0 (86.3-110.5)	106.3 (97.7-643.2)	102.8 (52.2-113.3)	108.5 (52.7-140.2)	82.5 (60.2-120.0)	112.7 (82.9-135.9)	126.3 (1.5-246.3)	101.2 (69.6-115.4)	88.7 (67.3-118.3)	101.7 (46.4-117.1)
	H	103.0 (80.4-121.5)	98.6 (87.6-108.4)	95.9 (89.6-728.7)	112.1 (83.8-156.3)	120.2 (75.9-177.4)	138.2 (93.6-287.9)	144.0 (69.6-250.6)	94.9 (36.6-240.3)	135.8 (76.4-313.5)	128.4 (86.2-295.1)	107.0 (86.4-197.1)
WP	C	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP
	H	106.4 (71.2-203.9)	93.3 (76.3-179.9)	88.1 (59.0-111.1)	109.8 (51.0-132.1)	110.9 (55.6-142.7)	115.5 (76.9-234.3)	93.0 (69.2-169.1)	89.9 (67.7-167.4)	97.3 (71.6-170.4)	112.1 (84.3-203.8)	114.5 (58.7-158.5)
HDS-1 [100]	C	107.5 (91.4-170.7)	104.1 (92.7-154.3)	89.2 (87.8-117.6)	101.9 (78.2-121.7)	99.8 (79.8-124.7)	155.7 (102.0-260.3)	123.0 (97.7-210.5)	156.5 (19.5-209.4)	154.3 (99.9-211.0)	149.8 (104.9-235.0)	106.0 (88.8-170.0)
	H	122.1 (82.3-217.1)	108.6 (90.0-162.6)	92.8 (37.4-123.8)	95.8 (76.1-139.5)	100.1 (61.6-144.6)	125.9 (100.7-183.6)	137.2 (94.7-230.2)	131.2 (86.6-381.0)	136.0 (92.3-176.7)	138.1 (97.4-180.5)	100.8 (83.2-129.8)

8.5.1.4 BALF cytology

The BALF neutrophil counts and ratios following challenge with HDS fractions are presented in Appendix 8.5, and summarised in Table 8.5, with data from saline and HDS-1[100] challenge being included for comparison.

Table 8.5 BALF neutrophil counts (x10^5/ml) and ratios (%) (median and range) in control (n=6) and heaves (n=7) horses at 6h following inhalation challenge with saline, WP, SUP, WF, WP/SUP[s], WP/SUP[m] and HDS-1[100]. NP = not performed.

	BALF neutrophil count (x10^5/ml)		BALF neutrophil ratio (%)	
	CONTROLS	HEAVES	CONTROLS	HEAVES
SALINE	0.06 (0.01-0.17)	0.06 (0.03-0.20)	1.3 (0.2-3.2)	2.3 (0.6-4.5)
WP	NP	0.19 (0.10-0.65)	NP	3.8 (2.4-13.0)
SUP	0.15 (0.04-0.25)	0.53 (0.28-1.64)	4.3 (1.4-6.1)	8.3 (4.2-22.2)
WF	NP	0.35 (0.05-1.54)	NP	7.9 (1.7-20.0)
WP/SUP[s]	NP	0.47 (0.12-9.29)	NP	14.9 (2.8-48.9)
WP/SUP[m]	NP	1.36 (0.43-4.55)	NP	25.7 (7.9-58.3)
HDS-1 [100]	0.28 (0.08-0.67)	2.17 (0.54-3.81)	6.3 (2.8-13.2)	50.7 (19.4-70.4)

Heaves horses developed a significant ($P<0.05$) BALF neutrophilia following challenge with all HDS fractions (Table 8.5; Figs. 8.2 and 8.3). When compared with saline inhalation, the BALF neutrophilia in the heaves group following HDS (36-fold increase in median count) (7.5.1.4) was significantly greater ($P<0.05$) than following SUP (8-fold increase), WP (3-fold increase) and WF (6-fold increase) challenges (Table 8.5; Figs. 8.2 and 8.3). Controls developed a significant ($P<0.05$) increase in BALF neutrophil count and ratio following SUP (Table 8.5, Figs. 8.4 and 8.5). In controls, when compared with saline inhalation, the increase in BALF neutrophil

count was also significantly ($P<0.05$) greater following HDS (5-fold increase in median count) (7.5.1.4) than SUP (2.5-fold increase) (Figs. 8.4 and 8.5).

Fig. 8.2: BALF neutrophil counts ($x10^5$/ml) in heaves (n=7) horses at 6h following inhalation challenge with saline, SUP, WP, WF and HDS-1[100]. * = outlier.

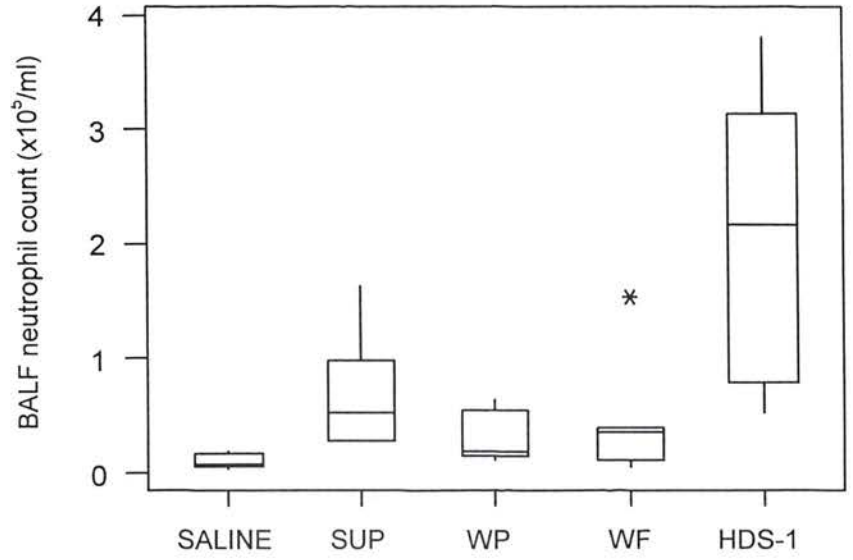


Fig. 8.3: BALF neutrophil ratio (%) in heaves (n=7) horses at 6h following inhalation challenge with saline, SUP, WP, WF and HDS-1[100]. * = outlier.

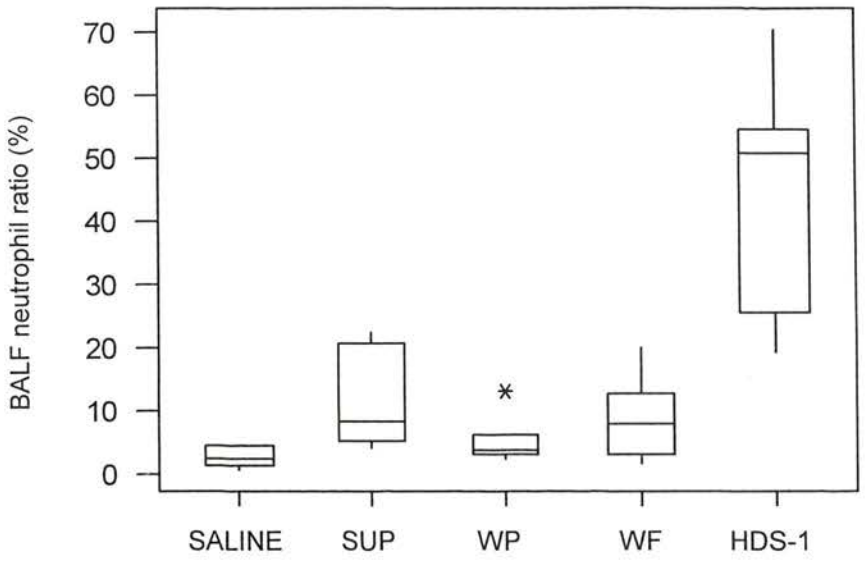


Fig. 8.4: BALF neutrophil counts (x10^5/ml) in control (n=6) horses at 6h following inhalation challenge with saline, SUP, and HDS-1[100].

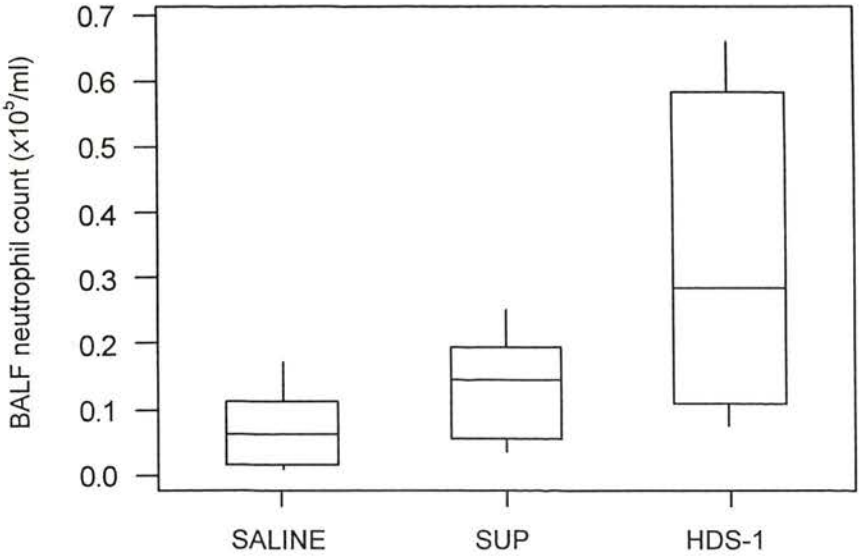
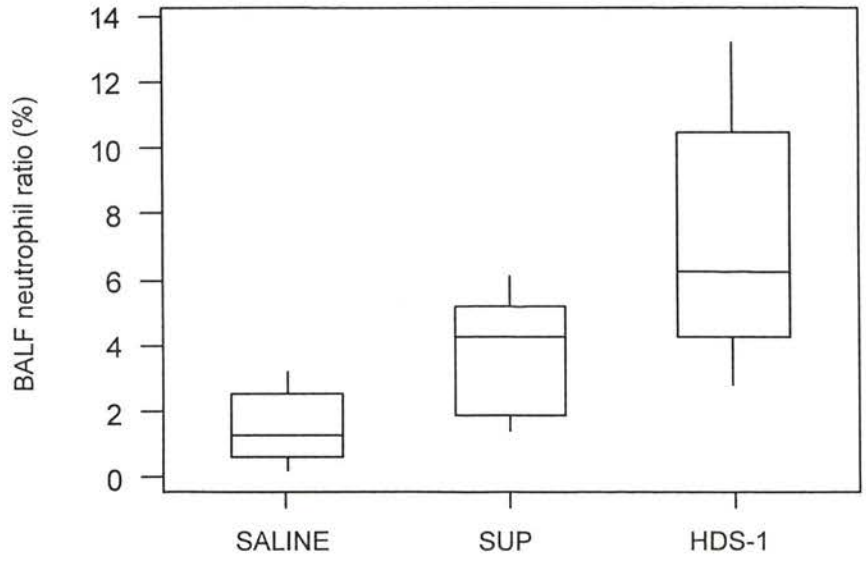


Fig. 8.5: BALF neutrophil ratio (%) in control (n=6) horses at 6h following inhalation challenge with saline, SUP, and HDS-1[100].



Following inhalation of SUP, heaves horses had a significantly higher BALF neutrophil count ($P<0.01$) and ratio ($P<0.05$) than controls, with no overlap in the neutrophil count between the groups (Figs. 8.6 and 8.7).

Fig. 8.6: BALF neutrophil counts ($x10^5$/ml) in heaves (n=7) and control (n=6) horses at 6h following inhalation challenge with SUP.

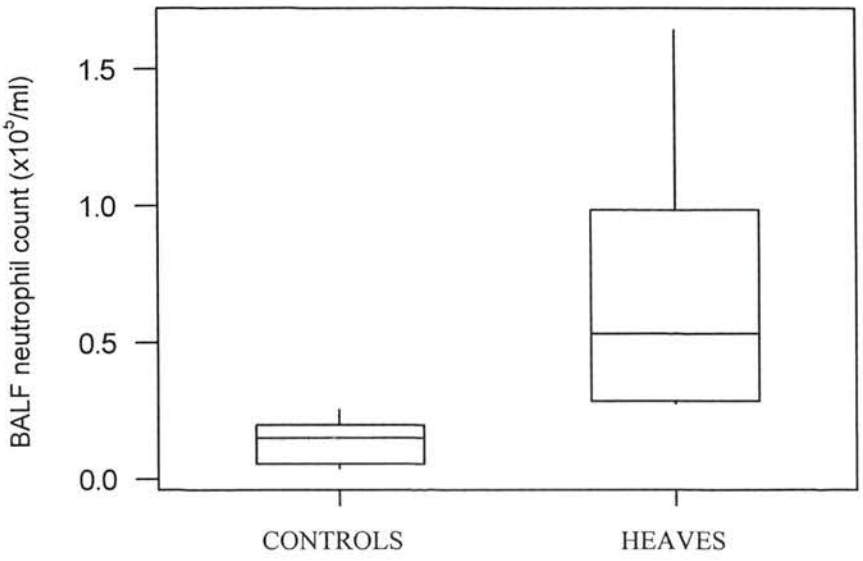
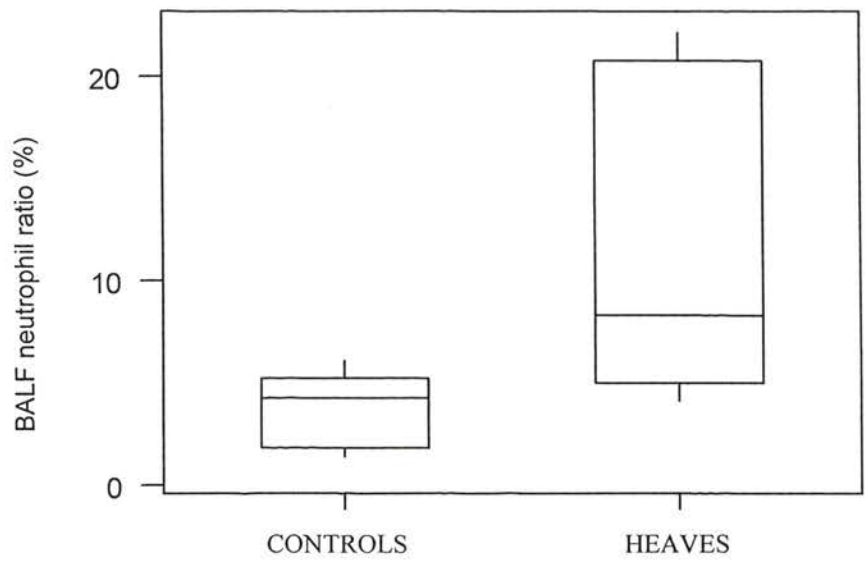


Fig. 8.7: BALF neutrophil ratio (%) in heaves (n=7) and control (n=6) horses at 6h following inhalation challenge with SUP.



Due to the influence of a BALF neutrophilia on the ratio of other BALF cell types, the latter were considered only as absolute numbers. The absolute number of other BALF cell types are summarised in Table 8.6.

Table 8.6: Total and differential BALF cell counts ($x10^5$/ml) (median and range) in heaves (H; n=7) and control (C; n=6) horses at 6h following inhalation challenge with saline, SUP, WP, WF, WP/SUP[s], WP/SUP[m] and HDS-1[100]. NP = not performed.

		TCC	Lymphocytes	Macrophages	Mast cells	Basophiloid cells	Eosinophils
SALINE	C	3.85 (2.00-9.60)	1.59 (0.99-6.22)	1.98 (0.73-2.80)	0.25 (0.06-0.39)	0.02 (0.00-0.22)	0.03 (0.00-0.06)
	H	3.80 (1.30-5.60)	2.09 (0.80-3.33)	1.58 (0.34-2.96)	0.13 (0.09-0.19)	0.01 (0.00-0.11)	0.01 (0.00-0.01)
SUP	C	3.30 (1.80-6.60)	1.28 (0.88-2.96)	1.43 (0.52-2.61)	0.16 (0.08-0.28)	0.02 (0.00-0.36)	0.03 (0.00-0.13)
	H	7.30 (3.40-9.40)	2.94 (1.63-5.61)	1.98 (0.55-4.21)	0.13 (0.02-0.23)	0.01 (0.00-0.03)	0.07 (0.01-0.33)
WP	C	NP	NP	NP	NP	NP	NP
	H	5.50 (2.00-9.10)	2.21 (0.95-5.20)	1.99 (0.71-3.92)	0.14 (0.09-0.23)	0.02 (0.00-0.04)	0.07 (0.00-1.54)
WF	C	NP	NP	NP	NP	NP	NP
	H	3.90 (2.30-7.70)	2.16 (0.68-3.08)	1.76 (0.65-2.98)	0.15 (0.03-0.19)	0.01 (0.00-0.11)	0.00 (0.00-0.06)
WP/SUP [s]	C	NP	NP	NP	NP	NP	NP
	H	5.90 (2.90-	2.42 (1.33-4.12)	1.78 (0.68-5.30)	0.19 (0.02-0.30)	0.00 (0.00-0.02)	0.03 (0.00-0.21)
WP/SUP [m]	C	NP	NP	NP	NP	NP	NP
	H	5.40 (2.30-	2.33 (1.09-2.83)	1.46 (0.38-4.81)	0.08 (0.02-0.24)	0.00 (0.00-0.02)	0.01 (0.00-0.20)
HDS-1 [100]	C	4.65 (1.60-8.00)	1.49 (0.66-2.29)	2.18 (0.51-4.19)	0.14 (0.11-0.24)	0.02 (0.00-1.04)	0.01 (0.00-0.23)
	H	4.10 (2.10-7.00)	1.19 (0.61-1.86)	1.16 (0.41-1.75)	0.07 (0.02-0.14)	0.00 (0.00-0.10)	0.01 (0.00-0.13)

SUP and WP significantly ($P<0.05$) increased total BALF cell counts in heaves horses, when compared with saline (Fig. 8.8). Heaves horses had a significant ($P<0.05$) increase in BALF eosinophil count after SUP (Fig 8.9), however median eosinophil numbers were always <1% of the median total BALF cell count following this challenge. None of the challenges significantly altered absolute BALF lymphocyte, macrophage or mast cell numbers, when compared with saline. In

comparison, BALF absolute counts of all of these cell types were significantly ($P<0.05$) lower following HDS challenge when compared with SUP and WP challenge.

Fig. 8.8: BALF total cell counts ($x10^5$/ml) in heaves (n=7) horses at 6h following inhalation challenge with saline, SUP, WP, WF and HDS[100].

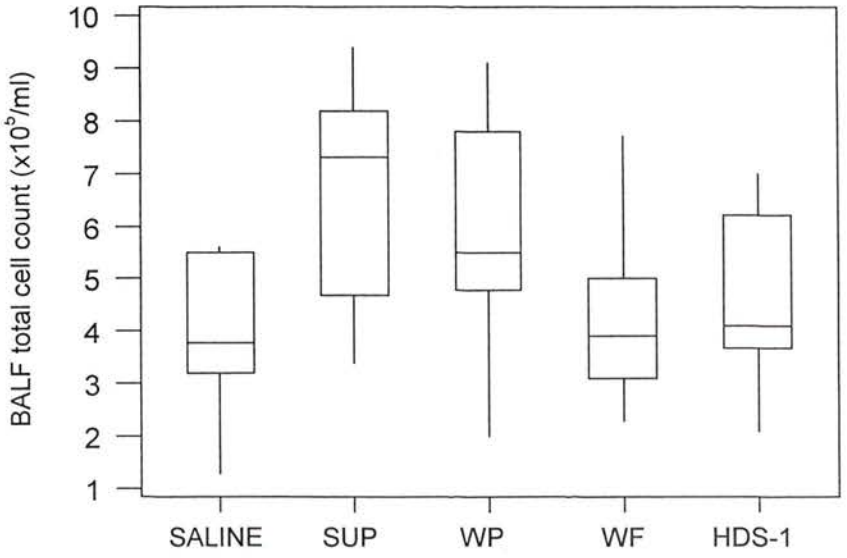
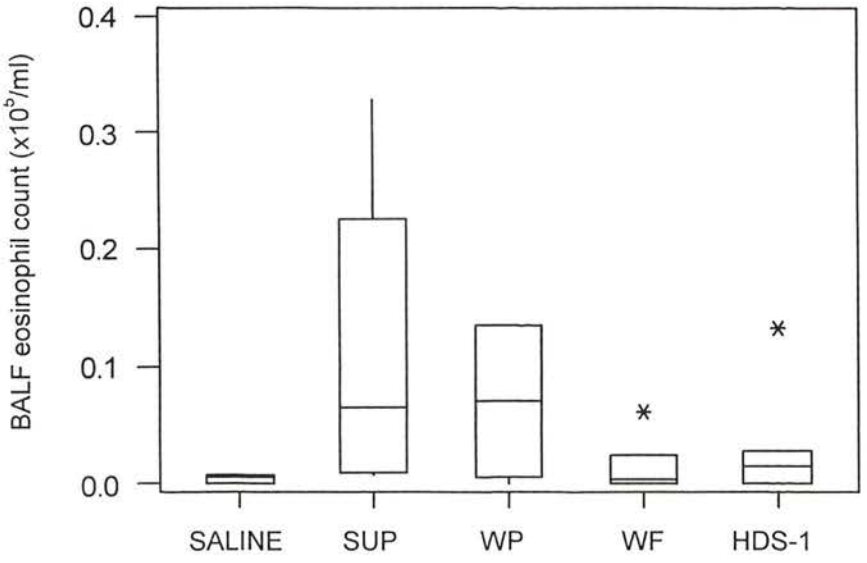
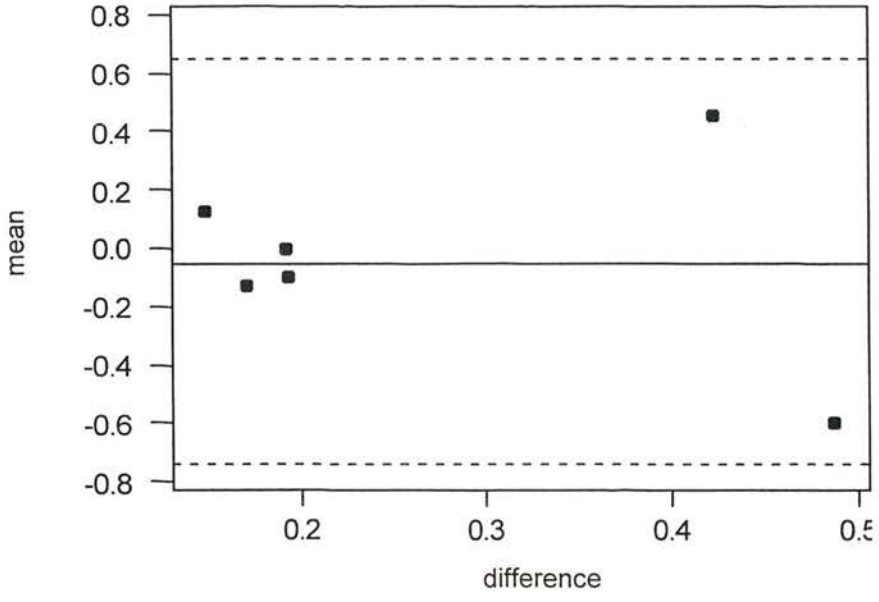


Fig. 8.9: BALF eosinophil counts ($x10^5$/ml) in heaves (n=7) horses at 6h following inhalation challenge with saline, SUP, WP, WF and HDS[100]. * = outlier.



There was no significant difference in BALF cytology between the repeated WP challenges, with the increase in median BALF neutrophil numbers following the two challenges being almost identical (3.0-fold *vs* 3.3-fold increases). Since the mean of the differences in paired values approximated to zero, and all of the 6 calculated differences in paired values fell within 2 standard deviations of the mean of the differences (Fig 8.10), the agreement between the neutrophilic response to both challenges was considered good (Bland and Altman 1986). In addition, no evidence of any carry over effects was detected.

Fig. 8.10: Difference between BALF neutrophil counts ($x10^5$/ml) plotted against the mean of the BALF neutrophil counts ($x10^5$/ml) in heaves (n=6) horses at 6h following both WP inhalation challenges. Solid line = mean of the differences; dotted line = mean of the differences + 2 standard deviations of the differences.



8.5.2 Response to inhalation challenge with combined WP/SUP

8.5.2.1 BALF cytology

When compared with saline inhalation, the BALF neutrophilia in the heaves group following the combined WP/SUP (21-fold increase in median) challenges was significantly greater ($P<0.05$) than that following SUP (8-fold increase in median), WP (3-fold increase in median) and WF (6-fold increase in median) challenges (Table 8.5; Figs. 8.11 and 8.12). There was no significant difference in BALF neutrophil counts following HDS and both the combined WP/SUP and separate WP/SUP challenges (Figs 8.11 and 8.12).

The absolute numbers of other BALF cell types are summarised in Table 8.6. Both WP/SUP[m] and WP/SUP[s] challenges resulted in significantly greater ($P<0.05$) BALF lymphocyte and macrophage counts than HDS inhalation (7.5.1.4) (Fig. 8.13 and Fig. 8.14).

Fig. 8.11: BALF neutrophil counts ($x10^5$/ml) in heaves (n=7) horses at 6h following inhalation challenge with SUP, WP, WP/SUP[s], WP/SUP[m] and HDS-1[100].

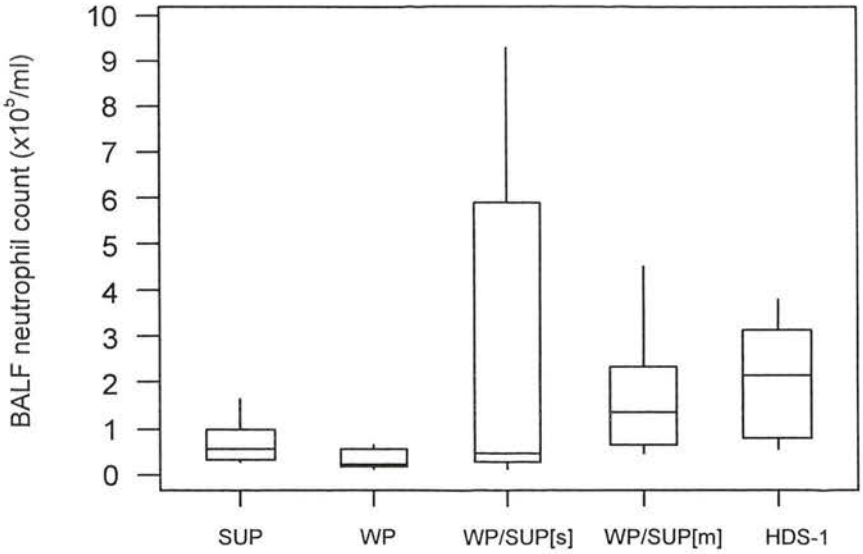


Fig. 8.12: BALF neutrophil ratio (%) in heaves (n=7) horses at 6h following inhalation challenge with SUP, WP, WP/SUP[s], WP/SUP[m] and HDS-1[100]. * = outlier.

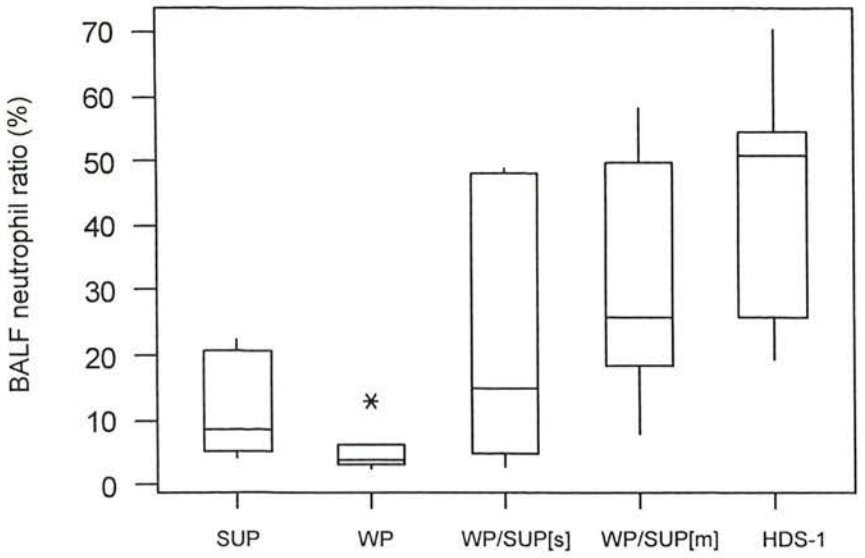


Fig. 8.13: BALF lymphocyte counts (x10^5/ml) in heaves (n=7) horses at 6h following inhalation challenge with SUP, WP, WP/SUP[s], WP/SUP[m] and HDS-1[100].

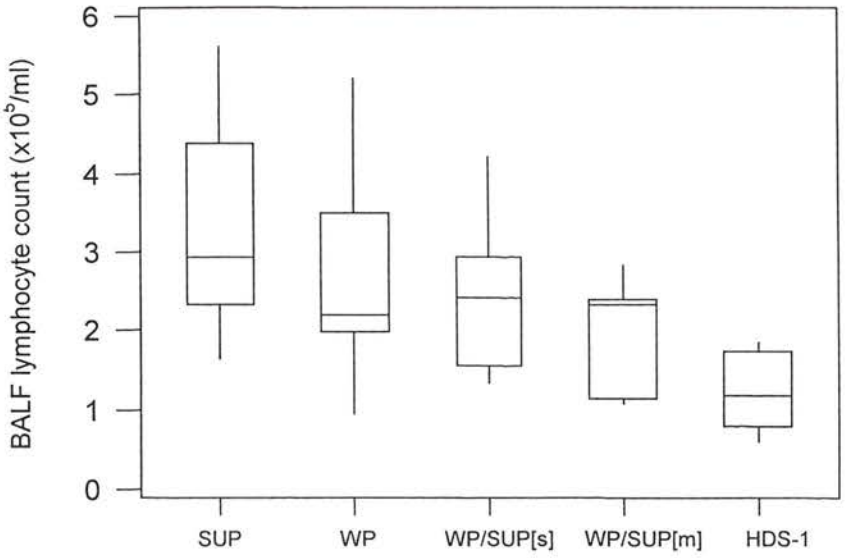
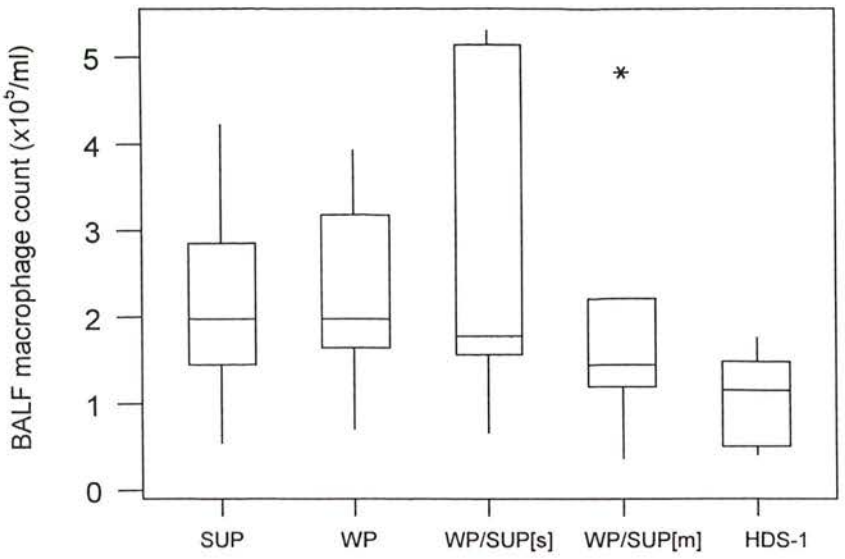


Fig. 8.14: BALF macrophage counts (x10^5/ml) in heaves (n=7) horses at 6h following inhalation challenge with SUP, WP, WP/SUP[s], WP/SUP[m] and HDS-1[100]. * = outlier.



8.6 Discussion

In addition to being a useful tool for the diagnosis of heaves (Chapter 7), hay dust suspensions (HDS) proved valuable in this study to investigate the relative importance of the soluble and particulate components of hay dust in inducing pulmonary inflammation in heaves horses. Inhalation challenges with each of the 3 HDS fractions induced airway neutrophilia in heaves horses, with the potency of the fractions being SUP > WF >> WP. The latter observation suggests that the soluble components of HDS are more important than particulates for inducing pulmonary neutrophil recruitment in heaves. While endotoxins and proteases may contribute to the pro-inflammatory effect of SUP (6.3.5.1 and 6.3.5.2) as they were present at only

low levels in WF (6.3.5.1 and 6.3.5.2), which also caused significant pulmonary inflammation, other soluble components appear also to be involved in the aetiopathogenesis of heaves.

It was considered likely that dust particulates also contributed to airway neutrophil recruitment since the magnitude of the airway neutrophilia induced by each of the 3 fractions was less marked than that induced by HDS, and unlike HDS, none of the fractions induced detectable pulmonary dysfunction. Consistent with the possibility that dust particulates potentiate the neutrophilic response to SUP, the combined WP/SUP challenges induced a neutrophilia of a magnitude approaching that induced by HDS. Although there was no significant difference between the neutrophil count following HDS (36-fold increase) and that following the mixed WP/SUP challenge (23-fold increase), the HDS challenge did result in an non significant greater median BALF neutrophil count and ratio. In addition, both the mixed and separate WP/SUP challenges differed significantly from HDS challenge with respect to the reduction in BALF macrophage (HDS – 1.16 x 10^5/ml, WP/SUP[m] – 1.46 x 10^5/ml, WP/SUP[s] – 1.78 x 10^5/ml) and lymphocyte counts (HDS – 1.19 x 10^5/ml, WP/SUP[m] – 2.33 x 10^5/ml, WP/SUP[s] – 2.42 x 10^5/ml). These findings suggest that fractionation and subsequent reconstitution of HDS may have resulted in a reduction in its overall pro-inflammatory properties. Alternatively, the fact that the WF fraction was not included in the reconstituted HDS (combined WP/SUP), may have resulted in a reduction in its pro-inflammatory capacity, despite the relatively low endotoxin, β-D-glucan and protease concentration of WF (6.3.5.1).

The importance of inhaled particulates, both organic and inorganic, in determining the type and magnitude of the pulmonary inflammatory response has previously been demonstrated in rodent inhalation studies (Kurup *et al.,* 1997; Li *et al.,* 1997), however the mechanism involved is unclear. Inhaled particulates may be pro-inflammatory *per se,* as evidenced by the mild airway neutrophilia induced by WP alone. However, it is possible that the pro-inflammatory effect of WP was partly due to adherent or particulate endotoxin not removed during the wash protocol, and undetected by the *Limulus* ameobocyte lysate assay, which mainly detects soluble endotoxin and underestimates particulate endotoxin content (Rylander *et al.,* 1989). Interestingly, in the present study the combined WP/SUP challenge was more potent than the separate WP/SUP challenge. This is possibly because in the combined WP/SUP challenge the particulates were coated with soluble components, resulting in enhanced activation of pulmonary inflammatory cells (Ning *et al.,* 2000), and/or increased pulmonary deposition, or reduced pulmonary clearance of pro-inflammatory dust components.

Consistent with these findings, pre- or co-exposure of rodent lungs *in vivo* or rodent lung derived cells *in vitro* to airborne particulates, such as concentrated airborne particles and residual oil fly ash, can alter the inflammatory response to pro-inflammatory agents, with the type of particulate determining the alteration in response (Goldsmith *et al.,* 1999; Hamada *et al.,* 1999; Imrich *et al.,* 1999b; Yang *et al.,* 1999; Ning *et al.,* 2000). In contrast, there is limited information available on the modulatory effects of inhaled mould spores. However, in one study the magnitude of the pulmonary eosinophilia induced in mice by inhaled soluble *Aspergillus fumigatus*

antigen was potentiated when the antigen was coupled to inert 3μm diameter polystyrene beads, resulting in a degree of eosinophilia comparable with that induced by inhalation of *A. fumigatus* spores (Kurup *et al.,* 1997).

Other possible reasons for the magnified response to combined WP/SUP may include an endotoxin-mediated enhanced neutrophilic response to allergen on the surface of spores, an effect which has previously been demonstrated in guinea pigs following chronic exposure to LPS and ovalbumin (Rylander and Holt, 1998). It is unlikely that the high β-D-glucan content of WP (6.3.5.1) was responsible for the magnified response when WP was combined with SUP, as β-D-glucan has been shown to markedly reduce the airway neutrophilic response to inhaled endotoxin following acute exposure in other species (Fogelmark *et al.,* 1994).

While there was no overlap in the BALF absolute neutrophil counts following SUP challenge in control and heaves horses, the magnitude of the difference between the two groups was less marked than after non-fractionated HDS challenge. This indicates that HDS is more useful than SUP as a diagnostic tool for differentiating heaves and control horses.

In summary, this study identified an important role for inhaled particulates in the inflammatory response of heaves horses. As the majority of particulates in HDS were mould spores (6.3.2.1) their involvement in the aetiopathogenesis of heaves may reflect not only their role as an antigenic source, but also the importance of their particulate structure which may potentiate the pulmonary inflammatory response to

other inhalants. Additionally, it is clear from this study that while aqueous dust extracts may aid the investigation of heaves, the inclusion of particulates in the challenge substance is important in optimising the resulting pulmonary inflammatory response.

CHAPTER 9: CONTRIBUTION OF LPS TO THE PULMONARY RESPONSE TO INHALED HDS

9.1 Summary

This chapter investigated the relative contribution of inhaled endotoxin and organic dust particulates (primarily mould spores) in the aetiopathogenesis of heaves. Depletion of endotoxin from an aqueous hay dust suspension (HDS) attenuated the airway neutrophilia and abrogated the airway dysfunction induced in heaves horses by inhaled HDS. The airway response was re-established by adding back LPS, confirming that the attenuation in airway response was specifically due to endotoxin depletion. Interestingly, the magnitude of alteration in airway response following endotoxin depletion and add-back was greater than that which could be attributed solely to endotoxin *per se,* suggesting that the activity of LPS was enhanced by other dust components. This indicates that inhaled organic dust components (soluble and/or particulates) and LPS have a synergistic pro-inflammatory action in heaves. It was concluded that inhaled endotoxin contributes to induction of airway inflammation and dysfunction in heaves, and that the airway response to inhaled endotoxin is synergised by co-challenge with other organic dust components.

9.2 Introduction

While previous work suggested that heaves is a hypersensitivity response to inhaled moulds (Halliwell *et al.,* 1979; Lawson *et al.,* 1979; McPherson *et al.,* 1979; McGorum *et al.,* 1993b), experimental aqueous mould extract inhalation induced

pulmonary neutrophilic inflammation and dysfunction, consistent with, but less severe than the natural disease (McGorum *et al.,* 1993c). This shortfall in response may reflect insufficient dose of mould, although the findings of Chapter 5 make this unlikely. Alternatively the shortfall in response may reflect a difference in the duration of challenge and/or the involvement of additional dust components, such as endotoxin (McGorum *et al.,* 1993c). Inhaled endotoxin is a likely candidate for involvement in heaves (Pirie *et al.,* 2001b) for the reasons highlighted earlier (2.2). However as the exposure level of inhaled LPS required to induce airway inflammation and dysfunction in short-term experimental inhalation challenges greatly exceeds that encountered in the stable, it is unlikely that LPS acts alone in disease induction (Chapter 2; Pirie *et al.,* 2001b).

Chapter 7 described the successful use of a nebulised aqueous hay dust suspension (HDS) to reproduce the features of heaves in susceptible horses and also to differentiate susceptible horses from control horses. Chapter 8 described the use of the same model to demonstrate the relative importance of both soluble and particulate components in the induction of heaves (Pirie *et al.,* 2001a, c and d). This HDS experimental model was also used in the present study, since it provided a method for investigating the *relative* importance of dust components, including endotoxin, in heaves. This chapter describes a series of inhalation challenge experiments performed to determine the importance of inhaled endotoxin in heaves. The effect of LPS depletion of HDS was studied to establish whether endotoxin contributes to the pulmonary functional and inflammatory response of heaves susceptible and control horses to inhaled HDS. In addition, heaves horses received a further series of

inhalation challenges following "LPS add-back" i.e. the addition of LPS to the LPS-depleted HDS, at a concentration equivalent to that which was previously removed.

9.3 Materials and methods

9.3.1 Subjects

Six previously described healthy control horses with no detectable respiratory tract disorders (2.3.1.2) and 6 previously described horses with a history and clinical diagnosis of heaves (3.3.1) were used. The disease status of all subjects was confirmed by mouldy hay/straw challenge as previously described (2.3.4.2). Throughout the study all horses were kept in a low dust environment (2.3.2.1).

9.3.2 Inhalation challenge material

The nebulised challenge material was endotoxin-depleted HDS-1[100] (HDS-LPS), prepared and characterised as described previously (Chapter 6).

9.3.2.1 Endotoxin analysis of HDS

Endotoxin analysis of HDS and HDS-LPS was performed using an endotoxin-specific *Limulus* amoebocyte assay as previously described (6.3.2.4).

9.3.2.2 Endotoxin depletion of HDS

Polymixin-coated agarose beads suspended in 50% glycerol were used to achieve endotoxin depletion, using a modification of the method previously described for

endotoxin depletion of *A. fumigatus* extract (5.3.2.2). 10ml HDS was centrifuged (1600g; 15min) to pellet the particulates, and the supernatant, which contained 83% of the HDS endotoxin activity (6.4.3.1), was decanted. 20ml of the polymixin-coated bead suspension was added to 25ml HDS supernatant in a sterile conical plastic tube. The LPS content of 25ml HDS-1[100] was calculated to be 540μg (25 x 21.6 [endotoxin content in 1ml HDS-1; 6.4.1.3]), and 20mls of polymixin-coated bead suspension was calculated to have the capacity to remove between 4000 and 10000μg LPS. The tube was rotated for 30min and the resulting mixture was then centrifuged (1600g; 15min) to pellet the beads. The LPS-depleted supernatant (HDS-LPS) was decanted, and used to re-suspend the previously pelleted HDS particulates to yield HDS-LPS, which was frozen at -80°C until used for the inhalation challenges. As it was not possible to pellet the glycerol along with the polymixin-coated beads, LPS depletion of 25ml HDS resulted in a final volume of 35ml of HDS-LPS, due to the added presence of 10ml glycerol in the final suspension.

9.3.2.3 Add-back of depleted LPS

The re-establishment of the original LPS concentration of HDS was achieved by adding back soluble LPS (*Salmonella typhimurium* Ra60) from a stock solution of 8.89mg/ml to HDS-LPS, at a quantity equivalent to that which had been removed during the depletion. This challenge material was termed HDS-LPS+LPS.

9.3.3 Inhalation challenges

9.3.3.1 Inhalation challenge protocol

Both the control and the heaves group received inhalation challenges with HDS-LPS. To ensure that any alteration in response following endotoxin depletion was due solely to depletion of endotoxin, heaves horses also received inhalation challenge with HDS-LPS+LPS. Because of the glycerol content of HDS-LPS and HDS-LPS+LPS (10ml glycerol per 25ml HDS), for these challenges, 1.4ml (35/25) of the suspension was nebulised in order to deliver the same quantity of soluble and particulate HDS components.

To facilitate subject cooperation, horses were sedated immediately prior to inhalation challenges as previously described (2.3.4.1). The aerosol was generated and delivered as previously described (2.3.4.1). The challenges were not randomised, therefore to minimise potential carry over effects of preceding challenges, all inhalation challenges on any one subject were conducted a minimum of 14 days apart and all horses were demonstrated to have normal clinical findings immediately prior to each inhalation challenge. In order to assess any carry-over effects, all baseline lung function values were compared.

9.3.3.2 Positive (HDS) and negative (saline) control challenges

To compare the HDS-LPS and HDS-LPS+LPS responses with those of placebo and HDS, comparisons were made with saline and HDS-1[100] challenge in the same horses. As only 6 heaves horses were used in this study, the response data of this

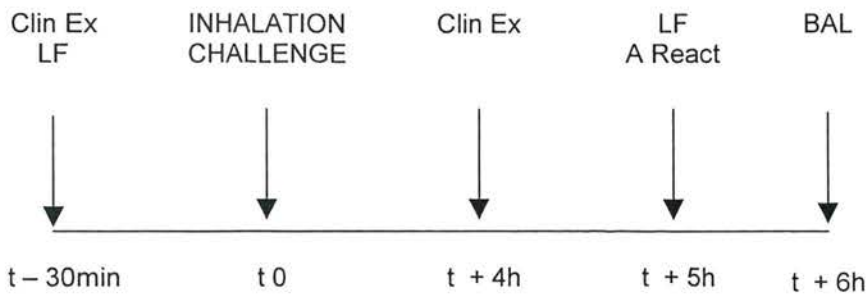
group to saline and HDS is presented fully in this chapter instead of referring to the results of Chapters 2 and 7 (where 7 heaves horses were used).

9.3.4 Monitoring the response to challenges

The method and timing of assessment of response to each challenge is summarised in Fig. 9.1. The response to challenges was assessed using clinical scoring, lung mechanics, airway reactivity and BALF cytology as previously described (2.3.7).

Fig. 9.1: Study design.

BAL = bronchoalveolar lavage; LF = lung function evaluation; Clin Ex = clinical examination; A React = airway reactivity evaluation.



9.4 Statistical analysis

As the data were either not normally distributed and/or the groups of compared data did not have equal variance, non-parametric tests were used. The effect of each inhalation challenge was determined by performing within-group analyses.

To check for the presence of any carry-over effects of one challenge on a subsequent challenge, pre-challenge (baseline) measurements of lung mechanics were compared using a Friedman test, and when significant, a Wilcoxon Rank Sum test was performed on paired data.

To check for any effects of challenge, where pre-challenge measurements were made at t-30min (lung mechanics), the post-challenge values were expressed as % of baseline value, except for clinical scores where actual values were used. As saline was the vehicle for delivery of all challenges, the effect of challenge was assessed by pairing and subtracting post HDS-LPS or HDS-LPS+LPS (% of baseline value) and post saline (% of baseline value) data. Where no pre-challenge data were collected (BALF cytology), comparisons were made with saline challenge data at equivalent time points. A Friedman test was performed on sets of paired data to reduce the risk of type 1 errors, and when significant, a Wilcoxon Rank Sum test was performed on paired data. Between group (heaves *vs* controls) analyses, for response to HDS-LPS, were performed for BALF neutrophil numbers, using the Mann Whitney test.

Significance was assumed if $P<0.05$. Following correction for any effect of saline inhalation, changes from baseline are expressed as increase/decrease or change in median values, with 95% confidence interval for the difference in median, calculated for non-parametric data (Campbell and Gardner 1994). Results in tables are expressed as median and range.

9.5 Results

9.5.1 LPS depletion of HDS

The endotoxin content of HDS was 21.6 μg/ml (6.4.1.3), while the endotoxin content of the HDS-LPS was 9.1μg/ml. Therefore polymixin treatment reduced the final endotoxin exposure present within the facemask from 21.6μg for HDS to 12.8μg for HDS-LPS (9.1μg x 1.4ml), equating to a 41% reduction in the endotoxin exposure.

9.5.2 Response to inhalation challenge

9.5.2.1 Clinical examination

All horses had a clinical score of zero prior to all challenges. When compared with baseline, no significant change in clinical scores was detected in either group following any of the challenges (Appendix 9.1).

9.5.2.2 Lung mechanics and airway reactivity

Raw data for lung mechanics measurements are presented in Appendix 9.2. There was a significant difference among the baseline data before the 3 challenges (HDS, HDS-LPS, HDS-LPS+LPS) with respect to dPpl, VT, $RL_{E75\%}$, $RL_{I25\%}$, TE, V'E, Wb_{el}, Wb_{res}, Wb_{Eres}, Wb_{Ires} and Wb_{Itot}, indicative of a slightly greater degree of pulmonary dysfunction prior to the HDS-LPS challenge. The percentage of baseline lung mechanics measurements are presented in Table 9.1. PCCdyn70 values following challenge are presented in Appendix 9.3.

Following correction for any effects of saline inhalation, HDS-LPS challenge did not result in detectable lung dysfunction in either group, however HDS-LPS+LPS induced a significant ($P<0.05$) increase in both $R_{LE25\%}$ (increase in median 142%, 95% CI 32-363) (Fig. 9.2) and $R_{LE75\%}$ (increase in median 103%, 95% CI 15-192) (Fig. 9.3) in the heaves group. For comparison in the same 6 heaves horses, HDS induced a significant ($P<0.05$) increase in expiratory resistive work of breathing (Wb_{E_res}) (increase in median 66%, 95% CI 8-157) (Fig. 9.4), and a non-significant ($P=0.059$) increase in $R_{LE75\%}$.

Fig. 9.2: Percent of baseline $RL_{E25\%}$ in heaves horses (n=6) at 5h following inhalation challenge with saline HDS-1[100], HDS-LPS and HDS-LPS+LPS minus percent of baseline $RL_{E25\%}$, at 5h following inhalation challenge with saline.

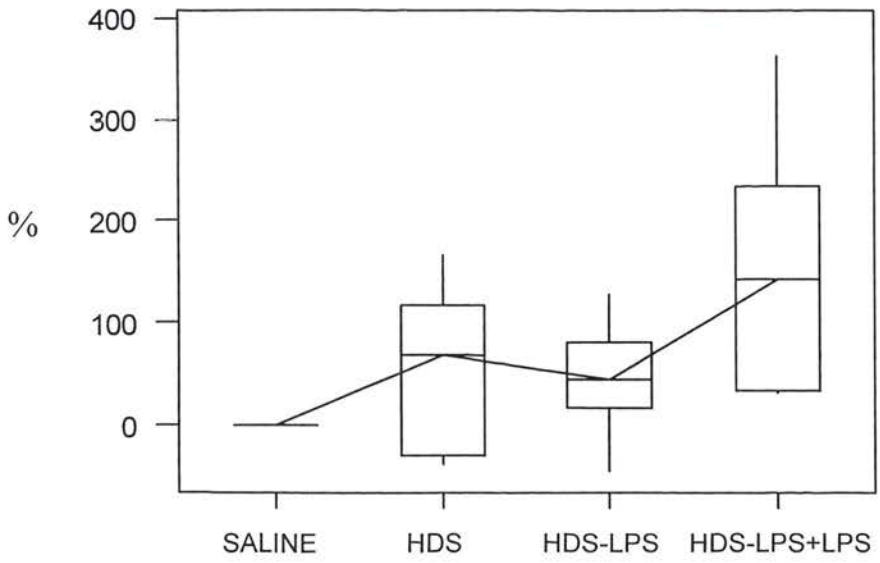


Fig. 9.3: Percent of baseline $RL_{E75\%}$ in heaves horses (n=6) at 5h following inhalation challenge with saline HDS-1[100], HDS-LPS and HDS-LPS+LPS minus percent of baseline $RL_{E75\%}$, at 5h following inhalation challenge with saline.

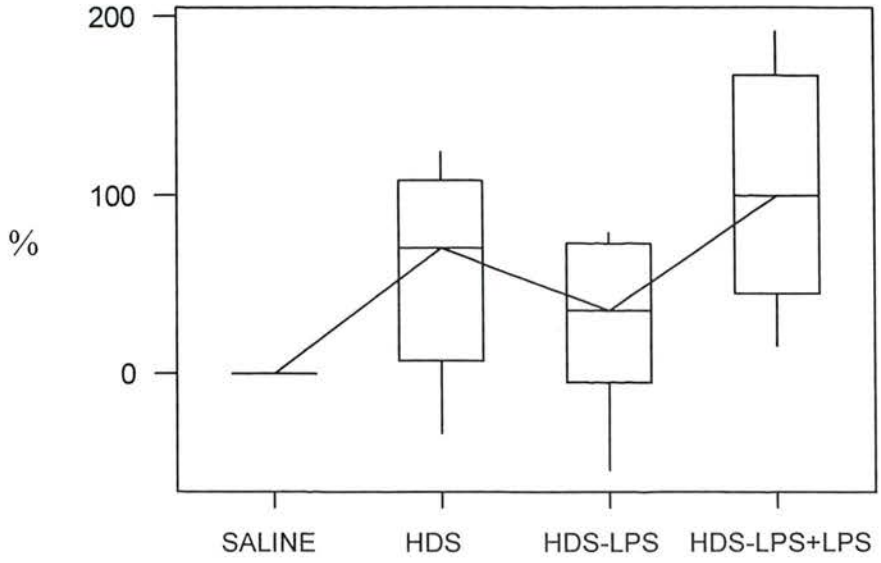


Fig. 9.4: Percent of baseline WB_{E_res} in heaves horses (n=6) at 5h following inhalation challenge with saline HDS-1[100], HDS-LPS and HDS-LPS+LPS minus percent of baseline WB_{E_res} at 5h following inhalation challenge with saline.

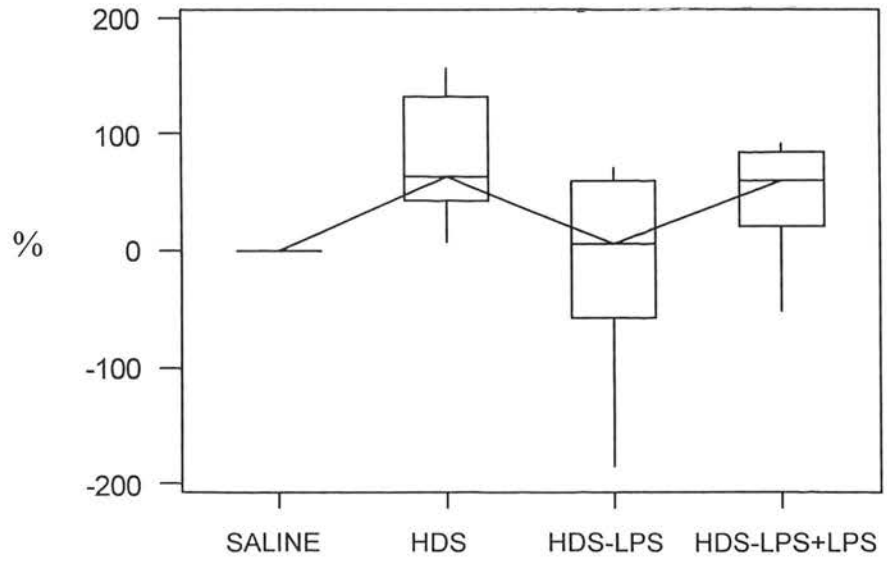


Table 9.1(a): Percent (%) of baseline lung function measurements (median and range) in heaves (H, n=7) and control (C, n=6) horses at 5h following inhalation challenge with saline, HDS-1[100], HDS-LPS and HDS-LPS+LPS. NP = not performed.

		Cdyn	dPpl	RL_{iso}	RR	VT	Wb	$RL_{E25\%}$	$RL_{E50\%}$	$RL_{E75\%}$	$RL_{I25\%}$	$RL_{I50\%}$	$RL_{I75\%}$
SALINE	C	68.4 (47.1-157.7)	134.3 (104.2-162.2)	137.0 (85.3-180.3)	91.5 (54.4-106.5)	110.3 (97.7-165.4)	127.9 (78.9-1345.7)	104.2 (26.9-2441.7)	105.9 (43.8-1689.6)	164.0 (132.0-217.6)	148.6 (115.9-190.5)	125.3 (102.8-152.9)	120.1 (106.2-141.9)
	H	96.9 (75.8-104.9)	108.1 (60.0-188.5)	88.7 (76.9-197.4)	93.0 (62.4-110.6)	111.3 (67.2-165.8)	62.1 (35.8-172.0)	65.0 (0.0-125.3)	70.3 (50.7-115.0)	124.8 (77.0-438.9)	101.3 (84.9-317.1)	89.4 (70.5-313.8)	95.8 (29.0-146.5)
HDS-1 [100]	C	92.7 (53.2-138.8)	140.8 (100.5-157.6)	110.9 (70.5-170.2)	93.8 (59.8-102.4)	120.6 (90.0-152.6)	136.3 (30.9-212.5)	155.0 (7.1-279.7)	103.9 (38.0-182.5)	140.2 (111.0-275.8)	136.2 (107.3-257.4)	116.5 (79.8-157.0)	118.3 (103.9-140.1)
	H	100.7 (70.1-139.8)	125.2 (97.6-172.6)	125.4 (101.3-193.2)	87.1 (66.8-110.6)	110.4 (98.0-159.9)	131.3 (8.2-274.1)	120.6 (20.8-148.7)	139.7 (81.1-175.2)	130.5 (69.7-180.8)	134.0 (83.2-181.2)	148.2 (118.7-159.4)	117.0 (79.3-196.1)
HDS-LPS	C	78.4 (54.9-110.2)	126.5 (69.9-171.6)	143.4 (68.5-235.1)	93.8 (63.3-214.1)	103.8 (56.0-174.1)	161.6 (101.8-3016.7)	87.8 (0.0-567.7)	96.0 (11.7-247.3)	146.7 (56.8-2031.3)	165.9 (86.1-792.3)	164.4 (78.6-439.5)	136.3 (53.7-169.7)
	H	100.3 (70.8-127.2)	99.6 (77.6-242.9)	107.2 (90.6-137.4)	90.8 (52.9-125.5)	98.6 (95.0-111.5)	114.1 (66.8-220.6)	127.2 (39.4-158.3)	109.6 (61.0-143.2)	89.0 (21.6-133.9)	105.2 (29.4-268.5)	132.1 (18.6-147.7)	106.0 (49.5-173.5)
HDS-LPS+LPS	C	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP
	H	67.2 (41.6-83.1)	135.2 (117.9-190.5)	176.1 (103.5-272.7)	91.4 (67.5-107.9)	101.1 (71.6-118.1)	186.9 (102.8-535.1)	146.8 (0.0-235.8)	168.6 (106.3-261.4)	183.3 (92.5-238.4)	158.2 (91.5-276.1)	159.2 (96.7-288.4)	124.9 (88.4-160.0)

Table 9.1(b): Percent (%) of baseline lung function measurements (median and range) in heaves (H, n=7) and control (C, n=6) horses at 5h following inhalation challenge with saline, HDS-1[100], HDS-LPS and HDS-LPS+LPS. NP = not performed.

		T_E	T_I	$T_I:T_E$	V'_E	V'_{Emax}	V'_{Imax}	Wb_{el}	Wb_{res}	Wb_{Eres}	Wb_{Ires}	Wb_{Itot}
SALINE	C	105.3 (90.7-175.9)	107.5 (92.0-179.8)	100.3 (40.1-253.8)	100.7 (89.8-103.3)	93.5 (79.5-116.5)	173.5 (119.5-194.8)	137.8 (110.3-204.7)	98.3 (6.7-222.4)	160.5 (134.3-199.4)	174.2 (130.6-183.7)	95.4 (89.1-116.8)
	H	101.4 (83.1-219.9)	110.2 (86.3-151.0)	102.2 (58.5-110.5)	104.9 (43.8-117.9)	115.8 (43.3-137.9)	127.6 (48.3-231.5)	92.2 (48.2-277.8)	76.5 (42.0-223.6)	108.3 (58.5-298.9)	117.2 (51.9-259.1)	102.0 (56.1-140.4)
HDS-1 [100]	C	107.5 (91.4-170.7)	104.1 (92.7-154.3)	89.2 (87.8-117.6)	101.9 (78.2-121.7)	99.8 (79.8-124.7)	155.7 (102.0-260.3)	123.0 (97.7-210.5)	156.5 (19.5-209.4)	154.3 (99.9-211.0)	149.8 (104.9-235.0)	106.0 (88.8-170.0)
	H	126.7 (82.3-217.1)	114.6 (90.0-162.6)	96.4 (37.4-123.8)	90.2 (76.1-139.5)	97.1 (61.6-144.6)	132.1 (100.7-183.6)	137.6 (111.1-230.2)	135.7 (122.8-381.0)	145.0 (92.3-176.7)	139.3 (97.4-180.5)	94.1 (83.2-129.8)
HDS-LPS	C	109.1 (65.7-319.4)	100.3 (51.0-419.4)	90.1 (85.6-122.1)	100.2 (57.6-109.4)	95.1 (56.2-129.3)	175.9 (39.2-487.7)	127.1 (58.1-487.3)	102.2 (44.9-3691.7)	149.2 (68.7-380.7)	156.6 (50.7-4970.2)	102.4 (62.3-132.9)
	H	101.6 (80.9-129.5)	104.9 (86.9-118.4)	115.8 (64.7-213.3)	95.1 (48.8-118.2)	93.6 (73.8-110.8)	96.3 (83.7-423.3)	105.3 (54.7-142.2)	96.2 (38.6-138.3)	108.9 (68.4-198.5)	100.7 (80.5-290.2)	96.8 (76.9-109.6)
HDS-LPS+LPS	C	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP
	H	113.8 (99.7-158.9)	107.9 (85.0-138.2)	92.2 (40.2-106.8)	81.8 (68.5-120.1)	81.5 (62.8-146.2)	150.8 (98.0-249.6)	142.3 (119.2-191.3)	140.9 (92.9-206.7)	139.9 (107.1-179.6)	136.0 (116.4-196.6)	76.7 (67.5-112.3)

9.5.2.3 BALF cytology

The BALF neutrophil counts and ratios following challenge with HDS-LPS and HDS-LPS+LPS are presented in Appendix 9.4, and summarised in Table 9.2, with data from saline and HDS-1[100] challenge included for comparison.

Table 9.2: BALF neutrophil counts ($x10^5$/ml) and ratios (%) (median and range) in control (n=6) and heaves (n=6) horses at 6h following inhalation challenge with saline, HDS and HDS-LPS and in heaves (n=6) horses at 6h following inhalation challenge with HDS-LPS+LPS. NP = not performed.

	BALF neutrophil count ($x10^5$/ml)		BALF neutrophil ratio (%)	
	CONTROLS	HEAVES	CONTROLS	HEAVES
SALINE	0.06 (0.01-0.17)	0.07 (0.03-0.20)	1.3 (0.2-3.2)	2.2 (0.6-4.5)
HDS	0.28 (0.08-0.67)	2.39 (0.80-3.81)	6.3 (2.8-13.2)	51.9 (19.4-70.4)
HDS-LPS	0.09 (0.04-0.18)	0.54 (0.44-2.48)	3.5 (1.2-6.6)	16.7 (9.8-60.4)
HDS-LPS+LPS	NP	1.87 (0.56-4.28)	NP	40.6 (16.5-71.3)

When compared with saline inhalation, heaves horses had significantly ($P<0.05$) increased BALF neutrophil ratios and counts after HDS (difference in median count 2.25 x 10^5/ml, 95% CI 0.76-3.74; 34-fold increase), HDS-LPS (difference in median count 0.92 x 10^5/ml, 95% CI 0.29-2.40; 8-fold increase) and HDS-LPS+LPS (difference in median count 1.68 x 10^5/ml, 95% CI 0.53-4.21; 27-fold increase) (Table 9.2, Figs. 9.5 and 9.6). While BALF neutrophil counts and ratios after HDS and HDS-LPS+LPS did not differ, they were significantly ($P<0.05$) higher than those after HDS-LPS (differences in median counts 1.27 x 10^5/ml, 95% CI 0.13-0.62 and 0.82 x 10^5/ml, 95%CI 0.07-2.65, respectively) (Table 9.2, Figs. 9.5 and 9.6).

Fig. 9.5: BALF neutrophil count ($x10^5$/ml) in heaves (n=6) horses at 6h following inhalation challenge with saline, HDS, HDS-LPS and HDS-LPS+LPS.

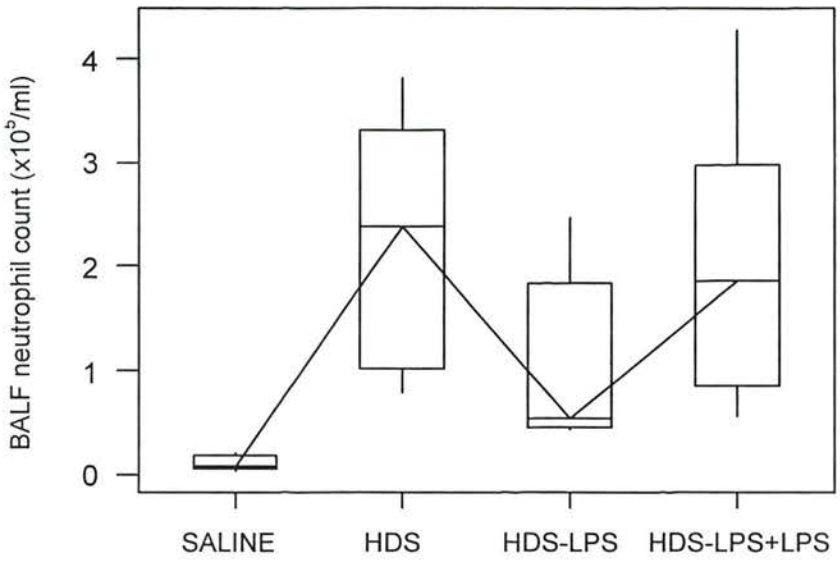
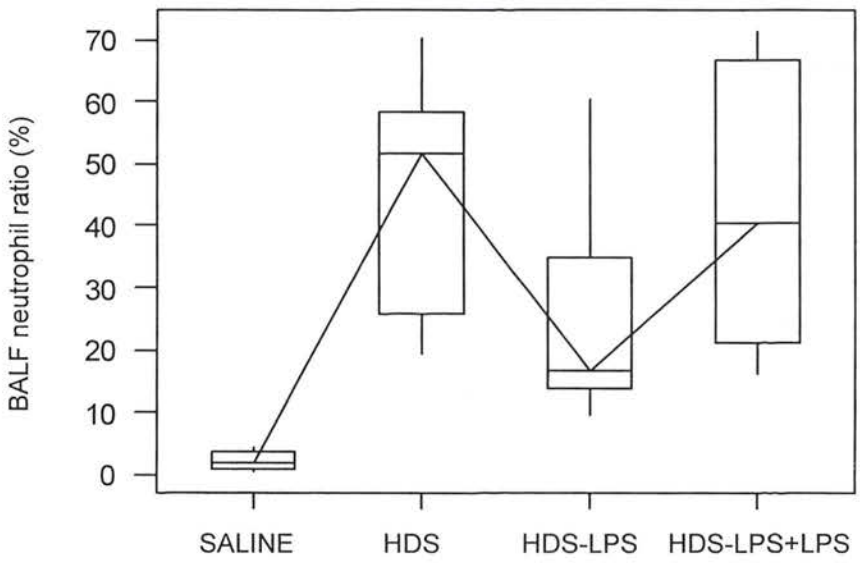


Fig. 9.6: BALF neutrophil ratio (%) in heaves (n=6) horses at 6h following inhalation challenge with saline, HDS, HDS-LPS and HDS-LPS+LPS.



After HDS challenge, controls had a slight, yet significant ($P<0.05$) increase in BALF neutrophil count and ratio, when compared with saline (difference in median count 0.26 x 10^5/ml, 95%CI 0.06-0.49). Controls also showed an increase in BALF neutrophil count and ratio, which approached significance ($P=0.059$), when compared with HDS-LPS (difference in median count 0.23 x 10^5/ml, 90% CI 0.03-0.49) (Table 9.2; Figs. 9.7 and 9.8). The BALF neutrophil count and ratio of controls following HDS-LPS and saline challenges did not differ significantly (Table 9.2; Figs. 9.7 and 9.8). The heaves group had a significantly ($P<0.01$) greater BALF neutrophil count and ratio following HDS-LPS challenge when compared with saline (Fig 9.9 and 9.10).

Fig. 9.7: BALF neutrophil count (x10^5/ml) in control (n=6) horses at 6h following inhalation challenge with saline, HDS and HDS-LPS.

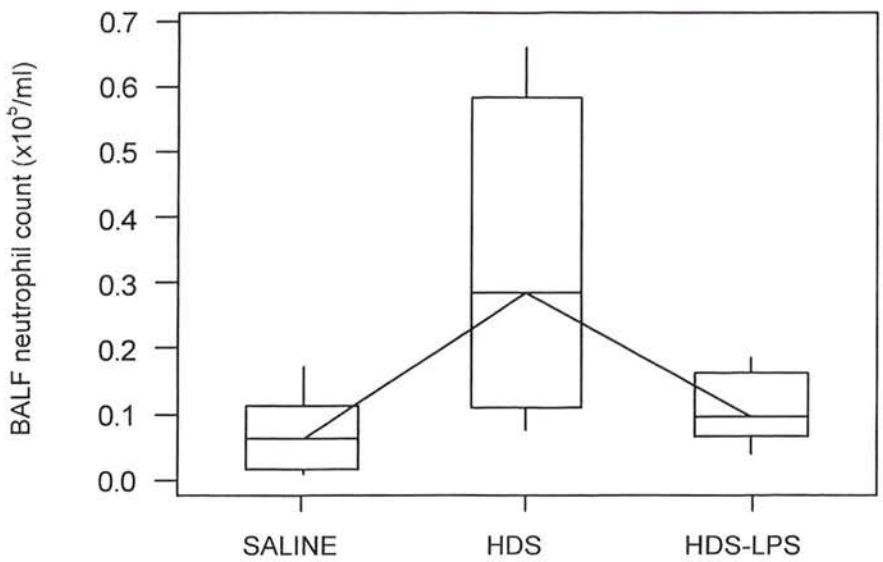


Fig. 9.8: BALF neutrophil ratio (%) in control (n=6) horses at 6h following inhalation challenge with saline, HDS and HDS-LPS.

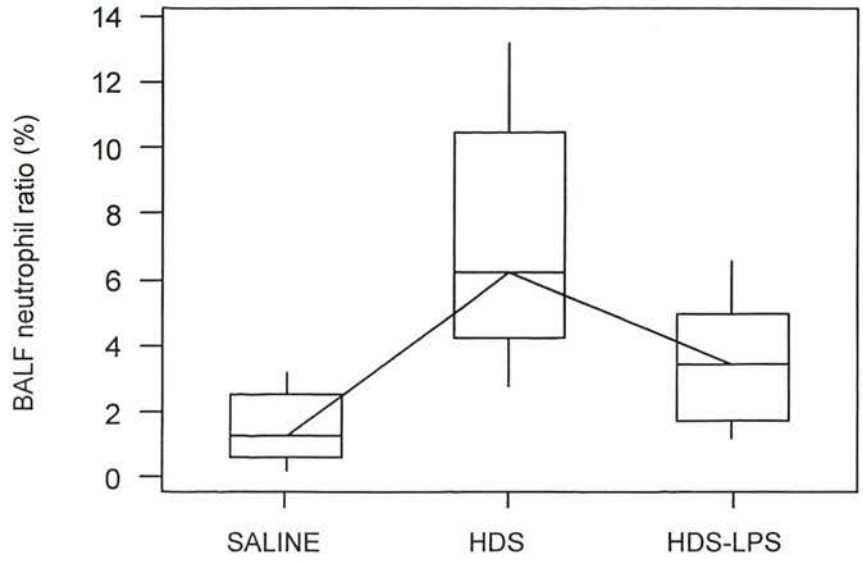


Fig. 9.9: BALF neutrophil count ($x10^5$/ml) in heaves (n=6) and control (n=6) horses at 6h following inhalation challenge with HDS-LPS.

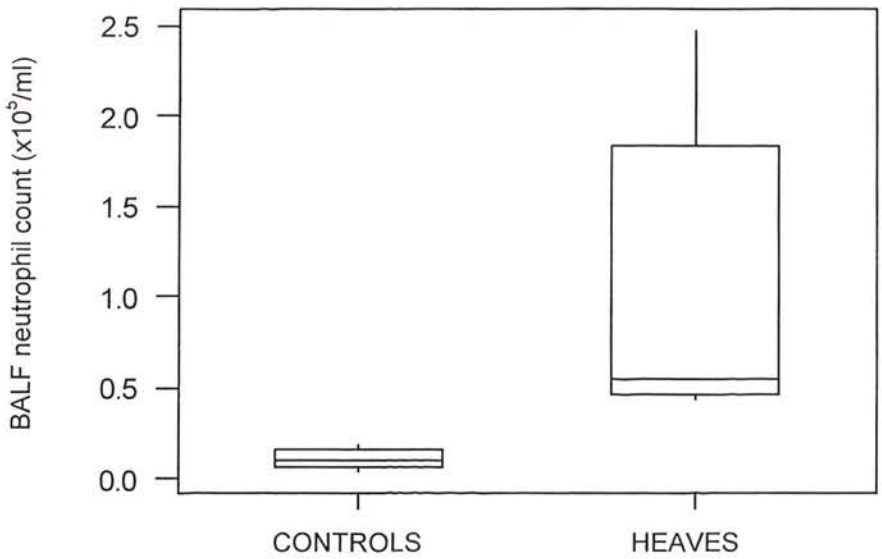
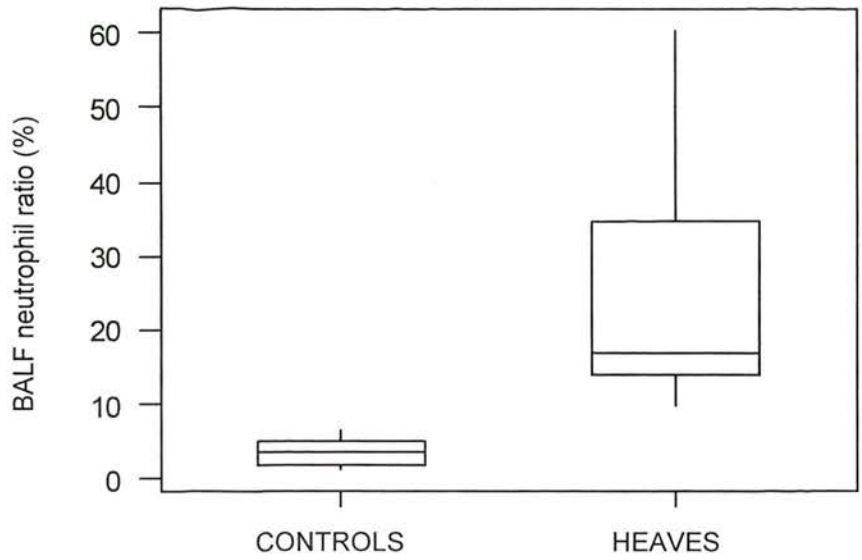


Fig. 9.10: BALF neutrophil ratio (%) in heaves (n=6) and control (n=6) horses at 6h following inhalation challenge with HDS-LPS.



Due to the influence of a BALF neutrophilia on the ratio of other BALF cell types, the latter were considered only as absolute numbers. The absolute number of other BALF cell types 6h following challenge is summarised in Table 9.3.

Table 9.3: Total and differential BALF cell counts ($x10^5$/ml) (median and range) in heaves (C; n=6) and control (H; n=6) horses at 6h following inhalation challenge with saline, HDS and HDS-LPS and in heaves (n=6) horses at 6h following inhalation challenge with HDS-LPS+LPS.
NP = not performed, TCC = total cell count.

		TCC	Lymphocytes	Macrophages	Mast cells	Basophiloid cells	Eosinophils
SALINE	C	3.85 (2.00-9.60)	1.59 (0.99-6.22)	1.98 (0.73-2.80)	0.25 (0.06-0.39)	0.02 (0.00-0.22)	0.03 (0.00-0.06)
	H	4.50 (3.20-5.60)	2.22 (1.54-3.33)	1.77 (1.24-2.96)	0.14 (0.09-0.19)	0.01 (0.01-0.11)	0.00 (0.00-0.01)
HDS	C	4.65 (1.60-8.00)	1.49 (0.66-2.29)	2.18 (0.51-4.19)	0.14 (0.11-0.24)	0.02 (0.00-1.04)	0.01 (0.00-0.23)
	H	4.10 (3.70-7.00)	1.41 (0.61-1.86)	1.17 (0.41-1.75)	0.06 (0.02-0.11)	0.00 (0.00-0.00)	0.01 (0.00-0.13)
HDS-LPS	C	3.20 (1.80-4.40)	1.20 (0.54-1.87)	1.75 (0.78-2.19)	0.15 (0.05-0.30)	0.04 (0.00-0.21)	0.00 (0.00-0.14)
	H	3.75 (3.00-6.20)	1.37 (0.90-2.43)	1.30 (0.67-2.16)	0.04 (0.01-0.14)	0.02 (0.00-0.08)	0.00 (0.00-0.01)
HDS-LPS+LPS	C	NP	NP	NP	NP	NP	NP
	H	4.05 (3.40-6.00)	1.07 (0.61-2.35)	1.07 (0.66-1.38)	0.07 (0.00-0.18)	0.01 (0.00-0.05)	0.01 (0.00-0.04)

In heaves horses, absolute BALF macrophage numbers after HDS-LPS+LPS were significantly ($P<0.05$) lower than those after saline (differences in median 0.85 x 10^5/ml, 95%CI 0.14-1.58) and HDS-LPS (difference in median 0.40 x 10^5/ml, 95%CI 0.01-1.10), but not significantly different from HDS (Fig. 9.11). In heaves horses, absolute BALF mast cell counts were significantly ($P<0.05$) reduced following challenge with HDS (difference in median 0.07 x 10^5/ml, 95% CI 0.03-0.14) and HDS-LPS (difference in median 0.08 x 10^5/ml, 95% CI 0.01-0.15), compared with saline (Fig. 9.12). In heaves horses, absolute BALF lymphocyte counts were reduced by HDS-LPS+LPS (difference in median 0.97 x 10^5/ml, 95% CI 0.58-1.30), and non-significantly ($P=0.059$) reduced by HDS (difference in median 0.91 x 10^5/ml, 90% CI 0.34-1.45), compared with saline (Fig. 9.13).

Fig. 9.11: BALF macrophage count (x10^5/ml) in heaves (n=6) horses at 6h following inhalation challenge with saline, HDS, HDS-LPS and HDS-LPS+LPS.

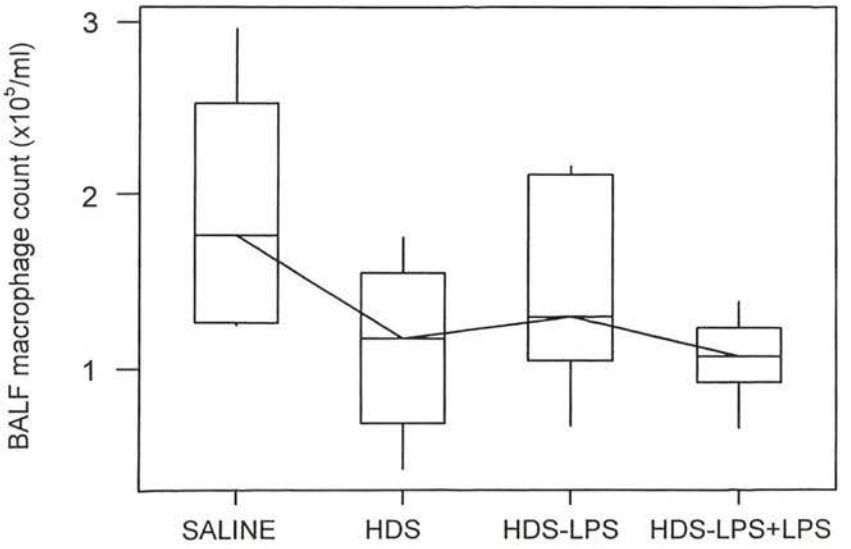


Fig. 9.12: BALF mast cell count ($x10^5$/ml) in heaves (n=6) horses at 6h following inhalation challenge with saline, HDS, HDS-LPS and HDS-LPS+LPS.

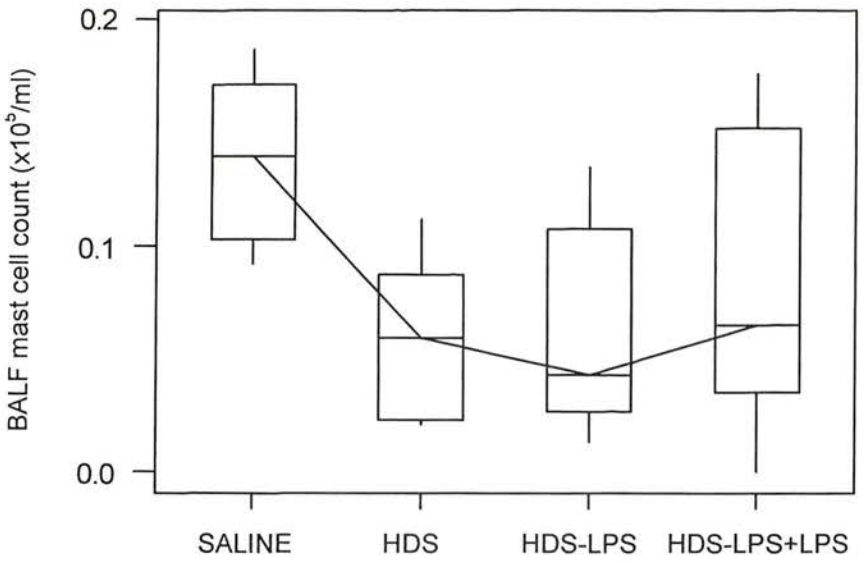
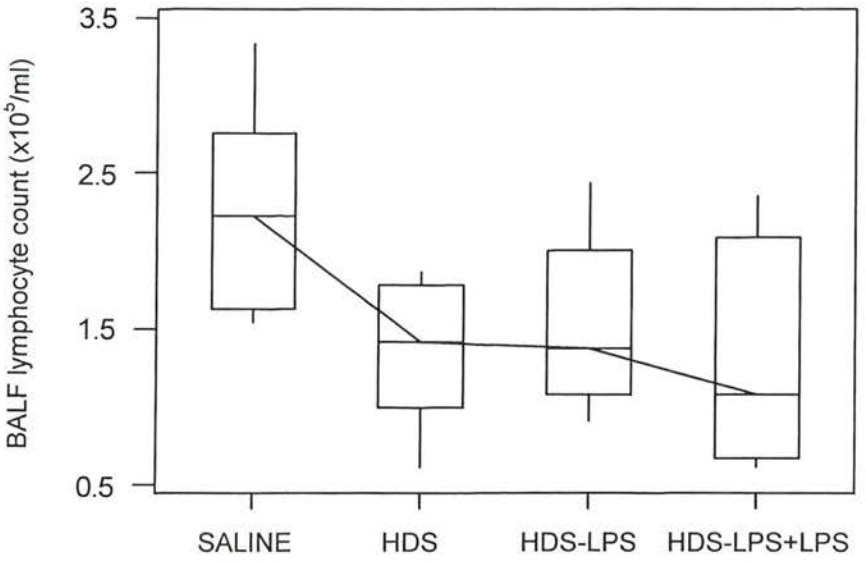


Fig. 9.13: BALF lymphocyte count ($x10^5$/ml) in heaves (n=6) horses at 6h following inhalation challenge with saline, HDS, HDS-LPS and HDS-LPS+LPS.



9.6 Discussion

This study investigated the contributing role of inhaled LPS to airway neutrophilic inflammation and dysfunction in equine heaves. Reduction of the LPS content of aqueous HDS significantly attenuated the airway neutrophilia and dysfunction induced by HDS inhalation challenge in heaves horses, and non-significantly reduced the airway neutrophilia in controls. The airway inflammatory and functional response was re-established in heaves horses after adding back endotoxin, confirming that the attenuation was due specifically to reduction in endotoxin and not to a reduction in other soluble components of HDS, or to the effect of glycerol contamination of the depleted suspension.

The attenuation of the pulmonary functional response was difficult to interpret considering that mild but significant pulmonary dysfunction was present prior to the HDS-LPS challenge. It is possible that this finding resulted in an underestimation of the degree of improvement in lung function following LPS depletion. Also, as percent change in baseline lung function, and not individual post-challenge lung function values were statistically analysed, it is equally possible that an overestimation of the effects the LPS depletion on lung function occurred. Mild pre-existing pulmonary obstruction may have resulted in a reduction in the percentage change of baseline lung function measurements at 5h following challenge. However, the improvement in $RL_{E25\%}$ noted following HDS-LPS challenge, despite there being no statistical difference between baseline measurements, is supportive of the attenuation in lung function following LPS depletion.

The reduction in airway neutrophilic inflammatory response following endotoxin depletion of HDS was less marked than that reported following endotoxin depletion of corn dust extract in a mouse model of organic dust-induced disease (Jagielo *et al.*, 1996a). Jagielo *et al.* (1996a) described that the LPS-depleted extract (initially suspended in pyrogen free water) was lyophilised and reconstituted in Hanks balanced salt solution (HBSS). It is therefore possible that the salts within the HBSS may have altered the solubility and biological activity of the LPS, resulting in a reduced biological effect in mice following inhalation (Galanos and Luderitz, 1984). However, despite this potential for a reduction in toxicity, the salt content should not affect the results of the *Limulus* amoebocyte lysate assay (Galanos and Luderitz, 1984), which did detect an 89% reduction in LPS activity in the Jagielo *et al.* study, compared with 41% in the present study. This greater depletion more likely explains the more marked reduction in neutrophilic response in the Jagielo *et al.* study. The reason for the difference between the two studies with respect to the efficiency of LPS-depletion is unclear. It may, however reflect a greater degree of LPS aggregation in sodium salt solution in the present study compared with pyrogen free water in Jagielo *et als.'* study, thus reducing exposure of the lipid A component of LPS to the polymixin-coated beads (Galanos and Luderitz, 1984; Makela and Stocker, 1984).

Other pro-inflammatory agents, such as dust particulates, which were present in HDS-LPS but not in the soluble corn dust extract, may have contributed to the airway response in the present study. HDS, containing both soluble and particulate dust components, was used since particulates have been shown to contribute to the airway response to HDS (Chapter 8). Endotoxin could only be removed from the HDS

supernatant and not from the particulates, since the removal of polymixin-coated beads following mixing with solution/suspension required centrifugation, a process which would also have removed the HDS particulates. This was not considered problematic since the supernatant contained 83% of the endotoxin activity of HDS (Chapter 6), although the particulate endotoxin content may have been underestimated (Rylander *et al.,* 1989).

Horses received an estimated total exposure of 12.8μg endotoxin during the HDS-LPS challenge, compared with 21.6μg during the HDS and HDS-LPS+LPS challenges. Interestingly, in the endotoxin depletion and add-back experiments, the magnitude of the airway response was altered to a greater extent than could be solely attributed to the direct action of 8.8μg (i.e. 21.6-12.8) LPS. Indeed, extrapolation from previously described data (2.5.2.6) indicates that inhalation of 20μg soluble LPS in these same heaves horses induced only mild airway neutrophilia (0.28 x 10^5/ml, 0.18-0.53). This equated to an increase in median neutrophil count of 0.20 x 10^5/ml (95% CI 0.06-0.48) neutrophils without airway dysfunction as compared with saline. Despite its limitations, this comparison suggests that other components of HDS enhance the activity of the LPS, resulting in a more severe airway response than would have resulted from inhalation of LPS alone. Therefore considering the apparent synergy between LPS and other HDS components described in this study, more significance may be attributed to airborne endotoxin when inhaled in concert with other dust components.

The endotoxin activity removed from HDS by polymixin adsorption likely comprised a variety of LPS types, in contrast to the *S. typhimurium* Ra60 mutant used in the LPS challenges (2.3.3.1) and this current "add-back" experiment. The *S. typhimurium* Ra60 mutant LPS (i.e. complete core oligosaccharide plus lipid A) was chosen because it was not possible to produce a pure endotoxin mix that is representative of those types of LPS encountered in equine stables. However, the complete core oligosaccharide plus lipid A is responsible for a major part of the biological activity of LPS and is likely to be present in high concentrations in HDS, since it is shared by many Enterobacteriaceae including all *Escherichia coli* and *Salmonella* species (Prof. IR Poxton, personal communication). It could however be argued that the homogenous preparation of *S. typhimurium* Ra60 mutant LPS may have a lower virulence following inhalation due to the truncated polysaccharide chain, resulting in a reduction in antiphagocytic properties (Taussig, 1984). However the measured responses to HDS and HDS-LPS+LPS inhalation were comparable, indicating that, as well as having similar activity in the *Limulus* amoebocyte lysate assay, the soluble LPS used for the add-back had similar biologic activity *in vivo* to the various endotoxins removed by the polymixin beads.

In conclusion, this study has demonstrated that inhaled endotoxin contributes markedly to the airway inflammatory and functional response to HDS inhalation in heaves. In addition, the response to inhaled endotoxin is enhanced when co-presented to the lung with other organic dust components. This may have major implications when response thresholds for endotoxin exposure in dusty environments are calculated by direct extrapolation from soluble LPS dose-response inhalation

experiments. Under such circumstances, consideration should also be given to the presence of other organic dust components, which may magnify the pulmonary response to a given dose of endotoxin. Such dust components appear to have greater significance when considering exposure levels in heaves-susceptible horses, possibly due to their exaggerated pulmonary response to *specific* agents, such as mould allergens.

CHAPTER 10: POTENTIATION OF THE RESPONSE TO INHALED LPS BY HAY DUST SUSPENSION PARTICULATES

10.1 Summary

This study investigated the relative contribution of inhaled LPS and organic dust particulates (primarily mould spores) in heaves. Washed particulates harvested from HDS enhanced the airway response to inhaled LPS in heaves horses. This indicates that inhaled organic dust particulates and LPS have a synergistic pro-inflammatory action in heaves. Interestingly, the magnitude of the enhancement in airway response following the addition of LPS to washed particulates was greater than that which could be attributed solely to LPS *per se,* indicating that the activity of LPS was enhanced by the dust particulates. It was concluded that inhaled endotoxin contributes to induction of airway inflammation and dysfunction in heaves and that the airway response to inhaled endotoxin is synergised by co-challenge with organic dust particulates.

10.2 Introduction

As with human organic dust-induced diseases (Popendorf, 1986; Rylander, 1988; Milanowski, 1997), the relative importance of different dust components in inducing heaves is unknown (Derksen, 1993; McGorum *et al.,* 1993c), and warrants further study (Robinson, 1998). A nebulised aqueous hay dust suspension (HDS)-induced disease model has successfully reproduced heaves in susceptible horses, differentiated susceptible horses from control horses and demonstrated the

importance of both soluble and particulate hay dust components in the induction of heaves (Chapters 7 and 8; (Pirie *et al.,* 2001a, c and d). In addition, synergy between the LPS content of HDS and other HDS components has been shown using the same model (Chapter 9). This chapter describes a further series of inhalation experiments performed to determine whether the *particulate* components HDS alone could potentiate the airway response to inhaled LPS in heaves-susceptible horses. This involved co-challenge with washed dust particulates harvested from HDS (WP as previously described 6.3.4) and soluble LPS.

10.3 Materials and methods

10.3.1 Subjects

Seven previously described horses with a history and clinical diagnosis of heaves (2.3.1.1) were used. Throughout the study all horses were kept in a low dust environment (2.3.2.1). The disease status of all subjects was confirmed by the previously described mouldy hay/straw challenge (2.3.4.2).

10.3.2 Inhalation challenge material

To determine whether co-inhalation with dust particulates enhanced the pulmonary inflammatory and functional response to inhaled endotoxin, heaves horses received a combined inhalation challenge with LPS and washed particulates (WP). This challenge was referred to as WP+LPS. WP was prepared from HDS as previously described (6.3.4). LPS, from a stock solution of 8.89mg/ml (as previously described 2.3.3.1), was added to the WP to give a final LPS concentration of the WP+LPS of

20μg/ml. This LPS concentration was comparable to that of the non-fractionated HDS (6.3.2.4). A constant 1ml volume of challenge substance was used for all challenges.

10.3.3 Inhalation challenges

10.3.3.1 Inhalation challenge protocol

To facilitate subject cooperation, horses were sedated immediately prior to inhalation challenge as previously described (2.3.4.1). The aerosol was generated and delivered as previously described (2.3.4.1). To minimise potential carry-over effects of preceding challenges, inhalation challenges on any one subject were conducted a minimum of 14 days apart and all horses were shown to have normal clinical findings immediately prior to each inhalation challenge. In order to assess any carry over effects, all baseline lung function values were compared those of any challenges to which comparisons were made, and 6 heaves horses received a repeat challenge with WP+LPS after completion of all other challenges. As well as assessing any potential carry-over effects, this also determined the repeatability of this challenge.

10.3.3.2 Positive (HDS) and negative (saline) control challenges

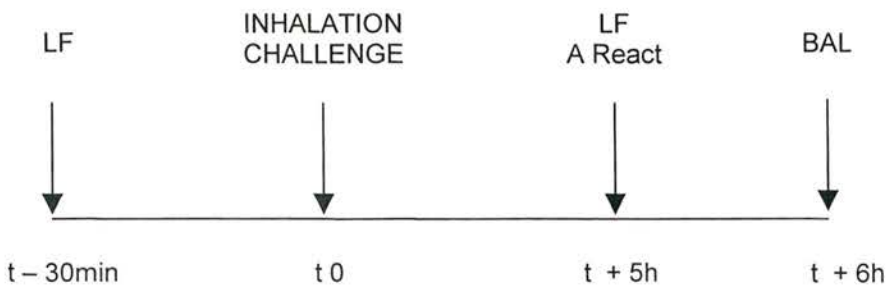
Comparisons of the responses to WP+LPS were made with previously described responses to inhalation challenge with saline and 20μg LPS (Chapter 2), HDS-1[100] (Chapter 7), WP (Chapter 8) and WP/SUP[m] (Chapter 8) in the same horses.

10.3.4 Monitoring the response to challenges

The method and timing of assessment of response to each challenge is summarised in Fig. 10.1. The response to WP+LPS was assessed using lung mechanics, airway reactivity and BALF cytology as previously described (2.3.7).

Fig. 10.1: Study design.

BAL = bronchoalveolar lavage; LF = lung function evaluation; A React = airway reactivity evaluation



10.4 Statistical analysis

As the data were either not normally distributed and/or the groups of compared data did not have equal variance, non-parametric tests were used. The effect of each inhalation challenge was determined by performing within-group analyses.

To check for the presence of any carry-over effects of one challenge on a subsequent challenge, pre-challenge (baseline) measurements of lung mechanics were compared using a Friedman test, and when significant, a Wilcoxon Rank Sum test was performed on paired data.

To check for any effects of challenge, where pre-challenge measurements were made at t-30min (lung mechanics), the post-challenge values were expressed as % of baseline value. As saline was the principle vehicle for delivery of all challenges, the effect of challenge was assessed by pairing and subtracting post-WP+LPS (% of baseline value) and post-saline (% of baseline value) data. Where no pre-challenge data was collected (BALF cytology), comparisons were made with saline (placebo) challenge data at equivalent time points. A Friedman test was performed on sets of paired data to reduce the risk of type 1 errors, and when significant, a Wilcoxon Rank Sum test was performed on paired data.

Significance was assumed if $P<0.05$. Following correction for any effect of saline inhalation, changes from baseline are expressed as increase/decrease or change in median values, with 95% confidence interval for the difference in median, calculated for non-parametric data (Campbell and Gardner 1994).

The two separate WP+LPS inhalation challenges were compared using a Wilcoxon Rank Sum test, and as an indication of repeatability, the differences in paired values were plotted against their mean (Bland and Altman 1986)(Bland and Altman, 1986). Good repeatability was assumed if the calculated differences in paired values fell within 2 standard deviations of the mean of the differences (British Standards Institution, 1979). Results are expressed as median and range.

10.5 Results

10.5.1 Response to inhalation challenge

10.5.1.1 Lung mechanics and airway reactivity

Lung function measurements raw data are presented in Appendix 10.1. With the exception of $RL_{E25\%}$, there was no significant difference in the baseline lung function measurements prior to each of the challenges, indicating a lack of carry-over effects. The percentage of baseline lung function measurements following challenge are presented in Table 10.1. PCCdyn70 values following challenge are presented in Appendix 10.2.

Following correction for any effects of saline inhalation, WP+LPS inhalation induced airway dysfunction as evidenced by a significant increase in $R_{LE25\%}$ (increase in median 103%, 95% CI 30-191) (Fig. 10.2), but did not alter airway reactivity.

Fig. 10.2: Percent of baseline $RL_{E75\%}$ in heaves horses (n=7) at 5h following inhalation challenge with saline, 20μg LPS, WP, WP+20μg LPS and HDS[100] minus percent of baseline $RL_{E75\%}$ at 5h following inhalation challenge with saline. * = outlier.

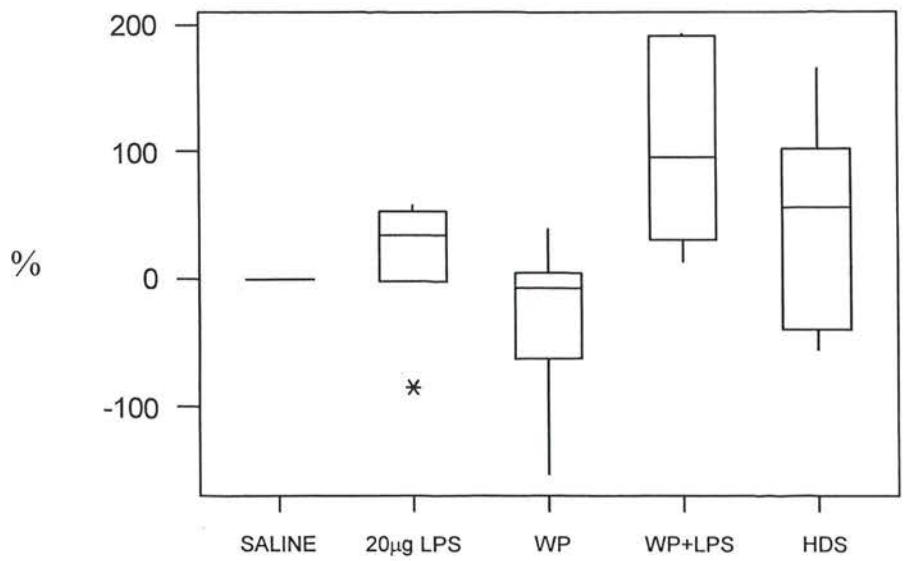


Table 10.1(a and b): Percent (%) of lung function measurements (median and range) in heaves (n=7) horses at 5h following inhalation challenge with saline, 20μg LPS, WP, WP+20μg LPS and HDS.

(a)

	Cdyn	dPpl	RL_{iso}	RR	VT	Wb	$RL_{E25\%}$	$RL_{E50\%}$	$RL_{E75\%}$	$RL_{I25\%}$	$RL_{I50\%}$	$RL_{I75\%}$
SALINE	96.4 (57.9-104.9)	111.6 (60.0-260.6)	95.9 (76.9-197.3)	90.1 (48.6-110.6)	113.2 (67.2-200.7)	99.2 (29.0-232.9)	70.8 (35.8-172.0)	66.1 (0.0-125.3)	69.3 (42.7-115.0)	129.4 (77.0-593.2)	102.5 (84.9-590.5)	89.4 (70.5-446.9)
20μg LPS	67.0 (51.3-100.6)	129.1 (89.7-169.9)	108.6 (63.9-160.3)	100.7 (62.2-126.3)	94.0 (87.9-151.1)	105.8 (78.3-154.5)	86.6 (51.1-169.3)	70.9 (0.8-185.5)	70.0 (60.6-211.1)	133.1 (59.3-620.9)	133.6 (66.5-419.5)	107.1 (74.6-400.0)
WP	83.7 (63.6-121.8)	96.9 (78.3-159.3)	111.5 (47.4-165.8)	97.0 (51.9-133.3)	98.9 (93.0-131.9)	83.4 (41.1-171.5)	57.8 (9.8-117.6)	72.7 (11.6-141.5)	93.7 (42.1-176.8)	117.3 (63.7-210.0)	119.1 (51.0-293.8)	100.7 (38.9-145.3)
WP+20μg LPS	79.0 (43.6-120.4)	93.0 (80.8-253.1)	128.8 (88.6-263.8)	92.0 (79.4-132.4)	95.2 (86.2-123.6)	118.0 (60.6-298.3)	149.4 (126.3-365.0)	131.6 (97.6-190.3)	117.0 (85.9-212.1)	108.2 (83.3-218.6)	113.6 (55.4-265.3)	113.1 (60.6-272.0)
HDS	105.4 (70.1-139.8)	123.0 (97.0-172.6)	118.2 (85.8-193.2)	87.6 (66.8-110.6)	111.0 (98.0-159.9)	115.3 (79.3-196.1)	108.9 (8.2-274.1)	120.2 (20.8-148.7)	132.9 (75.0-175.2)	128.0 (69.7-180.8)	126.4 (83.2-181.2)	142.5 (70.1-159.4)

(b)

	T_E	T_I	$T_I:T_E$	V'_E	V'_{Emax}	V'_{Imax}	Wb_{el}	Wb_{res}	Wb_{Eres}	Wb_{Ires}	Wb_{Itot}
SALINE	103.4 (83.1-219.9)	111.8 (86.3-263.8)	101.5 (41.3-110.5)	104.7 (43.8-117.9)	116.0 (43.3-137.9)	101.2 (56.1-140.4)	131.6 (48.3-646.1)	95.3 (48.2-505.7)	86.2 (42.0-439.0)	112.0 (58.5-545.3)	118.1 (51.9-597.2)
20μg LPS	102.1 (80.6-177.9)	93.4 (76.8-241.2)	93.1 (75.9-166.1)	99.2 (84.1-121.9)	112.2 (49.1-124.6)	111.6 (73.7-140.5)	162.2 (90.6-400.2)	105.5 (71.7-172.2)	86.5 (55.9-150.6)	120.7 (75.1-304.1)	146.8 (84.6-355.3)
WP	106.4 (71.2-203.9)	93.3 (76.3-179.9)	88.1 (59.0-111.1)	109.8 (51.0-132.1)	110.9 (55.6-142.7)	114.5 (58.7-158.5)	115.5 (76.9-234.3)	93.0 (69.2-169.1)	89.9 (67.7-167.4)	97.3 (71.6-170.4)	112.1 (84.3-203.8)
WP+20μg LPS	105.8 (71.0-123.5)	108.2 (79.1-117.3)	104.0 (72.9-112.2)	92.5 (73.1-114.3)	87.2 (73.7-116.3)	88.4 (77.4-99.5)	116.9 (62.5-314.8)	102.9 (71.7-318.5)	111.2 (72.7-288.6)	98.0 (70.7-359.1)	98.0 (75.9-340.1)
HDS	122.1 (82.3-217.1)	108.6 (90.0-162.6)	92.8 (37.4-123.8)	95.8 (76.1-139.5)	100.1 (61.6-144.6)	100.8 (83.2-129.8)	125.9 (100.7-183.6)	137.2 (94.7-230.2)	131.2 (86.6-381.0)	136.0 (92.3-176.7)	138.1 (97.4-180.5)

10.5.2.2 BALF cytology

The BALF neutrophil counts and ratios in heaves horses 6h following challenge with WP+LPS are presented in Appendix 10.3, and summarised in Table 10.2. Data from saline, 20μg LPS, HDS-1, WP and WP/SUP[m] challenge are included for comparison.

Table 10.2: BALF neutrophil counts ($x10^5$/ml) and ratios (%) (median and range) heaves (n=7) horses at 6h following inhalation challenge with saline, WP, SUP, 20μg LPS, WP+20μg LPS, WP/SUP[m] and HDS.

	BALF neutrophil count ($x10^5$/ml)	BALF neutrophil ratio (%)
SALINE	0.06 (0.03-0.20)	2.3 (0.6-4.5)
WP	0.19 (0.10-0.65)	3.8 (2.4-13.0)
SUP	0.53 (0.28-1.64)	8.3 (4.2-22.2)
20μg LPS	0.28 (0.18-0.53)	6.1 (5.6-7.2)
WP+LPS	**0.71 (0.4-1.62)**	**25.3 (13.0-46.7)**
WP/SUP[m]	1.36 (0.43-4.55)	25.7 (7.9-58.3)
HDS-1	2.17 (0.54-3.81)	50.7 (19.4-70.4)

WP+LPS induced an airway neutrophilia in heaves horses, which was significantly ($P<0.05$) greater than that for saline (increase in median count 0.89 x 10^5/ml, 95% CI 0.42-1.45), 20μg LPS (increase in median count 0.70 x 10^5/ml, 95% CI 0.13-1.27) and WP (0.67 x 10^5/ml, 95% CI 0.18-1.21), yet significantly ($P<0.05$) lower than that for HDS (Table 10.1; Figs. 10.3 and 10.4).

Fig. 10.3: BALF neutrophil count (x10^5/ml) in heaves (n=7) horses at 6h following inhalation challenge with saline, 20μg LPS, WP, WP+20μg LPS and HDS[100].

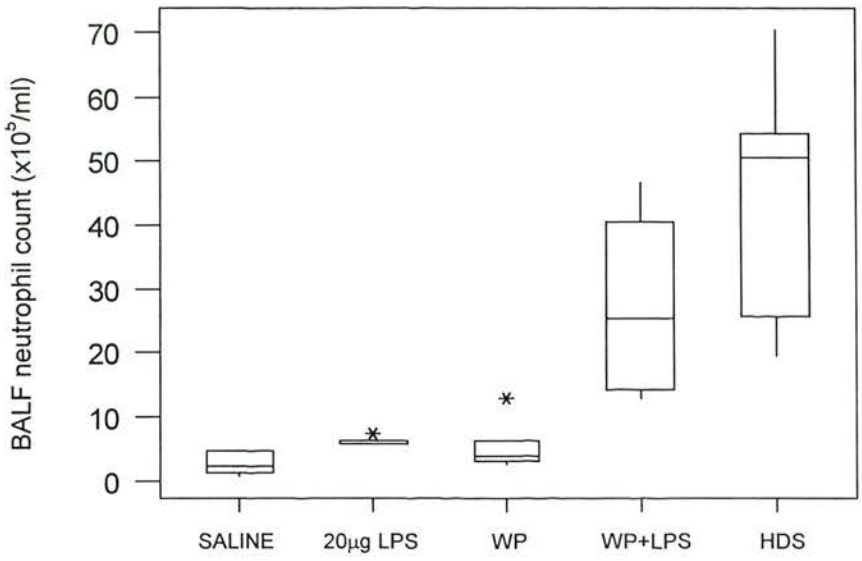
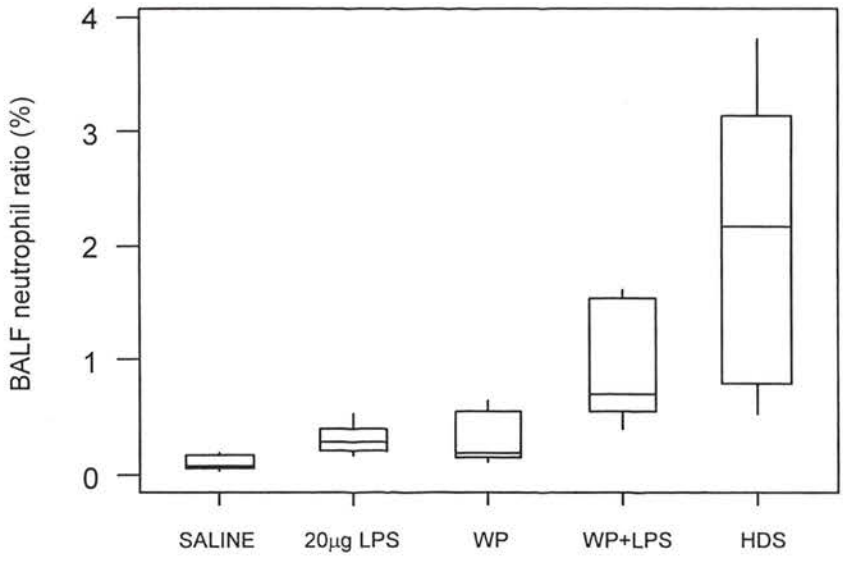


Fig. 10.4: BALF neutrophil ratio (%) in heaves (n=7) horses at 6h following inhalation challenge with saline, 20μg LPS, WP, WP+20μg LPS and HDS[100]. * = outlier.



There was no significant difference in BALF neutrophil numbers or ratio following WP/SUP[m] and WP+LPS inhalation challenges (Figs 10.5 and 10.6).

Fig. 10.5: BALF neutrophil count ($x10^5$/ml) in heaves (n=7) horses at 6h following inhalation challenge with WP/SUP[m] and WP+20μg LPS.

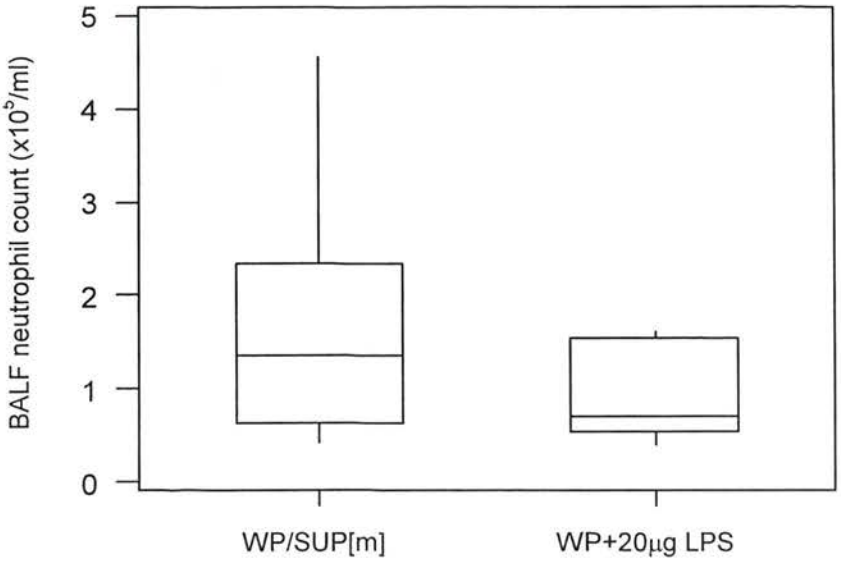
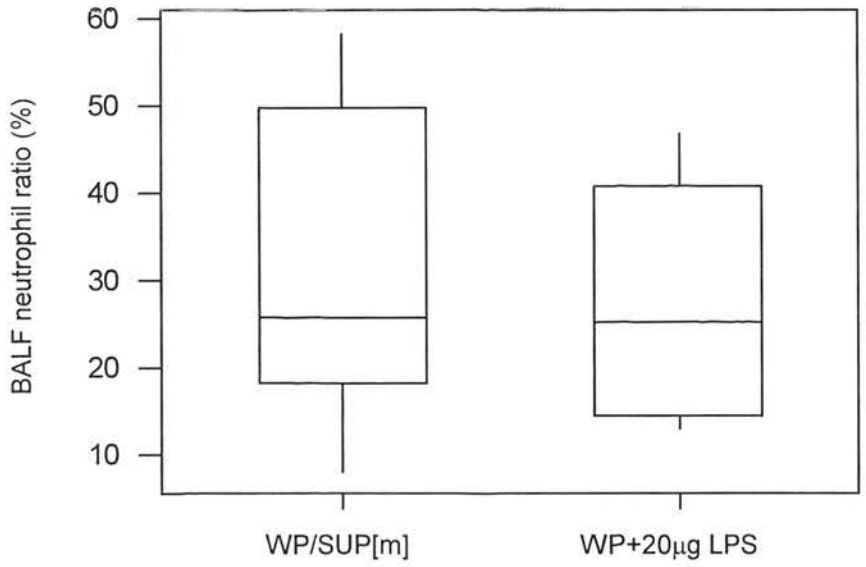


Fig. 10.6: BALF neutrophil ratio (%) in heaves (n=7) horses at 6h following inhalation challenge with WP/SUP[m] and WP+20μg LPS.



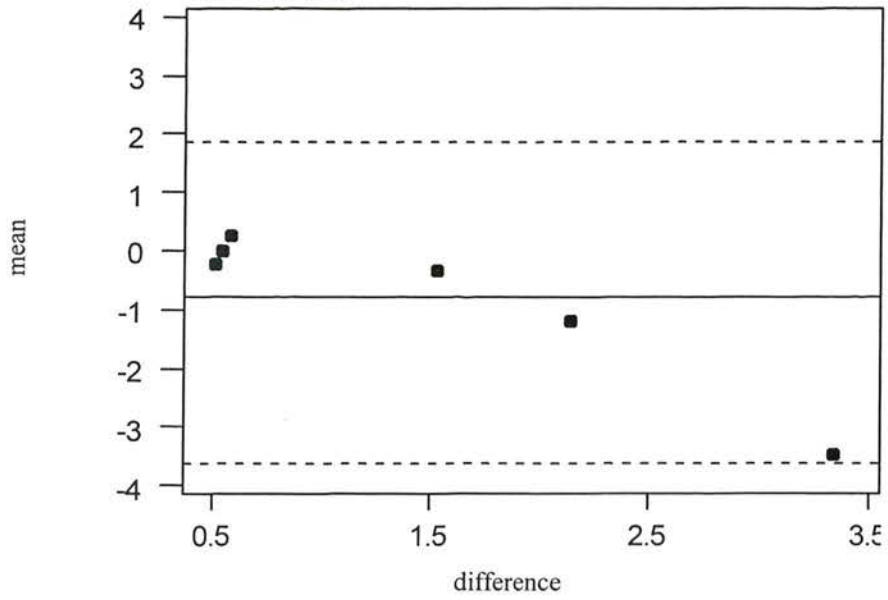
Due to the influence of a BALF neutrophilia on the ratio of other BALF cell types, the latter were considered only as absolute numbers. The absolute numbers of these cells are summarised in Table 10.3. The responses of other BALF cells to HDS and WP+LPS did not differ significantly.

Table 10.3: Total and differential BALF cell counts ($x10^5$/ml) (median and range) in heaves (n=7) horses at 6h following inhalation challenge with saline, WP, SUP, 20μg LPS, WP+20μg LPS, WP/SUP[m] and HDS[100]. TCC = total cell count.

	TCC	Lymphocytes	Macrophages	Mast cells	Basophiloid cells	Eosinophils
SALINE	3.80 (1.30-5.60)	2.09 (0.80-3.33)	1.58 (0.34-2.96)	0.13 (0.09-0.19)	0.01 (0.00-0.11)	0.01 (0.00-0.01)
WP	5.50 (2.00-9.10)	2.21 (0.95-5.20)	1.99 (0.71-3.92)	0.14 (0.09-0.23)	0.02 (0.00-0.04)	0.07 (0.00-1.54)
SUP	7.30 (3.40-9.40)	2.94 (1.63-5.61)	1.98 (0.55-4.21)	0.13 (0.02-0.23)	0.01 (0.00-0.03)	0.07 (0.01-0.33)
20μg LPS	4.60 (2.90-7.40)	2.45 (1.88-4.83)	1.68 (0.70-2.57)	0.07 (0.06-0.30)	0.01 (0.00-0.12)	0.00 (0.00-0.01)
WP+LPS	**3.80 (2.30-4.20)**	**1.38 (0.81-2.08)**	**0.89 (0.36-1.51)**	**0.09 (0.00-0.14)**	**0.01 (0.00-0.03)**	**0.01 (0.00-0.03)**
SUP/WP[m]	5.40 (2.30-10.20)	2.33 (1.09-2.83)	1.46 (0.38-4.81)	0.08 (0.02-0.24)	0.00 (0.00-0.02)	0.01 (0.00-0.20)
HDS	4.10 (2.10-7.00)	1.19 (0.61-1.86)	1.16 (0.41-1.75)	0.07 (0.02-0.14)	0.00 (0.00-0.10)	0.01 (0.00-0.13)

There was no significant difference in the number of any BALF cell type between the first and second WP+LPS challenges, except for a significant ($P<0.05$) elevation in macrophage count after the second challenge. As all of the 6 calculated differences in paired neutrophil values fell within 2 standard deviations of the mean of the differences, and the mean of the differences approximated zero (Fig 10.7), repeatability was considered good (Bland and Altman 1986).

Fig. 10.7: Difference between BALF neutrophil counts ($x10^5$/ml) plotted against the mean of the BALF neutrophil counts ($x10^5$/ml) in heaves (n=6) horses at 6h following both WP+LPS inhalation challenges. Solid line = mean of the differences; dotted line = mean of the differences ± 2 standard deviations of the differences.



10.6 Discussion

In agreement with the results of the study described in Chapter 9, this study highlights the contributing role of inhaled LPS to airway inflammation and dysfunction in equine heaves. Following LPS depletion (HDS-LPS) the residual neutrophilic response to HDS-LPS inhalation challenge (9.5.2.3) may have resulted from incomplete removal of LPS. However, it may also have resulted from the presence of other HDS pro-inflammatory agents present in HDS-LPS. The addition of 20μg soluble LPS to washed particulates (predominantly washed fungal spores) harvested

from HDS permitted the investigation of the potential synergistic effects of both of these components in the absence of the other soluble components of HDS.

The addition of LPS, at a concentration comparable to that present within the HDS supernatant (SUP; 6.3.5.1), enhanced the airway inflammatory response to WP harvested from HDS, to a greater extent than could be attributed to the effects of inhalation of 20ug LPS *per se*. Previous inhalation challenges with 20μg soluble LPS in the same heaves horses induced only mild airway neutrophilia (0.28 x 10^5/ml, [0.18-0.53]), equating to an increase in median neutrophil count of 0.20 x 10^5/ml (95% CI 0.06-0.48) compared with saline challenge (2.5.2.6). This finding of synergism is in agreement with previous *in vivo* and *in vitro* studies which demonstrate that particulates enhance the response to pro-inflammatory agents such as LPS, and conversely that the airway response to inhaled particulates is enhanced by LPS priming (Imrich *et al.,* 1999b; Yang *et al.,* 1999; Ning *et al.,* 2000; Oberdorster, 2000).

Synergy between LPS and particulates may be due to binding of LPS to the organic particulates, resulting in a more focal and concentrated LPS challenge, in comparison to the more likely diffuse pulmonary deposition of a solution with dilution throughout the respiratory tract (Urbain *et al.* 1996a). Alternatively, while challenges with WP alone induced only a mild airway neutrophilia (0.19 x 10^5/ml [0.10-0.65]), equating to an increase in median of 0.15 x 10^5/ml (95% CI 0.04-0.48) (8.5.1.4), they may prime target cells thereby enhancing LPS responsiveness. Both *in vitro* and *in vivo* priming of guinea pig alveolar macrophages has been reported following fungal

antigen and glucan challenge (Milanowski, 1996; Milanowski, 1997), both components of HDS (6.3.2) (Pirie *et al.,* 2001d).

In light of the results of previous inhalation studies (Eyre, 1972; Halliwell *et al.,* 1979; McPherson *et al.,* 1979; McPherson and Thomson, 1983; Robinson *et al.,* 1996), which have proposed that heaves is a hypersensitivity response, it is possible that the mould spores within the washed particulates acted as a source of allergen. Consequently mould spore inhalation may have resulted in an allergen-associated increase in LPS binding protein and soluble CD14 receptors in the airways as reported in asthmatic humans, thus magnifying the response to LPS (Martin *et al.,* 1992; Dubin *et al.,* 1996). This synergy may in part explain why the exposure level of experimentally inhaled LPS necessary to induce airway inflammation and dysfunction in heaves horses is markedly greater than that to which horses are exposed naturally (Pirie *et al.,* 2001b).

While this study has confirmed a major role for endotoxin in heaves, other findings indicate that other soluble dust components are also involved. For example, the airway response to LPS may have been partly enhanced by other soluble HDS components such as proteases (Pirie *et al.,* 2001d), since the response to WP+LPS was significantly less than that to HDS. However, as WP+LPS (0.71×10^5/ml [0.4-1.62]; 12-fold increase in median neutrophil count) resulted in an airway neutrophilic response approaching that following WP/SUP[m] (1.36×10^5/ml [0.43-4.55]; 23-fold increase in median), compared with saline, it is likely that LPS and particulates play a significant combined role compared with other HDS components. This combined

effect would have been further supported had BALF neutrophil ratios, rather than BALF neutrophil numbers, been used as the sole indicator of airway neutrophilia, since both WP+LPS and WP/SUP[m] resulted in almost identical BALF neutrophil ratios (25.3% and 25.7%, respectively).

The endotoxin activity removed from HDS by removal of HDS supernatant from the challenge material used in this reported study likely comprised a variety of LPS types, in contrast to the single *S. typhimurium* R60 mutant LPS which was used in the WP+LPS challenge. This Ra chemotype LPS was chosen for reasons highlighted previously (9.6), and although there are limitations with respect to the direct comparison of the *in vivo* effects of the two different sources of LPS, the LPS used in the WP+LPS challenges was considered to be representative of the LPS types encountered in equine stables.

In conclusion, this study has demonstrated that inhaled endotoxin and particulates contribute markedly to the airway inflammatory and functional response in heaves. In addition, the response to inhaled endotoxin is enhanced when co-presented to the lung with other organic dust components, especially hay dust particulates. This work has important implications for organic dust-induced disease in all species since it highlights the importance of quantifying both endotoxin and dust particulate exposure, and not considering each component in isolation.

CHAPTER 11: CONCLUDING ADDENDUM

The aim of this concluding addendum is to summarise the role of inhaled endotoxin in the aetiopathogenesis of equine heaves, in light of the findings of this study.

11.1 Heaves does not result solely from the inhalation of airborne endotoxin

The findings of the present study have demonstrated that the development of pulmonary inflammation and dysfunction associated with heaves in susceptible horses is not solely due to the inhalation of endotoxin. This evidence for this conclusion is based upon consideration of the following results:

The dose-response experiments detailed in Chapter 2 revealed that LPS inhalation did reproduce some of the features of heaves, namely airway neutrophilia and pulmonary dysfunction in heaves susceptible horses. However, in contrast to mouldy hay/straw exposure, none of the inhaled LPS doses induced a significant increase in the tracheal secretion score in susceptible horses. Although the tracheal secretion score was increased in 2 of the 7 heaves horses following the high doses of LPS, the increase was not significant when the data for the whole group was considered. These doses of LPS greatly exceeded the level encountered during a 5h exposure to mouldy hay and straw, which did significantly increase tracheal secretion score in all 7 heaves horses. Furthermore a significant increase in tracheal secretion score was detected in all 7 of the heaves group following HDS inhalation.

Despite the finding that the response threshold was lower in the heaves group for both pulmonary inflammation and dysfunction, following inhalation of the 2 higher doses of LPS, there was no statistically significant difference from controls with respect to airway neutrophilia. This is in contrast to 5h housing in a mouldy hay/straw environment, which induced a significantly greater airway neutrophilia in the heaves group. Furthermore, the soluble LPS exposure required to induce a similar degree of airway neutrophilia in the heaves group to that measured following a 5h mouldy hay/straw challenge was markedly greater than the endotoxin exposure encountered during this challenge.

There are, however, certain problems in making comparisons between a natural long-term endotoxin exposure and a short-term aerosolised purified LPS challenge. These include the likely underestimation of particulate endotoxin collected during the hay/straw challenge, a possible difference in the deposition and clearance of endotoxin/LPS between the 2 challenge systems, and the possible difference in biological activity of the variety of endotoxins in the stable dust compared with the pure LPS used in the aerosolised challenge.

The experiments detailed in Chapter 3 were conducted to overcome some of the highlighted problems associated with comparing the aforementioned challenge systems. These experiments involved measuring the pulmonary inflammatory and functional response of heaves horses to 2 separate dusty hay/straw exposures of equal duration. The results of this study indicated that disease severity did not relate to the level of endotoxin exposure. In fact, the challenge which resulted in a lesser degree of

pulmonary inflammation resulted in an, albeit non-significantly, greater exposure to airborne endotoxin within the respirable dust.

The response to HDS inhalation in the heaves group was dependent on the source of the stable dust used to produce the suspension. HDS-3 resulted in a greater degree of pulmonary inflammation and dysfunction, despite having a lower concentration of endotoxin than HDS-1 and HDS-2. This finding was further supported by the significantly lesser response to the soluble fraction of HDS (SUP) in the heaves group, compared with the non-fractionated HDS, despite the SUP containing almost all of the endotoxin activity.

The pulmonary inflammatory response to HDS inhalation was dose-dependent in both groups. However, the magnitude of response in the heaves group following inhalation of each dose of HDS could not be attributed purely to the endotoxin activity within the HDS, although it is likely that the particulate endotoxin concentration of the HDS was underestimated by the *Limulus* amoebocyte lysate assay. However, while the LPS dose response experiments demonstrated a 12-fold increase in median BALF neutrophil count over a 100–fold increase in LPS dose, a similar increase (15-fold) was seen with only a 3–fold increase in HDS dose.

11.2 Evidence for the role of inhaled moulds in heaves aetiopathogenesis

The challenges detailed in Chapter 2 indicated that a greater degree of pulmonary inflammation followed exposure to hay with visible mould contamination. The

presence of mould contamination of this hay was supported by the (albeit non-significantly) higher β-D-glucan concentration of the respirable dust collected throughout the challenge with the visibly mouldy hay.

In agreement with the findings of the studies detailed in Chapter 3, the response of heaves horses to HDS inhalation was also shown to be related to the β-D-glucan content of the HDS, which probably reflected the higher particulate (predominantly mould spores) content.

11.3 Evidence that endotoxin, in the presence of other inhalants, contributes to heaves aetiopathogenesis

Although there was sufficient evidence to indicate that inhaled endotoxin was not *solely* responsible for the induction of heaves in susceptible horses, it was considered likely that it would contribute to disease severity in the presence of other inhaled agents. Consequently, it was considered important to evaluate the response of heaves susceptible horses to inhalation of LPS in conjunction with other stable dust components.

The results of the inhalation challenges detailed in Chapter 5 suggested that LPS contamination of *A. fumigatus* extract contributed to the pulmonary neutrophilia induced by experimental inhalation challenge with *A. fumigatus* extract. However, this could only have been confirmed definitively had a further series of inhalation challenges been conducted following "add back" of the depleted LPS. Interestingly,

extrapolation from the LPS dose-response experiments detailed in Chapter 2 indicated that the reduction in the airway neutrophilia following depletion of LPS could not be solely attributed to the LPS activity removed during depletion, indicating that these agents acted in a *synergistic* fashion.

Inhalation challenge with fractionated HDS revealed that none of the fractions induced a neutrophilic response in the heaves group of the same magnitude to that induced by HDS. Given that previous challenges had indicated that the severity of response was related to β-D-glucan exposure (Chapters 3 and 7), this was an interesting finding as the particulate fraction contained almost all of the HDS β-D-glucan activity.

This work indicates that the response to HDS was dependent upon the presence of both the SUP and the WP, with a synergistic activity between these 2 fractions, as also demonstrated between the *A. fumigatus* extract and LPS (Chapter 5).

To establish whether the endotoxin content of the SUP acted in a synergistic fashion with the other components of the HDS, HDS was partially depleted of LPS as detailed in Chapter 9. LPS depletion resulted in a reduction in the pulmonary inflammation and dysfunction in the heaves group. Interestingly, the reduction in airway neutrophilia could not be attributed to the endotoxin activity that was removed if the contribution of the endotoxin activity to the response to HDS inhalation was solely *additive*. The re-establishment of the response following "add-back" of LPS, at a quantity equivalent to that which was removed, was an important finding. Firstly

this demonstrated that the reduction in response following LPS depletion was due specifically to the reduction in the LPS content. Secondly, it demonstrated that the biological activity of the various endotoxins that were removed was comparable to that of the soluble LPS used for the "add back" experiments, the LPS dose response experiments detailed in Chapter 2, and the WP+LPS challenges detailed in Chapter 10.

Therefore the inhalation challenges with HDS, HDS fractions and the LPS-depleted HDS indicated that soluble HDS components acted synergistically with the HDS particulates, and that at least some of this synergistic activity was due to the LPS content of the soluble components. The extent of the contribution of LPS to this response could not be fully assessed, as it was not possible to completely deplete the HDS of LPS. It was therefore possible that the remaining response to the HDS-LPS was due either to the remaining LPS or to other soluble components.

The response to challenge with a combination of WP and LPS was not significantly different from that following challenge with the WP re-suspended in the soluble HDS fraction, whereby the total endotoxin activity within both combinations was comparable. It could therefore be concluded that the endotoxin content of the SUP contributed greatly to the synergism between the SUP and the WP.

11.4 Summary

Firstly, endotoxin inhalation alone is not responsible for the pulmonary inflammation and dysfunction in heaves. Therefore heaves susceptibility does not represent an increased responsiveness to inhaled endotoxin.

Secondly, there is evidence to suggest that β-D-glucan exposure, and thus mould exposure, is related to the severity of disease. However the fact that the WP contained most of the HDS β-D-glucan and all of the mould spore content, yet only induced a minimal inflammatory response, suggests that β-D-glucan or mould spore inhalation alone is not solely responsible for the induction of heaves.

Thirdly, the endotoxin content of stable dust largely contributes to the pulmonary inflammation, and to a lesser extent the pulmonary dysfunction, of heaves, but only when inhaled in combination with dust particulates. As the dust particulates in HDS were primarily mould spores, it would appear that the combination of mould spores and endotoxin plays a major role in determining the severity of disease in susceptible horses.

BIBLIOGRAPHY

Alexis, N., Eldridge, M., Reed, W., Bromberg, P. and Peden, D.B. (2001) CD14-dependent airway neutrophil response to inhaled LPS: Role of atopy. *Journal of Allergy and Clinical Immunology* **107**, 31-35.

Allermann, L. and Poulsen, O.M. (2000) Inflammatory potential of dust from waste handling facilities measured as IL-8 secretion from lung epithelial cells in vitro. *Annals of Occupational Hygiene* **44**, 259-269.

Anon (1989) Respiratory symptoms in farmers. *Lancet* **1**, 1054-1055.

Arbour, N.C., Lorenz, E., Schutte, B.C., Zabner, J., Kline, J.N., Jones, M., Frees, K., Watt, J.L. and Schwartz, D.A. (2000) TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. *Nature Genetics* **25**, 187.

Bartz, J. and Hartung, J. (1993) Dust measurements in a horse using an "equine personal sampler". *Proceedings of 4th International Symposium of American Society of Agricultural Engineers* **4**, 742-746.

Becker, S., Clapp, W.A., QUAY, J., Frees, K.L., Koren, H.S. and Schwartz, D.A. (1999) Compartmentalization of the inflammatory response to inhaled grain dust. *American Journal of Respiratory and Critical Care Medicine* **160**, 1309-1318.

Beijer, L., Thorn, J. and Rylander, R. (1999) Inhalation of (1->3)-beta-D-glucan in humans. *American Journal of Respiratory and Critical Care Medicine* **159**, A856

Bland, J.M. and Altman, D.G. (1986) Statistical-methods for assessing agreement between 2 methods of clinical measurement. *Lancet* **1**, 307-310.

Blaski, C.A., Clapp, W.D., Thorne, P.S., Quinn, T.J., Watt, J.L., Frees, K.L., Yagla, S.J. and Schwartz, D.A. (1996) The role of atopy in grain dust-induced airway disease. *American Journal of Respiratory and Critical Care Medicine* **154**, 334-340.

Borm, P.J.A., Jetten, M., Keman, S. and Schins, R.P.F. (2000) Endotoxin-binding proteins in nasal lavage: Evaluation as biomarkers to occupational endotoxin exposure. *Biomarkers* **5**, 108-118.

Borm, P.J.A., Schins, R.P.F., Derhaag, J.J.M., Kant, I. and Jorna, T.H.J.M. (1996) Cross-shift changes in blood inflammatory markers occur in the absence of airway-obstruction in workers exposed to grain dust. *Chest* **109**, 1078-1085.

Brazil, T.J. (2000) Pulmonary neutrophil recruitment, activation and clearance in equine COPD. PhD Thesis, University of Edinburgh.

British Standards Institution (1979) Precision of test methods I: Guide for the determination and reproducibility for a standard test method. In: *BS 5497, part 1*, Edited by BSI, London, BSI.

Broadstone, R.V., Gray, P.R., Robinson, N.E. and Derksen, F.J. (1992) Effects of xylazine on airway function in ponies with recurrent airway-obstruction. *American Journal of Veterinary Research* **53**, 1813-1817.

Brown, J.F., Chafee, K.A. and Tepperman, B.L. (1998) Role of mast cells, neutrophils and nitric oxide in endotoxin-induced damage to the neonatal rat colon. *British Journal of Pharmacology* **123**, 31-38.

Brown, D.M. and Donaldson, K. (1996) Wool and grain dusts stimulate TNF secretion by alveolar macrophages in vitro. *Occupational and Environmental Medicine* **53**, 387-393.

Buick, J.B., Lowry, R.C. and Magee, T.R.A. (1994) Isolation, enumeration, and identification of gram-negative bacteria from flax dust with reference to endotoxin concentration. *American Industrial Hygiene Association Journal* **55**, 59-61.

Bureau, F., Bonizzi, G., Kirschvink, N., Delhalle, S., Desmecht, D., Merville, M.P., Bours, V. and Lekeux, P. (2000) Correlation between nuclear factor-kappa B activity in bronchial brushing samples and lung dysfunction in an animal model of asthma. *American Journal of Respiratory and Critical Care Medicine* **161**, 1314-1321.

Caillaud, D., Nguyen, L., Porthault, E. and Aiache, J.M. (1996) Endotoxins in agricultural respiratory tract disease. *Revue Francaise d'Allergologie et d'Immunologie Clinique* **36**, 946-949.

Cairns, J.A. and Walls, A.F. (1996) Mast cell tryptase is a mitogen for epithelial cells -Stimulation of IL-8 production and intercellular adhesion molecule-1 expression. *Journal of Immunology* **156**, 275-283.

Castranova, V., Robinson, V.A. and Frazer, D.G. (1996) Pulmonary reactions to organic dust exposures - development of an animal-model. *Environmental Health Perspectives* **104**, 41-53.

Cavagna, G., Foa, V. and Vigliani, E.C. (1969) Effects in man and rabbits of inhalation of cotton dusts or extracts and purified endotoxins. *British Journal of Industrial Medicine* **26**, 314-321.

Cavanaugh, M.J., Bronsky, E.A. and Buckley, J.M. (1977) Clinical value of bronchial provocation testing in childhood asthma. *Journal of Allergy and Clinical Immunology* **59**, 41-47.

Chew, G., Doekes, G., Douwes, J., Spithoven, J. and Brunekreef, B. (1999) Fungal extracellular polysaccharides and β-(1-3)-glucans in house dust: relation with culturable fungi. *American Journal of Respiratory and Critical Care Medicine* **159**, A129

Chignard, M. and Balloy, V. (2000) Neutrophil recruitment and increased permeability during acute lung injury induced by lipopolysaccharide. *American Journal of Physiology-Lung Cellular and Molecular Physiology* **279**, L1083-L1090.

Chow, J.C., Young, D.W., Golenbock, D.T., Christ, W.J. and Gusovsky, F. (1999) Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *Journal of Biological Chemistry* **274**, 10689-10692.

Chow, L.P., Liu, S.L., Yu, C.J., Liao, H.K., Tsai, J.J. and Tang, T.K. (2000) Identification and expression of an allergen *asp f* 13 from *Aspergillus fumigatus* and epitope mapping using human IgE antibodies and rabbit polyclonal antibodies. *Biochemical Journal* **346**, 423-431.

Christiani, D.C., Velazquez, A., Wilcox, M. and Olenchock, S.A. (1993) Airborne endotoxin concentrations in various work areas within a cotton mill in Central-America. *Environmental Research* **60**, 187-192.

Christiani, D.C., Ye, T., Zhang, S., Wegman, D.H., Eisen, E.A., Ryan, L.A., Olenchock, S.A., Pothier, L. and Dai, H. (1999) Cotton dust and endotoxin exposure and long-term decline in lung function: Results of a longitudinal study. *American Journal of Industrial Medicine* **35**, 321-331.

Clapp, W.D., Becker, S., Quay, J., Watt, J.L., Thorne, P.S., Frees, K.L., Zhang, X.O., Koren, H.S., Lux, C.R. and Schwartz, D.A. (1994) Grain dust-induced air-flow obstruction and inflammation of the lower respiratory-tract. *American Journal of Respiratory and Critical Care Medicine* **150**, 611-617.

Clapp, W.D., Thorne, P.S., Frees, K.L., Zhang, X., Lux, C.R. and Schwartz, D.A. (1993) The effects of inhalation of grain dust extract and endotoxin on upper and lower airways. *Chest* **104** (3), 825-830.

Clark, C.S., Rylander, R. and Larsson, L. (1983a) Levels of gram-negative bacteria, *Aspergillus-fumigatus*, dust, and endotoxin at compost plants. *Applied and Environmental Microbiology* **45**, 1501-1505.

Clark, S., Rylander, R. and Larsson, L. (1983b) Airborne bacteria, endotoxin and fungi in dust in poultry and swine confinement buildings. *American Industrial Hygiene Association* **44**, 537-541.

Clarke, A.F. (1987a) A review of environmental and host factors in relation to equine respiratory-disease. *Equine Veterinary Journal* **19**, 435-441.

Clarke, A.F. (1987b) Air hygiene and equine respiratory disease. *In Practice* **9**, 196-204.

Clarke, A.F. (1987c) Chronic pulmonary-disease - A multifaceted disease complex in the horse. *Irish Veterinary Journal* **41**, 258-264.

Clarke, A.F. (1993) Stable dust - Threshold limiting values, exposures variables and host risk-factors. *Equine Veterinary Journal* **25**, 172-174.

Clarke, A.F. and Madelin, T. (1987) Technique for assessing respiratory health hazards from hay and other source materials. *Equine Veterinary Journal* **19**, 442-447.

Clarke, A.F., Madelin, T. and Alpress, R.G. (1987) The relationship of air hygiene in stables to lower airway disease and pharyngeal lymphoid hyperplasia in two groups of Thoroughbred horses. *Equine Veterinary Journal* **19**, 524-530.

Clarke, J. (1788) A treatise on the prevention of diseases incidental to horses from bad management in regard to stables, food, water, air and exercise. W. Smellie, Edinburgh.

Clementsen, P., Milman, N., Struvechristensen, E., Petersen, B.N., Pedersen, M., Bisgaard, H., Permin, H. and Norn, S. (1991) Bacteria-induced histamine-release from human bronchoalveolar cells and blood leukocytes. *Allergy* **46**, 45-51.

Cormier, Y., Duchaine, C., IsraelAssayag, E., Bedard, G., Laviolette, M. and Dosman, J. (1997) Effects of repeated swine building exposures on normal naive subjects. *European Respiratory Journal* **10**, 1516-1522.

Coyle, A.J., Wagner, K., Bertrand, C., Tsuyuki, S., Bews, J. and Heusser, C. (1996) Central role of immunoglobulin (Ig) E in the induction of lung eosinophil infiltration and t helper 2 cell cytokine production: Inhibition by a non-anaphylactogenic anti-IgE antibody. *Journal of Experimental Medicine* **183**, 1303-1310.

Crameri, R. (2001) Antibodies against recombinant mould allergens and mould extracts in BALF and serum of RAO-affected horses. *Proceedings of 2nd International Symposium on Allergic Diseases of the Horse,* Hortobagy-Mata, Hungary, 17.

Crichlow, E.C., Yoshida, K. and Wallace, K. (1980) Dust levels in a riding stable. *Equine Veterinary Journal* **12**, 185-188.

Dallal, M.M. and Chang, S. (1992) Role of platelet-activating-factor in pulmonary vascular injury following inhaled endotoxin. *Clinical Research* **40**, A 278.

Darsow, U., Vieluf, D., Berg, B., Berger, J., Busse, A., Czech, W., Heese, A., Heidelbach, U., Peters, K.P., Przybilla, B., Richter, G., Rueff, F., Werfel, T.,

Wistokat-Wulfing, A. and Ring, J. (1999) Dose response study of atopy patch test in children with atopic eczema. *Pediatric Asthma Allergy & Immunology* **13**, 115-122.

Deetz, D.C., Jagielo, P.J., Quinn, T.J., Thorne, P.S., Bleuer, S.A. and Schwartz, D.A. (1997) The kinetics of grain dust-induced inflammation of the lower respiratory tract. *American Journal of Respiratory and Critical Care Medicine* **155**, 254-259.

Dentener, M.A., Vreugdenhil, A.C.E., Hoet, P.H.M., Vernooy, J.H.J., Nieman, F.H.M., Heumann, D., Janssen, Y.M.W., Buurman, W.A. and Wouters, E.F.M. (2000) Production of the acute-phase protein lipopolysaccharide-binding protein by respiratory type II epithelial cells -Implications for local defense to bacterial endotoxins. *American Journal of Respiratory Cell and Molecular Biology* **23**, 146-153.

Derksen, F.J. (1993) Chronic obstructive pulmonary-disease (heaves) as an inflammatory condition. *Equine Veterinary Journal* **25**, 257-258.

Derksen, F.J. and Robinson, N.E. (1981) Biphasic response to aerosol antigen challenge in unanesthetized ponies. *Federation Proceedings* **40**, 797.

Derksen, F.J., Robinson, N.E., Armstrong, P.J., Stick, J.A. and Slocombe, R.F. (1985a) Airway reactivity in ponies with recurrent airway obstruction (Heaves). *Journal of Applied Physiology* **58**, 598-604.

Derksen, F.J., Robinson, N.E., Scott, J.S. and Stick, J.A. (1988) Aerosolised *Micropolyspora faeni* antigen as a cause of pulmonary dysfunction in ponies with recurrent airway obstruction (Heaves). *American Journal of Veterinary Research* **49**, 933-938.

Derksen, F.J., Scott, J.S., Miller, D.C., Slocombe, R.F. and Robinson, N.E. (1985b) Broncho-alveolar lavage in ponies with recurrent airway obstruction (Heaves). *American Review of Respiratory Disease* **132**, 1066-1070.

Derksen, F.J., Slocombe, R.F., Brown, C.M., Rook, J. and Robinson, N.E. (1982) Chronic restrictive pulmonary-disease in a horse. *Journal of the American Veterinary Medical Association* **180**, 887-889.

Derochemonteixgalve, B., Marchatamoruso, B., Dayer, J.M. and Rylander, R. (1991) Tumor-necrosis-factor and interleukin-1 activities in free lung-cells after single and repeated inhalation of bacterial-endotoxin. *Infection and Immunity* **59**, 3646-3650.

Dillon, H.K., Miller, J.D., Sorenson, W.G., Douwes, J. and Jacobs, R.R. (1999) Review of methods applicable to the assessment of mold exposure to children. *Environmental Health Perspectives* **107**, 473-480.

Dixon, P.M., Railton, D.I. and McGorum, B.C. (1995a) Equine pulmonary disease: A case control study of 300 referred cases. Part 1: Examination techniques, diagnostic criteria and diagnoses. *Equine Veterinary Journal* **27**, 416-421.

Dixon, P.M., Railton, D.I., McGorum, B.C. and Todhill, S. (1995b) Equine pulmonary disease: A case control study of 300 referred cases. Part 4: Treatments and re-examination findings. *Equine Veterinary Journal* **27**, 436-439.

Dong, W.M., Lewtas, J. and Luster, M.I. (1996) Role of endotoxin in tumor-necrosis-factor-alpha expression from alveolar macrophages treated with urban air particles. *Experimental Lung Research* **22**, 577-592.

Donham, K.J., Cumro, D., Reynolds, S.J. and Merchant, J.A. (2000) Dose-response relationships between occupational aerosol exposures and cross-shift declines of lung function in poultry workers: Recommendations for exposure limits. *Journal of Occupational and Environmental Medicine* **42**, 260-269.

Douwes, J., Versloot, P., Hollander, A., Heederik, D. and Doekes, G. (1995) Influence of various dust sampling and extraction methods on the measurement of airborne endotoxin. Applied Environmental Microbiology **61**, 1763-1769.

Douwes, J., Doekes, G., Heinrich, J., Koch, A., Bischof, W. and Brunekreef, B. (1998) Endotoxin and beta-(1->3)-glucan in house dust and the relation with home characteristics: A pilot study in 25 German houses. *Indoor Air-International Journal of Indoor Air Quality and Climate* **8**, 255-263.

Douwes, J. and Heederik, D. (1997) Epidemiologic investigations of endotoxins. *International Journal of Occupational and Environmental Health* **3**, S26-S31.

Douwes, J., McLean, D., van der Maarl, E., Heederik, D., and Pearce, N. (2000a) Worker exposures to airborne dust, endotoxin and beta-(1,3)-glucan in two New Zealand sawmills. *American Journal of Industrial Medicine* **38**, 426-430.

Douwes, J., vanderSluis, B., Doekes, G., van Leusden, F., Wijnands, L., van Strien, R., Verhoeff, A. and Brunekreef, B. (1999) Fungal extracellular polysaccharides in house dust as a marker for exposure to fungi: Relations with culturable fungi, reported home dampness, and respiratory symptoms. *Journal of Allergy and Clinical Immunology* **103**, 494-500.

Douwes, J., Wouters, I., Dubbeld, H., vanZwieten, L., Steerenberg, P., Doekes, G. and Heederik, D. (2000b) Upper airway inflammation assessed by nasal lavage in compost workers: A relation with bio-aerosol exposure. *American Journal of Industrial Medicine* **37**, 459-468.

Dubin, W., Martin, T.R., Swoveland, P., Leturcq, D.J., Moriarty, A.R., Tobias, P.S., Bleecker, E.R., Goldblum, S.E. and Hasday, J.D. (1996) Asthma and endotoxin - lipopolysaccharide-binding protein and soluble CD14 in bronchoalveolar

compartment. *American Journal of Physiology-Lung Cellular and Molecular Physiology* **14**, L 736-L 744.

Dutkiewicz, J., Pomorski, Z.J.H., Sitkowska, J., Krysinskatraczyk, E., Skorska, C., Prazmo, Z., Cholewa, G. and Wojtowicz, H. (1994) Airborne microorganisms and endotoxin in animal houses. *Grana* **33**, 85-90.

Elder, A.C.P., Finkelstein, J., Johnston, C., Gelein, R. and Oberdorster, G. (2000a) Induction of adaptation to inhaled lipopolysaccharide in young and old rats and mice. *Inhalation Toxicology* **12**, 225-243.

Elder, A.C.P., Gelein, R., Finkelstein, J.N., Cox, C. and Oberdorster, G. (2000b) Endotoxin priming affects the lung response to ultrafine particles and ozone in young and old rats. *Inhalation Toxicology* **12**, 85-98.

Eldridge, M.W. and Peden, D.B. (2000) Airway response to concomitant exposure with endotoxin and allergen in atopic asthmatics. *Journal of Toxicology and Environmental Health-Part A* **61**, 27-37.

Eyre, P. (1972) Equine Pulmonary Emphysema: A bronchopulmonary mould allergy. *Veterinary Record* **91**, 134-140.

Fairbairn, S.M., Page, C.P., Lees, P. and Cunningham, F.M. (1993) Early neutrophil but not eosinophil or platelet recruitment to the lungs of allergic horses following antigen challenge. *Clinical and Experimental Allergy* **23**, 821-828.

Fogelmark, B., Goto, H., Yuasa, K., Marchat, B. and Rylander, R. (1992) Acute pulmonary toxicity of inhaled beta-1,3-glucan and endotoxin. *Agents and Actions* **35**, 50-56.

Fogelmark, B. and Rylander, R. (1997) (1->3)-beta-d-glucan in some indoor air fungi. *Indoor and Built Environment* **6**, 291-294.

Fogelmark, B., Sjostrand, M. and Rylander, R. (1994) Pulmonary inflammation-induced by repeated inhalations of beta-(1, 3)-D-glucan and endotoxin. *International Journal of Experimental Pathology* **75**, 85-90.

Fogelmark, B., Sjostrand, M., Wiliams, D. and Rylander, R. (1997) Inhalation toxicity of (1->3)-beta-d-glucan: recent advances. *Mediators of Inflammation* **6**, 263-265.

Fogelmark, B., Thorn, J. and Rylander, R. (2001) Inhalation of (1 -> 3)-beta-D-glucan causes airway eosinophilia. *Mediators of Inflammation* **10**, 13-19.

Folkesson, H.G., Westrom, B.R. and Karlsson, B.W. (1998) Effects of systemic and local immunization on alveolar epithelial permeability to protein in the rat. *American Journal of Respiratory and Critical Care Medicine* **157**, 324-327.

Frazer, D.G., Jones, W.G., Petsonk, E.L., Kullman, G.J., Barger, M.W., Afshari, A., Jones, T. and Castranova, V. (1993) Organic dust exposure from compost handling - response of an animal model. *American Journal of Industrial Medicine* **24**, 375-385.

Galanos, C. and Luderitz, O. (1984) Lipopolysaccharide: properties of an amphipathic molecule. In: *Handbook of Endotoxin* Vol. 1, pp46-58. Edited by Rietschel, E.Th., Amsterdam, Elsevier.

Gao, X.P., von Essen, S.G., Rennard, S.I., Oneill, D. and Rubinstein, I. (1993) Grain dust extract increases vascular permeability in the upper airway - Role of nk1 receptors. *American Review of Respiratory Disease* **147**, A662.

George, C.L.S., Jin, H., Wohlford-Lenane, C.L., O'Neill, M.E., Phipps, J.C., O'Shaughnessy, P., Kline, J.N., Thorne, P.S. and Schwartz, D.A. (2001) Endotoxin responsiveness and sub-chronic grain dust-induced airway disease. *American Journal of Physiology-Lung Cellular and Molecular Physiology* **280**, L203-L213.

Gilmour, M.I. (1995) Interaction of air pollutants and pulmonary allergic responses in experimental animals. *Toxicology* **105**, 335-342.

Goldsmith, C.A.W., Hamada, K., Ning, Y.Y., Qin, G.Z., Catalano, P., Murthy, G.G.K., Lawrence, J. and Kobzik, L. (1999) Effects of environmental aerosols on airway hyper-responsiveness in a murine model of asthma. *Inhalation Toxicology* **11**, 981-998.

Gordon, T. (1990) The role of inflammatory mediators in airway edema and obstruction produced in guinea-pigs exposed to inhaled endotoxin or cotton dusts. *American Journal of Industrial Medicine* **17**, 107-108.

Gordon, T. (1992) Dose-dependent pulmonary effects of inhaled endotoxin in guinea-pigs. *Environmental Research* **59**, 416-426.

Gordon, T. (1994) Role of the complement-system in the acute respiratory effects of inhaled endotoxin and cotton dust. *Inhalation Toxicology* **6**, 253-266.

Gordon, T. and Harkema, J. (1993) Mucous cell metaplasia in the respiratory-tract of rats after exposure to inhaled endotoxin. *American Review of Respiratory Disease* **147**, A 528.

Gordon, T. and Harkema, J.R. (1994) Effect of inhaled endotoxin on intraepithelial mucosubstances in f344 rat nasal and tracheobronchial airways. *American Journal of Respiratory Cell and Molecular Biology* **10**, 177-183.

Gordon, T. and Harkema, J.R. (1995) Cotton dust produces an increase in intraepithelial mucosubstances in rat airways. *American Journal of Respiratory and Critical Care Medicine* **151**, 1981-1988.

Gordon, T., Nadziejko, C., Plant, M., Rodger, I.W. and Pon, D.J. (1996) One-month exposure to inhaled endotoxin produces a dose-dependent increase in stored mucosubstances in rat intrapulmonary airways. *Experimental Lung Research* **22**, 509-523.

Halliwell, R.E., Fleischman, J.B., Mackay-Smith, M., Beech, J. and Gunson, D.E. (1979) The role of allergy in chronic pulmonary disease of horses. *Journal of the American Veterinary Medical Association* **174**, 277-281.

Halliwell, R.E.W., McGorum, B.C., Irving, P. and Dixon, P.M. (1993) Local and systemic antibody-production in horses affected with chronic obstructive pulmonary-disease. *Veterinary Immunology and Immunopathology* **38**, 201-215.

Halls, T.E. and Gudmundsson, B. (1985) Mites of stored hay in Iceland. *J Agr Res Icel* **17**, 31-37.

Hamada, K., Goldsmith, C.A. and Kobzik, L. (1999) Increased airway hyper-responsiveness and inflammation in a juvenile mouse model of asthma exposed to air-pollutant aerosol. *Journal of Toxicology and Environmental Health-Part A* **58**, 129-143.

Heederik, D., Douwes, J., Wouters, I. and Doekes, G. (2000) Organic dusts: Beyond endotoxin. *Inhalation Toxicology* **12**, 27-33.

Herbert, A., Carvalheiro, M., Rubenowitz, E., Bake, B. and Rylander, R. (1992) Reduction of alveolar-capillary diffusion after inhalation of endotoxin in normal subjects. *Chest* **102**, 1095-1098.

Holst, H., Edqvist, L.E., Kindahl, H. and Rylander, R. (1994) Hematological, blood biochemical, and cytological bronchoalveolar lavage studies in prepubertal gilts after endotoxin inhalation and ingestion. *Journal of Veterinary Medicine Series A-Zentralblatt fur Veterinarmedizin Reihe A-Physiology Pathology Clinical Medicine* **41**, 159-166.

Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K. and Akira, S. (1999) Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hypo-responsive to lipopolysaccharide: Evidence for TLR4 as the LPS gene product. *Journal of Immunology* **162**, 3749-3752.

Hsieh, V., Amorusomarchat, B., Rylander, R. and Polla, B.S. (1994) Oxygen metabolites from lavage and interstitial lung-cells after inhalation of endotoxin in guinea-pigs. *International Archives of Allergy and Immunology* **104**, 42-47.

Hsiue, T.R., Lei, H.Y., Hsieh, A.L., Wang, T.Y., Chang, H.Y. and Chen, C.R. (1997) Mite-induced allergic airway inflammation in guinea-pigs. *International Archives of Allergy and Immunology* **112**, 295-302.

Hunt, L.W., Gleich, G.J., Ohnishi, T., Weiler, D.A., Mansfield, E.S., Kita, H. and Sur, S. (1994) Endotoxin contamination causes neutrophilia following pulmonary allergen challenge. *American Journal of Respiratory and Critical Care Medicine* **149**, 1471-1475.

Hunt, L.W., Mansfield, E.S., Sur, S. and Gleich, G.J. (1992) Late neutrophilic response to bronchial allergen challenge - A response to endotoxin. *Journal of Allergy and Clinical Immunology* **89**, 334.

Iadarola, P., Lungarella, G., Martorana, P.A., Viglio, S., Guglielminetti, M., Korzus, E., Gorrini, M., Cavarra, E., Rossi, A., Travis, J. and Luisetti, M. (1998) Lung injury and degradation of extracellular matrix components by *Aspergillus fumigatus* serine proteinase. *Experimental Lung Research* **24**, 233-251.

Imrich, A., Koziel, H. and Kobzik, L. (1999a) LPS-priming amplifies lung macrophage TNF production in response to air particles. *American Journal of Respiratory and Critical Care Medicine* **159**, A699.

Imrich, A., Ning, Y.Y., Koziel, H., Coull, B. and Kobzik, L. (1999b) Lipopolysaccharide priming amplifies lung macrophage tumor necrosis factor production in response to air particles. *Toxicology and Applied Pharmacology* **159**, 117-124.

Ingalls, R.R., Heine, H., Lien, E., Yoshimura, A. and Golenbock, D. (1999) Lipopolysaccharide recognition, CD14, and lipopolysaccharide receptors. *Infectious Disease Clinics of North America* **13**, 341.

Iuvone, T., Van den Bossche, R., D'Acquisto, F., Carnuccio, R. and Herman, A.G. (1999) Evidence that mast cell degranulation, histamine and tumour necrosis factor alpha release occur in LPS-induced plasma leakage in rat skin. *British Journal of Pharmacology* **128**, 700-704.

Jacobs, R.R. (1997a) Endotoxins in the Environment. *International Journal of Occupational and Environmental Health* **3**, S3-S5.

Jacobs, R.R. (1997b) Environmental Monitoring of Endotoxins. *International Journal of Occupational and Environmental Health* **3**, S37-S41.

Jacobs, R.R., Boehlecke, B., Vanhagehamsten, M. and Rylander, R. (1993) Bronchial reactivity, atopy, and airway response to cotton dust. *American Review of Respiratory Disease* **148**, 19-24.

Jacobs, R.R., Heederik, D., Douwes, J. and Zahringer, U. (1997) Endotoxin structure. *International Journal of Occupational and Environmental Health* **3**, S6-S7.

Jagielo, P.J., Quinn, T.J., Qureshi, N. and Schwartz, D.A. (1998) Grain dust-induced lung inflammation is reduced by *Rhodobacter sphaeroides* diphosphoryl lipid A. *American Journal of Physiology-Lung Cellular and Molecular Physiology* **18**, L26-L31.

Jagielo, P.J., Thorne, P.S., Kern, J.A., Quinn, T.J. and Schwartz, D.A. (1996a) Role of endotoxin in grain dust-induced lung inflammation in mice. *American Journal of Physiology-Lung Cellular and Molecular Physiology* **14**, L1052-L1059.

Jagielo, P.J., Thorne, P.S., Watt, J.L., Frees, K.L., Quinn, T.J. and Schwartz, D.A. (1996b) Grain dust and endotoxin inhalation challenges produce similar inflammatory responses in normal subjects. *Chest* **110**, 263-270.

Jagielo, P.J., Watt, J.L., Quinn, T.J., Knapp, H.R. and Schwartz, D.A. (1997) Pentoxifylline does not alter the response to inhaled grain dust. *Chest* **111**, 1429-1435.

Jarreau, P.H., Dortho, M.P., Boyer, V., Harf, A. and Macquinmavier, I. (1994) Effects of capsaicin on the airway responses to inhaled endotoxin in the guinea-pig. *American Journal of Respiratory and Critical Care Medicine* **149**, 128-133.

Johnston, C.J., Finkelstein, J.N., Gelein, R. and Oberdorster, G. (1998) Pulmonary cytokine and chemokine mrna levels after inhalation of lipopolysaccharide in c57bl/6 mice. *Toxicological Sciences* **46**, 300-307.

Johnston, C.A. and Greisman, S.E. (1985) Mechanisms of endotoxin tolerance. In: *Handbook of Endotoxin*, Edited by Hinshaw, L.B. Amsterdam, Elsevier.

Jolie, R., Backstrom, L., Olson, L. and Chase, C. (1999) A 15-week experimental exposure of pigs to airborne dust with added endotoxin in a continuous flow exposure chamber. *Canadian Journal of Veterinary Research-Revue Canadienne de Recherche Veterinaire* **63**, 129-137.

Jones, R.D., McGreevy, P.D., Robertson, A., Clarke, A.F. and Wathes, C.M. (1987) Survey of the designs of racehorse stables in the southwest of England. *Equine Veterinary Journal* **19**, 454-457.

Jorna, T.H.J.M., Borm, P.J.A., Valks, J., Houba, R. and Wouters, E.F.M. (1994) Respiratory symptoms and lung-function in animal feed workers. *Chest* **106**, 1050-1055.

Kaup, F-J., Drommer, W., Damsch, S. and Deegan, E. (1990a) Ultrastructural findings in horses with chronic obstructive pulmonary disease (COPD) II: Pathomorphological change of the terminal airways and the alveolar region. *Equine Veterinary Journal* **22**, 349-355.

Kaup, F-J., Drommer, W. and Deegan, E. (1990b) Ultrastructural findings in horses with chronic obstructive pulmonary disease (COPD) I: Alterations of the larger conducting airways. *Equine Veterinary Journal* **22**, 343-348.

Kawabata, K., Hagio, T., Matsumoto, S., Nakao, S., Orita, S., Aze, Y. and Ohno, H. (2000) Delayed neutrophil elastase inhibition prevents subsequent progression of acute lung injury induced by endotoxin inhalation in hamsters. *American Journal of Respiratory and Critical Care Medicine* **161**, 2013-2018.

Keman, S., Jetten, M., Douwes, J. and Borm, P.J.A. (1998) Longitudinal changes in inflammatory markers in nasal lavage of cotton workers - Relation to endotoxin exposure and lung function changes. *International Archives of Occupational and Environmental Health* **71**, 131-137.

Kirkhorn, S.R. and Garry, V.F. (2000) Agricultural lung diseases. *Environmental Health Perspectives* **108**, 705-712.

Kline, J.N., Cowden, D.C., Hunninghake, G.W., Schutte, B.C., Watt, J.L., Wohlford-Lenane, C.L., Powers, L.S., Jones, M.P. and Schwartz, D.A. (1999) Variable airway responsiveness to inhaled lipopolysaccharide. *American Journal of Respiratory and Critical Care Medicine* **160**, 297-303.

KrausFilarska, M., Matolepszy, J., Medrala, W. and Dobek, R. (1998) Bacterial lipopolysaccharide-induced sulfidoleukotriene release from peripheral blood leukocytes in patients with asthma and chronic obstructive pulmonary disease. *Journal of Investigational Allergology & Clinical Immunology* **8**, 94-97.

Kuchuk, A.A., Basanets, A.V. and Louhelainen, K. (2000) Bronchopulmonary pathology in workers exposed to organic fodder dust. *Annals of Agricultural and Environmental Medicine* **7**, 17-23.

Kullman, G.J., Thorne, P.S., Waldron, P.F., Marx, J.J., Ault, B., Lewis, D.M., Siegel, P.D., Olenchock, S.A. and Merchant, J.A. (1998) Organic dust exposures from work in dairy barns. *American Industrial Hygiene Association Journal* **59**, 403-413.

Kurup, V.P., Choi, H., Murali, P.S., Resnick, A., Fink, J.N. and Coffman, R.L. (1997) Role of particulate antigens of *Aspergillus* in murine eosinophilia. *International Archives of Allergy and Immunology* **112**, 270-278.

Lacey, J. (1993) Inhalation hazards of fungal spoilage. *International Biodeterioration & Biodegradation* **32**, 187-189.

Lantz, R.C., Keller, G.E. and Burrell, R. (1991) The role of platelet-activating-factor in the pulmonary response to inhaled bacterial-endotoxin. *American Review of Respiratory Disease* **144**, 167-172.

Larsen, F.O., Clementsen, P., Hansen, M., Maltbaek, N., Gravesen, S., Skov, P.S. and Norn, S. (1996) The indoor microfungus *Trichoderma viride* potentiates histamine release from human bronchoalveolar cells. *Apmis* **104**, 673-679.

Larsson, B.M., Palmberg, L., Malmberg, P.O. and Larsson, K. (1997) Effect of exposure to swine dust on levels of IL-8 in airway lavage fluid. *Thorax* **52**, 638-642.

Larsson, K.A., Eklund, A.G., Hansson, L.O., Isaksson, B.N. and Malmberg, P.O. (1994) Swine dust causes intense airways inflammation in healthy-subjects. *American Journal of Respiratory and Critical Care Medicine* **150**, 973-977.

Larsson, R., Rocksen, D., Lilliehook, B., Jonsson, A. and Bucht, A. (2000) Dose-dependent activation of lymphocytes in endotoxin-induced airway inflammation. *Infection and Immunity* **68**, 6962-6969.

Lavoie, J. (2001) Expression of Th1/Th2-type cytokines in BALF cells from RAO-affected horses. *Proceedings of 2nd International Symposium on Allergic Diseases of the Horse,* Hortobagy-Mata, Hungary, 24

Lavoie, J.P., Pascoe, J.R. and Kurpershoek, C.J. (1992) Effect of head and neck position on respiratory mechanics in horses sedated with xylazine. *American Journal of Veterinary Research* **53**, 1652-1657.

Lawson, G.H.K., McPherson, E.A., Murphy, J.R., Nicholson, J.M., Wooding, P., Breeze, R.G. and Pirie, H. (1979) The presence of precipitating antibodies in the sera of horses with chronic obstructive pulmonary disease (COPD). *Equine Veterinary Journal* **11**, 172-176.

Ledebur, H.C. and Parks, T.P. (1995) Transcriptional regulation of the intercellular-adhesion molecule-1 gene by inflammatory cytokines in human endothelial-cells - Essential roles of a variant NF-kappa-b site and p65 homodimers. *Journal of Biological Chemistry* **270**, 933-943.

Lekeux, P., Art, T. and Kirschvink, N. (2001) Correlation between nuclear factor-kappa B activity in bronchial brushing samples and lung dysfunction. *Proceedings of 2nd International Symposium on Allergic Diseases of the Horse,* Hortobagy-Mata, Hungary, 21.

Li, D., Zhong, Y.N., Rylander, R., Ma, Q.Y. and Zhou, X.Y. (1995) Longitudinal-study of the health of cotton workers. *Occupational and Environmental Medicine* **52**, 328-331.

Li, X.Y., Donaldson, K. and Macnee, W. (1998) Lipopolysaccharide-induced alveolar epithelial permeability - The role of nitric oxide. *American Journal of Respiratory and Critical Care Medicine* **157**, 1027-1033.

Li, X.Y., Gilmour, P.S., Donaldson, K. and Macnee, W. (1997) *In vivo* and *in vitro* proinflammatory effects of particulate air pollution (PM10). *Environmental Health Perspectives* **105**, 1279-1283.

Ljungman, A.G., Leanderson, P. and Tagesson, C. (1998) (1->3)-beta-D-glucan stimulates nitric oxide generation and cytokine mRNA expression in macrophages. *Environmental Toxicology and Pharmacology* **5**, 273-281.

Loeffler, B.S., Arden, W.A., Fiscus, R.R. and Lee, L.Y. (1997) Involvement of tachykinins in endotoxin-induced airway hyper-responsiveness. *Lung* **175**, 253-263.

Louhelainen, K., Kangas, J., Reiman, M. and Kalliokoski, P. (1997) Farmers' exposure to dusts and gases in modern Finnish cubicle cow houses. *Agricultural and Food Science In Finland* **6**, 207-217.

Lundholm, M., Palmgren, U. and Malmberg, P. (1986) Exposure to endotoxin in the farm environment. *American Journal of Industrial Medicine* **10**, 314-315.

McGorum, B.C., Dixon, P.M. and Halliwell, R.E.W. (1993a) Evaluation of intradermal mold antigen testing in the diagnosis of equine chronic obstructive pulmonary-disease. *Equine Veterinary Journal* **25**, 273-275.

McGorum, B.C., Dixon, P.M. and Halliwell, R.E.W. (1993b) Quantification of histamine in plasma and pulmonary fluids from horses with chronic obstructive pulmonary-disease, before and after natural (hay and straw) challenges. *Veterinary Immunology and Immunopathology* **36**, 223-237.

McGorum, B.C., Dixon, P.M. and Halliwell, R.E. (1993c) Responses of horses affected with chronic obstructive pulmonary disease to inhalation challenges with mould antigens. *Equine Veterinary Journal* **25**, 261-267.

McGorum, B.C., Dixon, P.M., Halliwell, R.E.W. and Irving, P. (1993d) Comparison of cellular and molecular-components of bronchoalveolar lavage fluid harvested from different segments of the equine lung. *Research in Veterinary Science* **55**, 57-59.

McGorum, B.C., Ellison, J. and Cullen, R.T. (1998) Total and respirable airborne dust endotoxin concentrations in three equine management systems. *Equine Veterinary Journal* **30**, 430-434.

McPherson, E.A., Lawson, G.H.K., Murphy, J.R., Nicholson, J.M., Breeze, R.G. and Pirie, H. (1979) Chronic obstructive pulmonary disease (COPD) in horses: Aetiological studies: Response to intradermal and inhalation antigenic challenge. *Equine Veterinary Journal* **11**, 159-166.

McPherson, E.A. and Thomson, J.R. (1983) Chronic obstructive pulmonary-disease in the horse 1: Nature of the disease. *Equine Veterinary Journal* **15**, 203-206.

Madelin, T.M., Clarke, A.F. and Mair, T.S. (1991) Prevalence of serum precipitating antibodies in horses to fungal and thermophilic actinomycete antigens - Effects of environmental challenge. *Equine Veterinary Journal* **23**, 247-252.

Mair, T.S., Stokes, C.R. and Bourne, F.J. (1988) Distribution and ultrastructure of mast-cells in the equine respiratory-tract. *Equine Veterinary Journal* **20**, 54-58.

Makela, P.H. and Stocker, B.A.D. (1984) Genetics of lipopolysaccharide. In: *Handbook of Endotoxin* Vol. 1, pp59-137. Edited by Rietschel, E.Th., Amsterdam, Elsevier.

Malmberg, P. (1990) Health effects of organic dust exposure in dairy farmers. *American Journal of Industrial Medicine* **17**, 7-15.

Mandryk, J., Alwis, K.U. and Hocking, A.D. (2000) Effects of personal exposures on pulmonary function and work-related symptoms among sawmill workers. *Annals of Occupational Hygiene* **44**, 281-289.

Martin, T.R., Mathison, J.C., Tobias, P.S., Leturcq, D.J., Moriarty, A.M., Maunder, R.J. and Ulevitch, R.J. (1992) Lipopolysaccharide binding-protein enhances the responsiveness of alveolar macrophages to bacterial lipopolysaccharide -Implications for cytokine production in normal and injured lungs. *Journal of Clinical Investigation* **90**, 2209-2219.

Matsukura, S., Stellato, C., Plitt, J.R., Bickel, C., Miura, K., Georas, S.N., Casolaro, V. and Schleimer, R.P. (1999) Activation of eotaxin gene transcription by NF-kappa B and STAT6 in human airway epithelial cells. *Journal of Immunology* **163**, 6876-6883.

Mehlhop, P.D., vandeRijn, M., Goldberg, A.B., Brewer, J.P., Kurup, V.P., Martin, T.R. and Oettgen, H.C. (1997) Allergen-induced bronchial hyper-reactivity and eosinophilic inflammation occur in the absence of IgE in a mouse model of asthma. *Proceedings of The National Academy of Sciences of The United States of America* **94**, 1344-1349.

Michel, O. (1996) Endotoxin and asthma. *Revue Francaise d'Allergologie et d'Immunologie Clinique* **36**, 942-945.

Michel, O. (1997) Human challenge studies with endotoxins. *International Journal of Occupational and Environmental Health* **3**, S18-S25.

Michel, O. (2000) Systemic and local airways inflammatory response to endotoxin. *Toxicology* **152**, 25-30.

Michel, O., Duchateau, J., Plat, G., Cantinieaux, B., Hotimsky, A., Gerain, J. and Sergysels, R. (1995a) Blood inflammatory response to inhaled endotoxin in normal subjects. *Clinical and Experimental Allergy* **25**, 73-79.

Michel, O., Duchateau, J. and Sergysels, R. (1989) Effect of inhaled endotoxin on bronchial reactivity in asthmatic and normal subjects. *Journal of Applied Physiology* **66**, 1059-1064.

Michel, O., Ginanni, R., Duchateau, J., Vertongen, F., Lebon, B. and Sergysels, R. (1991) Domestic endotoxin exposure and clinical severity of asthma. *Clinical and Experimental Allergy* **21**, 441-448.

Michel, O., Ginanni, R., Lebon, B., Content, J., Duchateau, J. and Sergysels, R. (1992a) Inflammatory response to acute inhalation of endotoxin in asthmatic patients. *American Review of Respiratory Disease* **146**, 352-357.

Michel, O., Ginanni, R. and Sergysels, R. (1992b) Relation between the bronchial obstructive response to inhaled lipopolysaccharide and bronchial responsiveness to histamine. *Thorax* **47**, 288-291.

Michel, O., Ginanni, R. and Sergysels, R. (1995b) Protective effect of sodium cromoglycate on lipopolysaccharide-induced bronchial obstruction in asthmatics. *International Archives of Allergy and Immunology* **108**, 298-302.

Michel, O., Kips, J., Duchateau, J., Vertongen, F., Robert, L., Collet, H., Pauwels, R. and Sergysels, R. (1996) Severity of asthma is related to endotoxin in house dust. **154**, 1641-1646.

Michel, O., Nagy, A.M., Schroeven, M., Duchateau, J., Neve, J., Fondu, P. and Sergysels, R. (1997) Dose-response relationship to inhaled endotoxin in normal subjects. *American Journal of Respiratory and Critical Care Medicine* **156**, 1157-1164.

Michel, O., Olbrecht, J., Moulard, D. and Sergysels, R. (2000) Effect of anti-asthmatic drugs on the response to inhaled endotoxin. *Annals of Allergy Asthma & Immunology* **85**, 305-310.

Milanowski, J. (1996) The function of pulmonary phagocytes of guinea-pigs after the inhalation of organic dust-derived microbial agents. *Journal of Clean Technology Environmental Toxicology and Occupational Medicine* **5**, 91-102.

Milanowski, J. (1997) Experimental studies on the effects of organic dust-derived agents on respiratory system: Comparison between endotoxins and glucans. *Inhalation Toxicology* **9**, 369-388.

Milanowski, J. (1998) Effect of inhalation of organic dust-derived microbial agents on the pulmonary phagocytic oxidative metabolism of guinea pigs. *Journal of Toxicology and Environmental Health-Part A* **53**, 5-18.

Milanowski, J., Sorenson, W.G., Dutkiewicz, J. and Lewis, D.M. (1995a) Chemotaxis of alveolar macrophages and neutrophils in response to microbial products derived from organic dust. *Environmental Research* **69**, 59-66.

Milanowski, J., Sorenson, W.G., Lewis, D.M. and Dutkiewicz, J. (1995b) Chemotaxis of alveolar macrophages and neutrophils in response to microbial products derived from organic dust. *Journal of Investigational Allergology & Clinical Immunology* **5**, 221-227.

Milton, D.K., Amsel, J., Reed, C.E., Enright, P.L., Brown, L.R., Aughenbaugh, G.L. and Morey, P.R. (1995) Cross-sectional follow-up of a flu-like respiratory illness among fiberglass manufacturing employees - Endotoxin exposure associated with 2 distinct sequelae. *American Journal of Industrial Medicine* **28**, 469-488.

Monso, E., Magarolas, R., Radon, K., Danuser, B., Iversen, M., Weber, C., Opravil, U., Donham, K.J. and Nowak, D. (2000) Respiratory symptoms of obstructive lung disease in European crop farmers. *American Journal of Respiratory and Critical Care Medicine* **162**, 1246-1250.

Moreland, J.G., Fuhrman, R.M., Wohlford-Lenane, C.L., Quinn, T.J., Benda, E., Pruessner, J.A. and Schwartz, D.A. (2001) TNF-alpha and IL-1 beta are not essential to the inflammatory response in LPS-induced airway disease. *American Journal of Physiology-Lung Cellular and Molecular Physiology* **280**, L173-L180.

Mosbach, M., WienerSchmuck, M. and Seidel, A. (1996) Influence of co-exposure of ozone with quartz, latex, albumin, and LPS on TNF-alpha and chemotactic factor release by bovine alveolar macrophages in vitro. *Inhalation Toxicology* **8**, 625-638.

Nagai, H., Tsuji, F., Shimazawa, T., Goto, S., Yoshitake, K. and Koda, A. (1991) Participation of collagenase and elastase in LPS-induced airway hyper-responsiveness in guinea-pigs. *Inflammation* **15**, 317-330.

Nicholls, J.M. (1978) A pathological study of chronic obstructive pulmonary disease in the horse. PhD Thesis, University of Glasgow.

Nightingale, J.A., Rogers, D.F., Hart, L.A., Kharitonov, S.A., Chung, K.F. and Barnes, P.J. (1998) Effect of inhaled endotoxin on induced sputum in normal, atopic, and atopic asthmatic subjects. *Thorax* **53**, 563-571.

Ning, Y.Y., Imrich, A., Goldsmith, C.A., Qin, C.Z. and Kobzik, L. (2000) Alveolar macrophage cytokine production in response to air particles in vitro: Role of endotoxin. *Journal of Toxicology and Environmental Health-Part A* **59**, 165-180.

Norn, S. (1994) Microorganism-induced or enhanced mediator release - A possible mechanism in organic dust related diseases. *American Journal of Industrial Medicine* **25**, 91-95.

Norn, S., Clementsen, P., Kristensen, K.S., Skov, P.S., Bisgaard, H. and Gravesen, S. (1994) Examination of mechanisms responsible for organic dust-related diseases - Mediator release induced by microorganisms - A review. *Indoor Air-International Journal of Indoor Air Quality and Climate* **4**, 217-222.

Nowak, D. (1998) Health effects of airborne pollutants particularly in swine confinement houses - A review focusing on aspects of occupational medicine. *Deutsche Tierarztliche Wochenschrift* **105**, 225-234.

Oberdorster, G. (2000) Toxicology of ultrafine particles: *In vivo* studies. *Philosophical Transactions of The Royal Society of London Series A-Mathematical Physical and Engineering Sciences* **358**, 2719-2739.

Ohkawara, Y., Lei, X.F., Stampfli, M.R., Marshall, J.S., Xing, Z. and Jordana, M. (1997) Cytokine and eosinophil responses in the lung, peripheral blood, and bone marrow compartments in a murine model of allergen-induced airways inflammation. *American Journal of Respiratory Cell and Molecular Biology* **16**, 510-520.

Olenchock, S.A., May, M.M., Pratt, D.S. and Morey, P.R. (1986) Endotoxins in the agricultural environment. *American Journal of Industrial Medicine* **10**, 323-324.

Olenchock, S.A., Murphy, S.A., Mull, J.C. and Lewis, D.M. (1992) Endotoxin and complement activation in an analysis of environmental dusts from a horse barn. *Scandinavian Journal of Work & Environmental Health* **18** (suppl. 2), 58-59.

Park, H.S., Jung, K.S., Hwang, S.C., Nahm, D.H. and Yim, H.E. (1998) Neutrophil infiltration and release of IL-8 in airway mucosa from subjects with grain dust-induced occupational asthma. *Clinical and Experimental Allergy* **28**, 724-730.

Park, J.H., Gold, D.R., Spiegelman, D.L., Burge, H.A. and Milton, D.K. (2001) House dust endotoxin and wheeze in the first year of life. *American Journal of Respiratory and Critical Care Medicine* **163**, 322-328.

Pickrell, J.A. (1991) Hazards in confinement housing - Gases and dusts in confined animal houses for swine, poultry, horses and humans. *Vet. Hum. Toxicol.* **33** (1), 32-39.

Pirie, R.S., Collie, D.D.S., Dixon, P.M. and McGorum, B.C. (2001a) Evaluation of nebulised hay dust suspensions (HDS) for the diagnosis and investigation of heaves. 2: Effects of inhaled HDS on control and heaves horses . *Equine Veterinary Journal* In press.

Pirie, R.S., Dixon, P.M., Collie, D.D.S. and McGorum, B.C. (2001b) Pulmonary and systemic effects of inhaled endotoxin in control and heaves horses . *Equine Veterinary Journal* **33**, (3) 311-318.

Pirie, R.S., Dixon, P.M. and McGorum, B.C. (2001c) Evaluation of nebulised hay dust suspensions (HDS) for the diagnosis and investigation of heaves. 3: Effect of fractionation of HDS. *Equine Veterinary Journal* In press.

Pirie, R.S., McLachlan, G. and McGorum, B.C. (2001d) Evaluation of nebulised hay dust suspensions (HDS) for the diagnosis and investigation of heaves. 1: Preparation and composition of HDS . *Equine Veterinary Journal* In press.

PlattsMills, T.A.E., Sporik, R.B., Chapman, M.D. and Heymann, P.W. (1997) The role of domestic allergens. *Ciba Foundation Symposia* **206**, 173-189.

Popendorf, W. (1986) Report on agents. *American Journal of Industrial Medicine* **10**, 251-259.

Preller, L., Heederik, D., Boleij, J.S.M., Vogelzang, P.F.J. and Tielen, M.J.M. (1995a) Lung-function and chronic respiratory symptoms of pig farmers - Focus on exposure to endotoxins and ammonia and use of disinfectants. *Occupational and Environmental Medicine* **52**, 654-660.

Preller, L., Heederik, D., Kromhout, H., Boleij, J.S.M. and Tielen, M.J.M. (1995b) Determinants of dust and endotoxin exposure of pig farmers - Development of a control strategy using empirical modeling. *Annals of Occupational Hygiene* **39**, 545-557.

Qureshi, S.T., Lariviere, L., Leveque, G., Clermont, S., Moore, K.J., Gros, P. and Malo, D. (1999) Endotoxin-tolerant mice have mutations in toll-like receptor 4 (TLR4). *Journal of Experimental Medicine* **189**, 615-625.

Raymond, S.L., Curtis, E.F. and Clarke, A.F. (1994) Comparative dust challenges faced by horses when fed alfalfa cubes or hay. *Equine Practice* **16**, 42-47.

Reitmeyer, H., Klein, H.-J. and Deegen, E. (1986) The effect of sedatives on lung function in horses. *Acta Vet. Scand.* **82**, 111-120.

Reynolds, S.J., Donham, K.J., Whitten, P., Merchant, J.A., Burmeister, L.F. and Popendorf, W.J. (1996) Longitudinal evaluation of dose-response relationships for environmental exposures and pulmonary-function in swine production workers. *American Journal of Industrial Medicine* **29**, 33-40.

Rietschel E.Th., Brade, H, Brade, L. et al (1987). Lipid A, the endotoxin center of bacterial lipopolysaccharides: relation of chemical structure to biological activity. In: *Detection of Bacterial Endotoxins with the Limulus Amebocyte Lysate Test*. pp 25-53. Edited by Stanley, W.W. Levin, J. New York, Alan R. Liss.

Rietschel E.Th., Brade, L, Brade, H. et al (1990). Bacterial lipopolysaccharides: relationship of structure of conformation of endotoxin activity, serological specificity and biological function. Advances in Experimental Medical Biology **256**, 81-99.

Rizzo, M.C., Naspitz, C.K., FernandezCaldas, E., Lockey, R.F., Mimica, I. and Sole, D. (1997) Endotoxin exposure and symptoms in asthmatic children. *Pediatric Allergy and Immunology* **8**, 121-126.

Robinson, N.E. (1998) COPD: Newer Concepts. *Proceedings of 1st World Equine Airways Symposium,* Guelph, Canada.

Robinson, N.E. (2001) International workshop on equine chronic airway disease. *Equine Veterinary Journal* **33**, 5-20.

Robinson, N.E., Derksen, F.J., Olszewski, M.A. and Buechner-Maxwell, V.A. (1996) The pathogenesis of chronic obstructive pulmonary disease of horses. *British Veterinary Journal* **152**, 283-305.

Rolla, G., Bucca, C., Brussino, L., Dutto, L., Colagrande, P. and Polizzi, S. (1997) Pentoxifylline attenuates LPS-induced bronchial hyper-responsiveness but not the increase in exhaled nitric oxide. *Clinical and Experimental Allergy* **27**, 96-103.

Ryan, L.K., Jin, R.H., Boggs, S.S., Karol, M.H. and Day, B.W. (1994) Mouse model for assessing endotoxin involvement in the lung inflammation and cytokine production resulting from inhaled organic dust. *Inhalation Toxicology* **6**, 485-499.

Rylander, R. (1986) Lung-diseases caused by organic dusts in the farm environment. *American Journal of Industrial Medicine* **10**, 221-227.

Rylander, R. (1987) Effects after acute and chronic exposure to cotton dust: The Manchester criteria. *British Journal of Industrial Medicine* **44**, 577-579.

Rylander, R. (1988) Plant constituents of cotton dust and lung effects after inhalation. *European Respiratory Journal* **1**, 812-817.

Rylander, R. (1990) Health effects of cotton dust exposures. *American Journal of Industrial Medicine* **17**, 39-45.

Rylander, R. (1992) Diseases associated with exposure to plant dusts - Focus on cotton dust. *Tubercle and Lung Disease* **73**, 21-26.

Rylander, R. (1994) Organic dusts - from knowledge to prevention. *Scandinavian Journal of Work Environment & Health* **20**, 116-122.

Rylander, R. (1996) Airway responsiveness and chest symptoms after inhalation of endotoxin or (1-3)-beta-D-glucan. *Indoor Built Environ* **5**, 106-111.

Rylander, R. (1997a) Airborne (1->3)-beta-d-glucan and airway disease in a day-care center before and after renovation. *Archives of Environmental Health* **52**, 281-285.

Rylander, R. (1997b) Evaluation of the Risks of Endotoxin Exposures. *International Journal of Occupational and Environmental Health* **3**, S32-S36.

Rylander, R. (1997c) Investigations of the relationship between disease and airborne (1- >3)-beta-D-glucan in buildings. *Mediators of Inflammation* **6**, 275-277.

Rylander, R. (1998) Microbial cell wall constituents in indoor air and their relation to disease. *Indoor Air-International Journal of Indoor Air Quality and Climate* 59-65.

Rylander, R., Bake, B., Fischer, J.J. and Helander, I.M. (1989) Pulmonary-function and symptoms after inhalation of endotoxin. *American Review of Respiratory Disease* **140**, 981-986.

Rylander, R. and Beijer, L. (1987) Inhalation of endotoxin stimulates alveolar macrophage production of platelet-activating-factor. *American Review of Respiratory Disease* **135**, 83-86.

Rylander, R. and Bergstrom, R. (1993) Bronchial reactivity among cotton workers in relation to dust and endotoxin exposure. *Annals of Occupational Hygiene* **37**, 57-63.

Rylander, R. and Fogelmark, B. (1994) Inflammatory responses by inhalation of endotoxin and (1-3)-beta-D-glucan. *American Journal of Industrial Medicine* **25**, 101-102.

Rylander, R. and Holt, P.G. (1998) (1->3)-beta-D-glucan and endotoxin modulate immune response to inhaled allergen. *Mediators of Inflammation* **7**, 105-110.

Rylander, R., Norrhall, M., Engdahl, U., Tunsater, A. and Holt, P.G. (1998) Airways inflammation, atopy, and (1->3)-beta-D-glucan exposures in two schools. *American Journal of Respiratory and Critical Care Medicine* **158**, 1685-1687.

Rylander, R., Thorn, J. and Attefors, R. (1999) Airways inflammation among workers in a paper industry. *European Respiratory Journal* **13**, 1151-1157.

Sandstrom, T., Bjermer, L. and Rylander, R. (1992) Lipopolysaccharide (LPS) inhalation in healthy-subjects increases neutrophils, lymphocytes and fibronectin levels in bronchoalveolar lavage fluid. *European Respiratory Journal* **5**, 992-996.

Sandstrom, T., Bjermer, L. and Rylander, R. (1994) Lipopolysaccharide (LPS) inhalation in healthy-subjects causes bronchoalveolar neutrophilia, lymphocytosis, and fibronectin increase. *American Journal of Industrial Medicine* **25**, 103-104.

Schimming, A., Gleich, G.J. and Kita, H. (1997) Inhibition of eosinophil infiltration into peritoneal cavities of allergen-challenged mice by lipopolysaccharide (LPS). *Journal of Allergy and Clinical Immunology* **99**, 1466.

Schulte, R., Grassl, G.A., Preger, S., Fessele, S., Jacobi, C.A., Schaller, M., Nelson, P.J. and Autenrieth, I.B. (2000) *Yersinia enterocolitica* invasin protein triggers IL-8 production in epithelial cells via activation of Rel p65-p65 homodimers. *Faseb Journal* **14**, 1471-1484.

Schuyler, M., Gott, K. and Cherne, A. (1998) Effect of glucan on murine lungs. *Journal of Toxicology and Environmental Health-Part A* **53**, 493-505.

Schwartz, D.A., Donham, K.J., Olenchock, S.A., Popendorf, W.J., Vanfossen, D.S., Burmeister, L.F. and Merchant, J.A. (1995a) Determinants of longitudinal changes in spirometric function among swine confinement operators and farmers. *American Journal of Respiratory and Critical Care Medicine* **151**, 47-53.

Schwartz, D.A., Thorne, P.S., Jagielo, P.J., White, G.E., Bleuer, S.A. and Frees, K.L. (1994) Endotoxin responsiveness and grain dust-induced inflammation in the lower respiratory-tract. *American Journal of Physiology-Lung Cellular and Molecular Physiology* **11**, L 609-L 617.

Schwartz, D.A., Thorne, P.S., Yagla, S.J., Burmeister, L.F., Olenchock, S.A., Watt, J.L. and Quinn, T.J. (1995b) The role of endotoxin in grain dust-induced lung-disease. *American Journal of Respiratory and Critical Care Medicine* **152**, 603-608.

Shahan, T.A., Sorenson, W.G. and Lewis, D.M. (1994) Superoxide anion production in response to bacterial lipopolysaccharide and fungal spores implicated in organic dust toxic syndrome. *Environmental Research* **67**, 98-107.

Sherson, D., Hansen, I. and Sigsgaard, T. (1989) Occupationally related respiratory symptoms introut-processing workers. *Allergy* **44**, 336-341.

Shimada, M., Tsukada, H., Ishizuka, O., Kon, Y., Hasegawa, T., Suzuki, E. and Gejyo, F. (2000) Lipopolysaccharide tolerance in relation to intrabronchial influx of neutrophils in the rat. *Lung* **178**, 235-248.

Shvedova, A.A., Satoh, T., Tollerud, D., Guevarra, L. and Karol, M.H. (1996) Elevated levels of IL-6, INF-gamma, and TNF-alpha in mice in response to cotton dust are modulated by anti-TNF-alpha antiserum. *Experimental Lung Research* **22**, 149-161.

Sigsgaard, T., Bonefeld-Jorgensen, E.C., Kjaergaard, S.K., Mamas, S. and Pedersen, O.F. (2000) Cytokine release from the nasal mucosa and whole blood after experimental exposures to organic dusts. *European Respiratory Journal* **16**, 140-145.

Sigsgaard, T., Pedersen, O.F., Juul, S. and Gravesen, S. (1992) Respiratory disorders and atopy in cotton, wool, and other textile mill workers in Denmark. *American Journal of Industrial Medicine* **22**, 163-184.

Sjogren, B., Wang, Z.P., Larsson, B.M., Larsson, K., Larsson, P.H. and Westerholm, P. (1999) Increase in interleukin-6 and fibrinogen in peripheral blood after swine dust inhalation. *Scandinavian Journal of Work Environment & Health* **25**, 39-41.

Slater, J.E., Paupore, E. and Truscott, W. (1998) Lipopolysaccharide (LPS) augments IgG and IgE responses of mice to the latex allergen *hev b 5*. *Journal of Allergy and Clinical Immunology* **101**, 851.

Smid, T., Heederik, D., Houba, R. and Quanjer, P.H. (1994) Dust-related and endotoxin-related acute lung-function changes and work-related symptoms in workers in the animal feed-industry. *American Journal of Industrial Medicine* **25**, 877-888.

Snella, M.C., Venaille, T., Holt, P.G. and Rylander, R. (1987) Distribution and chemotactic activity of cells in bronchoalveolar lavage and lung interstitium of guinea-pigs after inhalation of endotoxin. *Chest* **91**, 297.

Soukup, J.M. and Becker, S. (2001) Human alveolar macrophage responses to air pollution particulates are associated with insoluble components of coarse material, including particulate endotoxin. *Toxicology and Applied Pharmacology* **171**, 20-26.

Stewart, G.A., Kollinger, M.R., King, C.M. and Thompson, P.J. (1994) A comparative-study of 3 serine proteases from *dermatophagoides pteronyssinus* and *dermatophagoides farinae*. *Allergy* **49**, 553-560.

Stewart, G.A., Lake, F.R. and Thompson, P.J. (1991) Fecally derived hydrolytic enzymes from *dermatophagoides pteronyssinus* - Physicochemical characterization of potential allergens. *International Archives of Allergy and Applied Immunology* **95**, 248-256.

Stewart, G.A., Vanhagehamsten, M., Krska, K., Thompson, P.J. and Olsson, S. (1998) An enzymatic analysis of the storage mite *lepidoglyphus destructor*. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology* **119**, 341-347.

Strohmeier, G.R., Walsh, J.H., Klings, E.S., Farber, H.W., Cruikshank, W.W., Center, D.M. and Fenton, M.J. (2001) Lipopolysaccharide binding protein potentiates airway reactivity in a murine model of allergic asthma. *Journal of Immunology* **166**, 2063-2070.

Sur, S., Crotty, T.B., Kephart, G.M., Hyma, B.A., Colby, T.V., Reed, C.E., Hunt, L.W. and Gleich, G.J. (1993) Sudden-onset fatal asthma - A distinct entity with few eosinophils and relatively more neutrophils in the airway submucosa. *American Review of Respiratory Disease* **148**, 713-719.

Suzuki, T., Wang, W., Lin, J.T., Shirato, K., Mitsuhashi, H. and Inoue, H. (1996) Aerosolized human neutrophil elastase induces airway constriction and hyper-

responsiveness with protection by intravenous pretreatment with half-length secretory leukoprotease inhibitor. *American Journal of Respiratory and Critical Care Medicine* **153**, 1405-1411.

Tanner, M.K., Swinker, A.M., Beard, M.L., Cosma, G.N., TraubDargatz, J.L., Martinez, A.B. and Olenchock, S.A. (1998) Effect of phone book paper versus sawdust and straw bedding on the presence of airborne gram-negative bacteria, fungi and endotoxin in horse stalls. *Journal of Equine Veterinary Science* **18**, 457-461.

Taussig, M.J. (1984) Infection: Bacteria. In: *Processes in Pathology and Microbiology* 2nd Edition, pp 394-396, Edited by Taussig, M.J., London, Blackwell Scientific Publications.

Teeuw, K.B., van den Brouckegrauls, C.M.J.E. and Verhoef, J. (1994) Airborne gram-negative bacteria and endotoxin in sick building syndrome - a study in Dutch governmental office buildings. *Archives of Internal Medicine* **154**, 2339-2345.

Tesfaigzi, Y., Fischer, M.J., Martin, A.J. and Seagrave, J. (2000) BCL-2 in LPS and allergen-induced hyperplastic mucous cells in airway epithelia of Brown Norway rats. *American Journal of Physiology-Lung Cellular and Molecular Physiology* **279**, L1210-L1217.

Thomson, J.R. and McPherson, E.A. (1984) Effects of environmental-control on pulmonary-function of horses affected with chronic obstructive pulmonary-disease. *Equine Veterinary Journal* **16**, 35-38.

Thorn, J., Beijer, L. and Rylander, R. (1998) Airways inflammation and glucan exposure among household waste collectors. *American Journal of Industrial Medicine* **33**, 463-470.

Thorn, J. and Rylander, R. (1998a) Airways inflammation and glucan in a rowhouse area. *American Journal of Respiratory and Critical Care Medicine* **157**, 1798-1803.

Thorn, J. and Rylander, R. (1998b) Inflammatory response after inhalation of bacterial endotoxin assessed by the induced sputum technique. *Thorax* **53**, 1047-1052.

Thorne, P.S. (2000) Inhalation toxicology models of endotoxin- and bioaerosol-induced inflammation. *Toxicology* **152**, 13-23.

Tomee, J.F.C., van Weissenbruch, R., de Monchy, J.G.R., and Kauffman, H.F. (1998) Interactions between inhalant allergen extracts and airway epithelial cells: Effect on cytokine production and cell detachment. *Journal of Allergy and Clinical Immunology* **102**, 75-85.

Toward, T.J. and Broadley, K.J. (2000) Airway reactivity, inflammatory cell influx and nitric oxide in guinea-pig airways after lipopolysaccharide inhalation. *British Journal of Pharmacology* **131**, 271-281.

Townley, R.G., Dennis, M. and Itkin, I.H. (1965) Comparative action of acetyl-beta-methacholine, histamine and pollen antigens in subjects with hay fever and patients with bronchial asthma. *Journal of Allergy* **36**, 121-137.

Trapp, J.F., Watt, J.L., Frees, K.L., Quinn, T.J., Nonnenmann, M.W. and Schwartz, D.A. (1998) The effect of glucocorticoids on grain dust induced airway disease. *Chest* **113**, 505-513.

Tulic, M.K., Wale, J.L., Holt, P.G. and Sly, P.D. (2000) Modification of the inflammatory response to allergen challenge after exposure to bacterial lipopolysaccharide. *American Journal of Respiratory Cell and Molecular Biology* **22**, 604-612.

Ulich, T.R., Yin, S., Remick, D.G., Russell, D., Eisenberg, S.P. and Kohno, T. (1993) Intracheal administration of endotoxin and cytokines .4: The soluble tumor-necrosis-factor receptor type-1 inhibits acute-inflammation. *American Journal of Pathology* **142**, 1335-1338.

Ulmer, A.J. (1997) Biochemistry and cell biology of endotoxins. *International Journal of Occupational and Environmental Health* **3**, S8-S17.

Uno, T., Tanaka, H. and Nagai, H. (1996) Cysteinyl leukotrienes do not mediate lipopolysaccharide-induced airway hyper-responsiveness in guinea pigs. *Prostaglandins* **52**, 447-461.

Uno, T., Tanaka, H., Nakai, N. and Nagai, H. (1997) Participation of leukotriene D-4 and tumor necrosis factor on lipopolysaccharide-induced airway hyper-responsiveness in guinea pigs. *Biological & Pharmaceutical Bulletin* **20**, 332-337.

Urbain, B., Prouvost, J.F., Beerens, D., Ansay, M. and Gustin, P. (1996a) Acute effects of endotoxin inhalation on the respiratory tract in pigs: Interaction with ammonia. *Inhalation Toxicology* **8**, 947-968.

Urbain, B., Prouvost, J.F., Beerens, D., Michel, O., Nicks, B., Ansay, M. and Gustin, P. (1996b) Chronic exposure of pigs to airborne dust and endotoxins in an environmental chamber - Technical note. *Veterinary Research* **27**, 569-578.

van der Haegen, A., Gerber, V., Zurbriggen, A., Robinson, E. and Marti, E. (2001) IgE-protein and IgE-mRNA positive cells in lung tissue samples from RAO-affected and control horses after mouldy hay challenge. *Proceedings of 2nd World Equine Airways Symposium,* Edinburgh, Scotland, 58.

van der Veen, M.J., Lopuhaa, C.E., Aalberse, R.C., Jansen, H.M. and van der Zee, J.S. (1998) Bronchial allergen challenge with isolated major allergens of *Dermatophagoides pteronyssinus*: The role of patient characteristics in the early asthmatic response. *Journal of Allergy and Clinical Immunology* **102**, 24-31.

Vanhagehamsten, M. and Johansson, E. (1998) Clinical and immunologic aspects of storage mite allergy. *Allergy* **53**, 49-53.

Vincent, D., Lefort, J., Chatelet, F., Bureau, M.F., Dry, J. and Vargaftig, B.B. (1993) Intratracheal *Escherichia coli* lipopolysaccharide induces platelet- dependent bronchial hyper-reactivity. *Journal of Applied Physiology* **74**, 1027-1038.

Vogelzang, P.F.J., van der Gulden, J.W.J., Folgering, H., Heederik, D., Tielen, M.J.M. and van Schayck, C.P. (2000) Longitudinal changes in bronchial responsiveness associated with swine confinement dust exposure. *Chest* **117**, 1488-1495.

Vogelzang, P.F.J., van der Gulden, J.W.J., Folgering, H., Kolk, J.J., Heederik, D., Preller, L., Tielen, M.J.M. and van Schayck, C.P. (1998) Endotoxin exposure as a major determinant of lung function decline in pig farmers. *American Journal of Respiratory and Critical Care Medicine* **157**, 15-18.

von Essen, S.G., Oneill, D.P., McGranaghan, S., Olenchock, S.A. and Rennard, S.I. (1995a) Neutrophilic respiratory-tract inflammation and peripheral-blood neutrophilia after grain-sorghum dust extract challenge. *Chest* **108**, 1425-1433.

von Essen, S.G., Oneill, D.P., Olenchok, S.A., Robbins, R.A. and Rennard, S.I. (1995b) Grain dusts and grain plant-components vary in their ability to recruit neutrophils. *Journal of Toxicology and Environmental Health* **46**, 425-441.

Votion, D., Ghafir, Y., Munsters, K., Duvivier, D.H., Art, T. and Lekeux, P. (1997) Aerosol deposition in equine lungs following ultrasonic nebulisation versus jet aerosol delivery system. *Equine Veterinary Journal* **29**, 388-393.

Wan, G.H., Li, C.S. and Lin, R.H. (2000) Airborne endotoxin exposure and the development of airway antigen-specific allergic responses. *Clinical and Experimental Allergy* **30**, 426-432.

Wang, Z., Larsson, K., Palmberg, L., Malmberg, P., Larsson, P. and Larsson, L. (1997) Inhalation of swine dust induces cytokine release in the upper and lower airways. *European Respiratory Journal* **10**, 381-387.

Wang, Z.P., Malmberg, P., Larsson, B.M., Larsson, K., Larsson, L. and Saraf, A. (1996a) Exposure to bacteria in swine-house dust and acute inflammatory reactions in humans. *American Journal of Respiratory and Critical Care Medicine* **154**, 1261-1266.

Wang, Z.P., Malmberg, P., Larsson, P., Larsson, B.M. and Larsson, K. (1996b) Time-course of interleukin-6 and tumor-necrosis-factor-alpha increase in serum following inhalation of swine dust. *American Journal of Respiratory and Critical Care Medicine* **153**, 147-152.

Wang, Z., Manninen, A., Malmberg, P. and Larsson, K. (1998) Inhalation of swine-house dust increases the concentrations of interleukin-1 beta (IL-1 beta) and interleukin-1 receptor antagonist (IL-1ra) in peripheral blood. *Respiratory Medicine* **92**, 1022-1027.

Webster, A., Clarke, A.F., Madelin, T. and Wathes, C.M. (1987) Air hygiene in stables 1: Effects of stable design, ventilation and management on the concentration of respirable dust. *Equine Veterinary Journal* **19**, 448-453.

Wesselius, L.J., Nelson, M.E., Bailey, K. and O'Brien Ladner, A.R. (1997) Rapid lung cytokine accumulation and neutrophil recruitment after lipopolysaccharide inhalation by cigarette smokers and nonsmokers. *Journal of Laboratory and Clinical Medicine* **129**, 106-114.

Widmer, F., Hayes, P.J., Whittaker, R.G. and Kumar, R.K. (2000) Substrate preference profiles of proteases released by allergenic pollens. *Clinical and Experimental Allergy* **30**, 571-576.

Wiegand, B., Hartung, J., Hinz, T. and Wiemann, H.D. (1993) Air-quality in Louisiana type broiler houses .2. Bacteria and endotoxin levels in airborne dust. *Landbauforschung Volkenrode* **43**, 236-241.

Williams, P.B. and Halsey, J.F. (1997) Endotoxin as a factor in adverse reactions to latex gloves. *Annals of Allergy Asthma & Immunology* **79**, 303-310.

Winder, N.C. and von Fellenberg, R. (1990) Mast-cells in normal and pathological specimens of the equine lung. *Journal of Veterinary Medicine Series A-Zentralblatt fur Veterinarmedizin Reihe A-Physiology Pathology Clinical Medicine* **37**, 641-650.

WohlfordLenane, C.L., Deetz, D.C. and Schwartz, D.A. (1999) Cytokine gene expression after inhalation of corn dust. *American Journal of Physiology-Lung Cellular and Molecular Physiology* **20**, L736-L743.

Woods, P.S.A., Robinson, N.E., Swanson, M.C., Reed, C.E., Broadstone, R.V. and Derksen, F.J. (1993) Airborne dust and aeroallergen concentration in a horse stable under two different management systems. *Equine Veterinary Journal* **25**, 208-213.

Wouters, I.M., Douwes, J., Doekes, C., Thorne, P.S., Brunekreef, B. and Heederik, D.J.J. (2000) Increased levels of markers of microbial exposure in homes with indoor storage of organic household waste. *Applied and Environmental Microbiology* **66**, 627-631.

Yamashiro, S., Viel, L., Bast, T. and Harris, W. (1985) The morphological-study of airways and cells recovered by bronchoalveolar lavage in a horse with chronic obstructive pulmonary- disease (COPD). *Anatomical Record* **211**, A219.

Yang, H.M., Barger, M.W., Castranova, V., Ma, J.K.H., Yang, J.J. and Ma, J.Y.C. (1999) Effects of diesel exhaust particles (DEP), carbon black, and silica on macrophage responses to lipopolysaccharide: evidence of DEP suppression of macrophage activity. *Journal of Toxicology and Environmental Health-Part A* **58**, 261-278.

Zejda, J.E., Barber, E., Dosman, J.A., Olenchock, S.A., McDuffie, H.H., Rhodes, C. and Hurst, T. (1994) Respiratory health-status in swine producers relates to endotoxin exposure in the presence of low dust levels. *Journal of Occupational Medicine* **36**, 49-56.

Zock, J.P., Heederik, D. and Kromhout, H. (1995) Exposure to dust, endotoxin and microorganisms in the potato processing-industry. *Annals of Occupational Hygiene* **39**, 841-854.

Zuskin, E., Mustajbegovic, J., Schachter, E.N., Kern, J., Rienzi, N., Goswami, S., Marom, Z. and Maayani, S. (1995) Respiratory-function in poultry workers and pharmacological characterization of poultry dust extract. *Environmental Research* **70**, 11-19.

PUBLICATIONS

9.1 Papers

9.1.1 Papers resulting directly from the thesis

Pirie, R.S., Dixon, P.M., Collie, D.D.S. and McGorum, B.C. (2001) Pulmonary and systemic effects of inhaled endotoxin in control and heaves horses. *Equine Veterinary Journal* **33**, (3) 311-318.

Pirie, R.S., Collie, D.D.S., Dixon, P.M. and McGorum, B.C. (2001) Evaluation of nebulised hay dust suspensions (HDS) for the diagnosis and investigation of heaves. 2: Effects of inhaled HDS on control and heaves horses . *Equine Veterinary Journal* In press.

Pirie, R.S., McLachlan, G. and McGorum, B.C. (2001) Evaluation of nebulised hay dust suspensions (HDS) for the diagnosis and investigation of heaves. 1: Preparation and composition of HDS . *Equine Veterinary Journal* In press.

Pirie, R.S., Dixon, P.M. and McGorum, B.C. (2001) Evaluation of nebulised hay dust suspensions (HDS) for the diagnosis and investigation of heaves. 3: Effect of fractionation of HDS. *Equine Veterinary Journal* In press.

9.1.2 Papers resulting indirectly from the thesis

Nevalainen M, Raulo RS, Brazil TJ, **Pirie RS**, Sorsa T, McGorum BC and Maisi P Inhalation of organic dusts and lipopolysaccharide increases gelatinolytic metalloproteinase activity in the lungs of heaves affected horses. *Equine Veterinary Journal* In press.

Pickles KJ, **Pirie RS**, Rhind S, Dixon PM and McGorum BC. Cytological analysis of equine bronchoalveolar lavage fluid. Part 1: Comparison of sequential and pooled aliquots. *Equine Veterinary Journal* In press.

Pickles KJ, **Pirie RS**, Rhind S, Dixon PM and McGorum BC. Cytological analysis of equine bronchoalveolar lavage fluid. Part 2: Comparison of smear and cytocentrifuged preparations. *Equine Veterinary Journal* In press.

Pickles KJ, **Pirie S**, Rhind S, Dixon PM and McGorum BC. Cytological analysis of equine bronchoalveolar lavage fluid. Part 3: The effect of time, temperature and fixatives. *Equine Veterinary Journal* In press.

9.2 Abstracts

9.2.1 Abstracts resulting directly from the thesis

Pirie, R.S., McGorum, B.C., Brazil, T.J. and Dixon, P.M. (1998) The role of inhaled endotoxin in the aetiopathogenesis of COPD. Proceedings of the Dorothy Russel Havermeyer Foundation, Inc. Workshop on Allergic Diseases of the Horse. 20th – 22nd April, Lipica, Slovenia.

Pirie, R.S., McGorum, B.C., Dixon, P.M. and Brazil, T.J. (1998) Inhaled endotoxin – A possible role in equine COPD? Proceedings of 1st World Equine Airways Symposium, 3rd – 6th August, Guelph, Canada.

Pirie, R.S., McGorum, B.C., Brazil, T.J. and Dixon, P.M. (1998) The role of inhaled endotoxin in the aetiopathogenesis of equine COPD. AVTRW Symposium, Glasgow, November 1998.

Pirie, R.S., Collie, D.D.S., Dixon, P.M. and McGorum, B.C. (2000) Pulmonary and systemic effects of inhaled endotoxin in heaves and control horses. Comparative Respiratory Society Annual Congress, Melbourne, November 2000. ***Winner of the Joan O'Brien Research Award 2000.***

Pirie, R.S., Collie, D.D.S., Dixon, P.M. and McGorum, B.C. (2001) The use of nebulised endotoxin and hay dust suspension in establishing the role of endotoxin in RAO. Proceedings of the Dorothy Russel Havermeyer Foundation, Inc. Workshop on Allergic Diseases of the Horse. 25th – 29th April, Hortobagy-Mata, Hungary.

Pirie, R.S., Dixon, P.M. and McGorum, B.C. (2001) The role of inhaled endotoxin in the aetiopathogenesis of heaves. Proceedings of 2nd World Equine Airways Symposium, 19th – 23rd July, Edinburgh, Scotland.

Pirie, R.S., Dixon, P.M. and McGorum, B.C. (2001) *Aspergillus fumigatus* extract dose response inhalation challenges in heaves horses and the contribution of endotoxin contamination to the pulmonary inflammatory and functional response to *Aspergillus fumigatus* extract inhalation. Proceedings of 2nd World Equine Airways Symposium, 19th – 23rd July, Edinburgh, Scotland.

Pirie, R.S., McLachlan, G., Collie, D.D.S., Dixon, P.M. and McGorum, B.C. (2001) Evaluation of nebulised hay dust suspensions (HDS) for the diagnosis and investigation of heaves. Proceedings of 2nd World Equine Airways Symposium, 19th – 23rd July, Edinburgh, Scotland. ***Winner of the Joan O'Brien Research Award 2001.***

9.2.2 Abstracts resulting indirectly from the thesis

Brazil, T.J., Dagleish, M.P., **Pirie, R.S.**, McGorum, B.C., Dixon, P.M., Haslett, C. and Chilvers, E.R. (1998) Neutrophil activation, apoptosis and clearance in COPD. Proceedings of the Dorothy Russel Havermeyer Foundation, Inc. Workshop on Allergic Diseases of the Horse. 20th – 22nd April, Lipica, Slovenia.

Brazil, T.J., Dagleish, M.P., **Pirie, R.S.**, McGorum, B.C., Dixon, P.M., Haslett, C. and Chilvers, E.R. (1998) Neutrophil activation and clearance and the resolution of airway inflammation in acute equine chronic obstructive pulmonary disease. Proceedings 37th British Equine Veterinary Association Congress.

Pickles, K.J., **Pirie, R.S.,** Rhind, S., McGorum, B.C. and Dixon, P.M. (2001) The effect of time, temperature and fixatives on cytological analysis of equine bronchoalveolar lavage fluid. Proceedings of 2nd World Equine Airways Symposium, 19th – 23rd July, Edinburgh, Scotland.

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Pulmonary and systemic effects of inhaled endotoxin in control and heaves horses

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Summary

To investigate whether inhaled endotoxin contributes to airway inflammation and dysfunction in stabled horses, control (n = 6) and asymptomatic heaves (previously termed chronic obstructive pulmonary disease)-susceptible (n = 7) horses were given inhalation challenges with 20, 200 and 2000 µg of soluble *Salmonella typhimurium* Ra60 lipopolysaccharide (LPS). LPS inhalation induced a dose-dependent neutrophilic airway inflammatory response in both groups. Inhalation with 2000 µg of LPS also induced detectable lung dysfunction in the heaves group. LPS inhalation did not alter clinical score, tracheal secretion volume or airway reactivity in either group. The no-response thresholds were lower for the heaves group (<20 µg for airway inflammation; 200 to 2000 µg for lung dysfunction) than for the control group (20 to 200 µg for airway inflammation; >2000 µg for lung dysfunction). To enable comparison of these threshold levels with airborne endotoxin concentrations in stables, horses also received a 5 h duration hay/straw challenge, during which the total and respirable airborne endotoxin concentrations were determined. Comparison of the effects of acute LPS inhalation and hay/straw challenges suggest that inhaled endotoxin is not the sole cause of heaves. However, it is likely that it contrlbutes to airway inflammation, both in heaves horses in concert with other inhalants, and in normal horses when they are exposed to high levels in poor stable environments.

Introduction

Inhaled endotoxins are an important cause of human pulmonary disease (Jacobs 1997a), with the severity of pulmonary inflammation and clinical symptoms experienced by subjects exposed to organic dusts being related to the endotoxin concentration of the inhaled dust (Rylander and Bergstrom 1993; Smid *et al.* 1994; Zejda *et al.* 1994; Schwartz *et al.* 1995; Vogelzang *et al.* 1998). Additionally, the severity of human asthma has been related to the level of endotoxin exposure (Michel *et al.* 1991, 1996; Rizzo *et al.* 1997), suggesting that inhaled endotoxin may potentiate the inflammatory response to allergens in atopic subjects. In man, considerable efforts have been made to establish no-response threshold levels for inhaled endotoxin (Michel *et al.* 1997) and to identify safety guidelines for occupational endotoxin exposure (Rylander 1997). Since horse stables contain high concentrations of airborne endotoxin (Olenchock *et al.* 1992; Dutkiewicz *et al.* 1994; McGorum *et al.* 1998; Tanner *et al.* 1998) and, given the similarities between heaves and inhaled endotoxin mediated lung disease in other species (McGorum *et al.* 1998), it is surprising that the role of endotoxin in heaves is unknown. The aims of the present study were (a) to investigate the response of control and heaves horses to increasing doses of inhaled LPS, (b) to determine no-response threshold levels for both control and heaves horses and (c) to compare no response threshold levels of inhaled soluble LPS with endotoxin levels encountered in equine stables.

Materials and methods

Horses

Six healthy control horses with no detectable respiratory tract disorders (all female, median age 6 years, range 4–9 years; median 320 kg bwt, range 316–356 kg bwt) and 7 horses (3 geldings, 4 mares; age 17 years, range 8–28 years; 434 kg bwt, 323–594 kg bwt) with a history and clinical diagnosis of heaves were used. The disease status of all subjects was confirmed by hay/straw challenge (*vide infra*). This challenge induced BALF neutrophilia (>20%), increased volume of tracheal secretions and a reduction in Pa_{O_2} in all heaves horses and, in some heaves horses, induced coughing, nasal discharge, hyperpnoea, double expiratory lift, increased isovolumetric and expiratory lung resistance and decreased dynamic compliance (Cdyn). All of the above clinical and laboratory abnormalities reverted to normal when the heaves horses were moved to a low dust environment (i.e. a well-ventilated stable with wood shavings bedding and haylage feeding, or at pasture). Hay/straw challenge did not induce detectable pulmonary inflammation and dysfunction, or detectable tracheal secretions, in control horses. Throughout the study all horses were kept in a low dust environment. The study was approved by the Home Office, and conducted under a Home Office project licence.

Challenge protocol

All horses received an initial control inhalation challenge with sterile isotonic saline (Vetivex 0.9%)[1], followed by 3 separate increasing doses (20, 200 and 2000 µg) of purified *Salmonella typhimurium* Ra60 LPS, followed by a hay/straw challenge. Although randomisation of the inhalation challenges was

TABLE 1: Baseline BALF total cell counts (x 10^5), neutrophil ratios (%) and absolute neutrophil counts (x 10^5) (median and range) obtained at t>-7days before LPS (20, 200 and 2000 µg) inhalation challenges and hay/straw exposure

Challenge	Total BALF cell count (x 10^5/ml)		BALF neutrophil ratio (%)		BALF neutrophil count (x 10^5/ml)	
	Heaves	Controls	Heaves	Controls	Heaves	Controls
20 µg LPS	4.4 (2.9–9.1)	3.3 (2.4–4.6)	2.3 (0.7–4.1)	1.0 (0.5–1.6)	0.09 (0.04–0.25)	0.03 (0.01–0.06)
200 µg LPS	5.0 (1.5–7.2)	5.4 (3.7–7.0)	2.3 (1.8–3.6)	1.1 (0.6–2.5)	0.11 (0.03–0.15)	0.06 (0.02–0.18)
2000 µg LPS	4.4 (1.8–9.7)	4.1 (2.4–7.3)	1.9 (0.7–3.2)	1.5 (0.6–2.9)	0.08 (0.04–0.18)	0.07 (0.03–0.08)
Hay/straw	4.2 (2.4–9.1)	2.1 (1.0–2.4)	1.5 (0.4–5.5)	0.9 (0.7–1.9)	0.06 (0.03–0.13)	0.02 (0.01–0.03)

TABLE 2: Clinical scoring system

Clinical variable	Response	Score
Cough	Present	0
	Absent	1
Nasal discharge	Present	0
	Absent	1
Dyspnoea	Absent	0
	Mild	1
	Moderate	2
	Severe	3
Respiratory rate	<20 breaths/min	0
	20–30 breaths/min	1
	>30 breaths/min	2
Thoracic auscultation	Normal	0
	Increased normal	1
	Adventitious sounds	2
	Marked adventitious noise	3
Pulse rate	<50 beats/min	0
	50–70 beats/min	1
	>70 beats/min	2
Rectal temperature	Normal	0
	Elevated (>39.5°C)	1
Total score		**13**

considered, this order was chosen for safety reasons due to the unknown effects of LPS inhalation in the horse. Several procedures were performed to minimise potential carry-over effects of a preceding challenge on subsequent challenges. Firstly, inhalation challenges were conducted a minimum of 14 days apart and, secondly, all horses were shown to have normal BALF cytology at least 7 days prior to challenges (Table 1) and normal clinical findings and lung function immediately prior to each inhalation challenge. In addition, 6 heaves and 4 control horses received a repeat inhalation challenge of 200 µg LPS following completion of the other challenges, both to assess potential carry-over effects and to determine the repeatability of LPS inhalation challenge.

LPS was diluted from a stock solution (8.89 mg/ml) in sterile isotonic saline[1] immediately prior to nebulisation. To facilitate nebulisation, horses were sedated with 20 µg/kg bwt romifidine (Sedivet)[2] and 10 µg/kg bwt butorphanol (Torbugesic)[3], i.v. The aerosol was generated using a compressor (Parimaster)[4] with a calibrated output of 7 l/min, connected to a nebuliser cup (Sidestream)[5], the manufacturers of which state that 80% of aerosol is in the respirable range (<5 µm). The nebuliser cup contained 2 ml of challenge solution. The aerosol passed via a 'T piece' system into an airtight facemask, with inspiratory and expiratory valves to minimise aerosol loss. One ml solution was delivered to the facemask for each challenge.

For the hay/straw challenge, horses were housed for 5 h in a poorly ventilated stable (3.7 x 3.7 m) with the bottom and top doors and all air vents closed, fed a mixture of good quality hay and hay with visible mould growth, and bedded on deep litter straw. This environment has previously been shown to induce airway inflammation, clinical signs and lung dysfunction in heaves horses (McGorum *et al.* 1993). During this challenge, time zero (t0) represented the time when the horse entered the stable. To quantify endotoxin exposure during the hay/straw challenge, total and respirable stable dusts were collected using personal samplers (AFC124 High Flow Personal Sampler)[6] from the breathing zones of all horses. Samples were collected and prepared for analysis as described previously (McGorum *et al.* 1998), except that samples were not filtered after elution of endotoxin from the filter membrane. The endotoxin content of the filter eluent was determined using an endotoxin-specific assay (Endospecy)[7] as described by Thorn (1999). Ten µl of appropriately diluted filter eluent was placed in a microwell plate, 100 µl specific endotoxin lysate was added, the mixture incubated at 37°C for 30 min and the reaction stopped by the addition of 200 µl 0.6 mol/l acetic acid. The absorbance of the resulting colour reaction was read photometrically at 405 nm, and compared to a standard curve. All samples were analysed in duplicate and the mean value calculated. Analysis was repeated if (a) the paired values differed from their mean by >10% of the mean, (b) either of the paired values exceeded the value of undiluted standard solution, or (c) either of the paired values were less than the value obtained from a 1:8 dilution of the standard solution.

Assessment of response to challenges

The response to challenges was assessed as indicated in Figure 1, including use of a clinical scoring system (Table 2). Lung mechanics were determined as previously described (McGorum and Dixon 1992), except that raw data were recorded using data acquisition software (Labview)[8]. The following lung mechanics indices were calculated: Cdyn; maximum transpulmonary pressure change ($dPpl_{max}$); isovolumetric lung resistance (RL_{iso}); inspiratory and expiratory lung resistance at 25, 50 and 75% tidal volume ($RL_{I25\%}$, $RL_{I50\%}$, $RL_{I75\%}$, $RL_{E25\%}$, $RL_{E50\%}$ and $RL_{E75\%}$, respectively); and total, resistive and elastic work of breathing (Wb_t, Wb_{res} and Wb_{el}, respectively). Airway reactivity was evaluated by inhalation of saline followed by doubling concentrations of methacholine chloride[9] solution (beginning with 0.4 mg/ml) for 60 s, with 2 min of data recording following each inhalation. The provocative concentration of methacholine (PCCdyn70) was the inhaled concentration (mg/ml) that reduced Cdyn to 70% of the value recorded following saline inhalation. All horses were sedated for airway reactivity assessment to ensure subject safety and compliance. Arterial blood samples were collected as previously described (Dixon *et al.* 1995) and analysed using a blood gas analyser (AVL Opti CCA)[10]. Venous

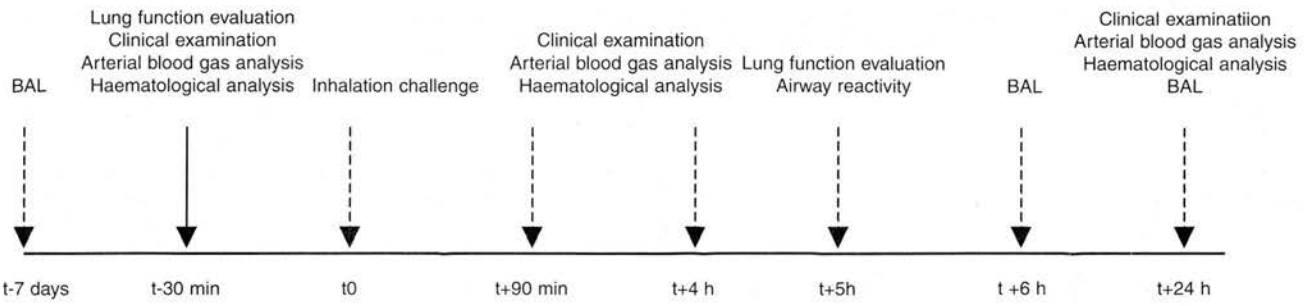

Fig 1: Study design. For the 5 h hay/straw challenges, the horses entered the stable at t = 0. BAL = bronchoalveolar lavage.

blood was collected by jugular venipuncture for total leucocyte and neutrophil counts. BALF was collected transendoscopically as previously described (Dixon *et al.* 1995), but without local anaesthesia of the tracheal carina, and analysed as previously described (McGorum and Dixon 1992), except differential cell counts included 1000 cells. The t-7 days and 6 h BALF samples were both collected from the right accessory lobe, and the 24 h BALF samples collected from the left ventral segment. Both sampled lung segments were cranioventral, thereby maximising the chance of them receiving an aerosol challenge of similar magnitude. Prior to BALF collection, the volume of tracheal secretions was graded 0–5 as previously described (Dixon *et al.* 1995).

Statistical analyses

The effects of each challenge were determined mostly by performing within-group analyses. Where prechallenge measurements were made at t-30 min (arterial blood gas analyses, peripheral blood leucocyte and neutrophil counts, and lung mechanics) the postchallenge values were expressed as % of baseline value, except for clinical scores where actual values were used. The effect of LPS challenge was assessed, by pairing and subtracting post-LPS and postsaline data. Where no prechallenge data was collected, comparisons were made with saline challenge data. A Friedman test was performed on sets of paired data and, when significant, a Wilcoxon Rank Sum test was performed on paired data. Between-group analyses were performed for BALF neutrophil numbers, using the Mann Whitney test. Significance was assumed if $P<0.05$. The 2 separate 200 µg LPS inhalation challenges were compared using a Wilcoxon Rank Sum test and, as an indication of repeatability, the differences in paired values were plotted against their mean as described by Bland and Altman (1986). Good repeatability was assumed if the calculated differences in paired values fell within 2 s.d. of the mean of the differences (Anon 1979). Results are expressed as median and range. Following correction for any effect of saline inhalation, changes from baseline are expressed as increase/decrease in median values, with 95% confidence interval for the difference in median, calculated for nonparametric data as described by Campbell and Gardner (1994).

Results

Clinical scores

When compared with baseline values, no significant increase in clinical scores was detected in either group following any of the challenges.

Lung mechanics and airway reactivity

LPS inhalation challenges had no significant effect on lung function of controls. The heaves group had increased $RL_{E50\%}$ (increase in median 106%, 95% confidence interval (CI) 18–2017; $P<0.05$) and $RL_{E75\%}$ (increase 116%, 34–595; $P<0.05$) at 5 h following inhalation of 2000 µg when compared with baseline values. None of the challenges altered airway reactivity in either group when compared with saline inhalation challenge. There was no significant difference in airway reactivity between the first (median 6.9 mg/ml, range 1.5–19.4) and second (5.2 mg/ml, 2.8–17.1) 200 µg LPS inhalation challenges. There was good agreement of PCCdyn70 between the 2 challenges, since all the calculated differences in paired values fell within 2 s.d. of the mean of the differences.

Arterial blood analyses

LPS challenges did not significantly alter arterial pH, Pa_{O_2} or Pa_{CO_2} when compared with baseline values in either group. In the heaves group, hay/straw challenge reduced Pa_{O_2} at 90 min (decrease 6%, 1–17; $P<0.05$), and increased arterial pH at 4 h (increase 0.2%, 0.1–0.6; $P<0.05$). Pa_{CO_2} was reduced in the control group at 24 h following hay/straw challenge (decrease 9%, 3–15; $P<0.05$).

Peripheral blood leucocytes and neutrophils

Inhalation of 2000 µg LPS significantly reduced peripheral blood total leucocyte counts at 4 h when compared with baseline in both groups (heaves: reduction 14%, 5–24; $P<0.05$; controls: 23%, 12–36; $P<0.05$). Control horses also had a significant, but minor, reduction in peripheral blood total leucocyte counts at 90 min following inhalation of 200 µg LPS (8%, 0–17; $P<0.05$). Compared with baseline values, hay/straw challenge significantly increased peripheral blood total leucocyte counts in the controls at 90 min (increase 13%, 1–24; $P<0.05$). A marked and significant increase in peripheral blood neutrophil counts was also noted in the heaves group at 24 h following hay/straw challenge (increase 34%, 13–75; $P<0.05$).

BALF cytology

Prechallenge BALF cytology is shown in Table 1. Saline inhalation did not alter BALF cytology in either group. The BALF neutrophil counts and ratios at 6 and 24 h after each challenge are summarised in Table 3. LPS induced a dose-dependent BALF neutrophilia in both groups (Fig 2). In the

TABLE 3: Absolute BALF neutrophil counts and BALF neutrophil ratios (median and range) at 6 and 24 h after saline and LPS (20, 200 and 2000 μg) inhalation challenges and hay/straw exposure in heaves group (n = 7) and control group (n = 6)

	BALF neutrophil count (x 10^5/ml)				BALF neutrophil ratio (%)			
	Heaves		Controls		Heaves		Controls	
	6 h	24 h	6 h	24 h	6 h	24 h	6 h	24 h
Saline	0.06 (0.03–0.20)	0.08 (0.04–0.16)	0.06 (0.01–0.17)	0.04 (0.02–0.60)	2.3 (0.6–4.5)	1.7 (1.1–11.5)	1.3 (0.2–3.2)	1.7 (0.6–17.7)
20 μg LPS	0.28[a,b] (0.18–0.53)	0.20[a,b] (0.13–1.04)	0.09[b] (0.01–0.17)	0.03[b] (0.02–0.18)	6.1[a,c] (5.6–7.2)	4.7[b] (2.4–23.6)	1.7[c] (0.3–6.2)	1.8[b] (0.6–2.6)
200 μg LPS	1.45[a] (0.42–2.22)	0.77[a] (0.26–1.26)	0.57[a] (0.08–2.59)	0.39 (0.27–3.43)	23.2[a] (10.5–41.8)	13.7[a] (6.1–21.9)	13.9[a] (3.1–28.4)	12.3 (5.7–41.3)
2000 μg LPS	3.25[a] (0.57–4.34)	1.28[a] (0.76–6.24)	1.44[a] (0.52–2.70)	1.69[a] (1.12–2.70)	34.2[a] (28.4–65.8)	33.4[a] (17.8–39.6)	36.7[a] (10.9–64.2)	37.9[a] (19.0–51.0)
Hay/straw	2.05[a,b] (0.74–9.83)	0.67[a,b] (0.27–1.41)	0.17[b] (0.01–0.40)	0.09[b] (0.06–0.22)	36.0[a,b] (21.0–60.7)	17.6[a,b] (5.4–31.5)	7.0[a,b] (0.3–11.2)	4.3[b] (1.9–7.4)

[a] = Significantly different from postsaline challenge (P<0.05); [b] = Significantly different from other group at same time point following same challenge (P<0.01); [c] = Significantly different from other group at same time point following same challenge (P<0.05).

heaves group, when compared with saline inhalation, absolute BALF neutrophil count was significantly increased (P<0.05) at both 6 and 24 h after 20, 200 and 2000 μg LPS inhalation. These significant increases were also seen in the BALF neutrophil ratio, with the exception of the 24 h values following 20 μg LPS inhalation. In controls, BALF neutrophil count and ratio was significantly increased (P<0.05) at 6 h after inhalation of 200 and 2000 μg LPS, and at 24 h after inhalation of 2000 μg LPS. BALF neutrophil count and ratio was significantly increased (P<0.05) in the heaves group at 6 and 24 h after hay/straw challenge (Fig 2). No increase in BALF neutrophil count was seen in the control group at 6 or 24 h after hay/straw challenge; however, a slight, yet significant (P<0.05) increase in BALF neutrophil ratio was noted in this group at 6 h. Absolute BALF neutrophil count was significantly greater (P<0.01) at both 6 and 24 h in the heaves group compared with the control group, following inhalation of 20 μg LPS and after hay/straw challenge (Fig 2). In addition, the heaves group also had a significantly greater BALF neutrophil ratio following 20 μg LPS inhalation (P<0.05 at 6 h, P<0.01 at 24 h) and hay/straw challenge (P<0.01). There was no significant difference in BALF neutrophil counts between the first (1.3 x 10^5/ml, 0.3–2.2) and the second (0.9 x 10^5/ml, 0.6–1.5) 200 μg LPS inhalation challenges. Nine of the 10 calculated differences in paired values fell within 2 s.d. of the mean of the differences. As the data point falling out with this range was a clear outlier (out with the lower limit defined as: first quartile minus 1.5 x [third quartile minus first quartile]), repeatability was considered good (Bland and Altman 1986).

Due to the influence of a BALF neutrophilia on the ratio of other BALF cell types, they were considered only as absolute numbers. Reduced absolute BALF macrophage (1.98 x 10^5/ml, 0.73–2.80 to 0.91 x 10^5/ml, 0.17–1.76; P<0.05) and mast cell (0.25 x 10^5/ml, 0.06–0.39 to 0.08 x 10^5/ml, 0.01–0.23; P<0.05) numbers followed 2000 μg LPS inhalation only in controls, with heaves horses showing a similar but nonsignificant reduction. None of the challenges induced significant changes in BALF total cell count, or absolute lymphocyte, epithelial cell, basophiloid or eosinophil counts. There was no difference between the 6 and 24 h BALF total or absolute cell counts following all challenges, although there was a trend towards a reduction in all cell types at 24 h.

Tracheal secretions

Compared with saline challenge, LPS challenges did not significantly increase tracheal secretion scores at 6 h in either group; however, 2 horses in the heaves group had increased scores following inhalation of 200 and 2000 μg LPS. The heaves group had significantly (P<0.05) increased tracheal secretion scores after hay/straw challenge (median score 2, range 1–3), when compared with saline (score 0, 0–0).

Dust and endotoxin measurements in hay/straw stable

Total and respirable airborne dust endotoxin concentrations and dust endotoxin content in the hay/straw challenge stable are given in Table 4. The biologically active endotoxin dose received during the 5 h challenge was calculated using the formula: airborne endotoxin concentration (ng/m^3) x ventilation rate of 3.1 m^3/h x duration of challenge (h) x 3 (correction for approximate 3-fold underestimation of biologically active endotoxin content in dust by the limulus method [Rylander *et al.* 1989]).

Discussion

The systemic and pulmonary effects of inhalation with soluble LPS in control and asymptomatic heaves horses are reported here for the first time. Consistent with endotoxin inhalation studies in man and other species (Gordon 1992; Schwartz *et al.* 1994; Urbain *et al.* 1996; Michel *et al.* 1997), inhalation of 20, 200 and 2000 μg soluble *Salmonella typhimurium* Ra60 LPS induced a dose-dependent airway neutrophilia, with BALF neutrophil numbers increasing approximately 50-fold in heaves horses at 6 h after the high dose challenge. After inhalation of 2000 μg LPS, absolute BALF macrophage and mast cell numbers were significantly reduced in controls and nonsignificantly reduced in the heaves group. A reduction in BALF macrophage numbers occurs following LPS inhalation in other species, possibly due to LPS-induced macrophage apoptosis (Michel *et al.* 1997), or to migration of macrophages from the lung following antigenic stimulation or phagocytosis and clearance of apoptotic neutrophils (Brazil 2000).

Peripheral blood total leucocyte counts were reduced at 4 h following inhalation of 2000 μg LPS in both groups, consistent

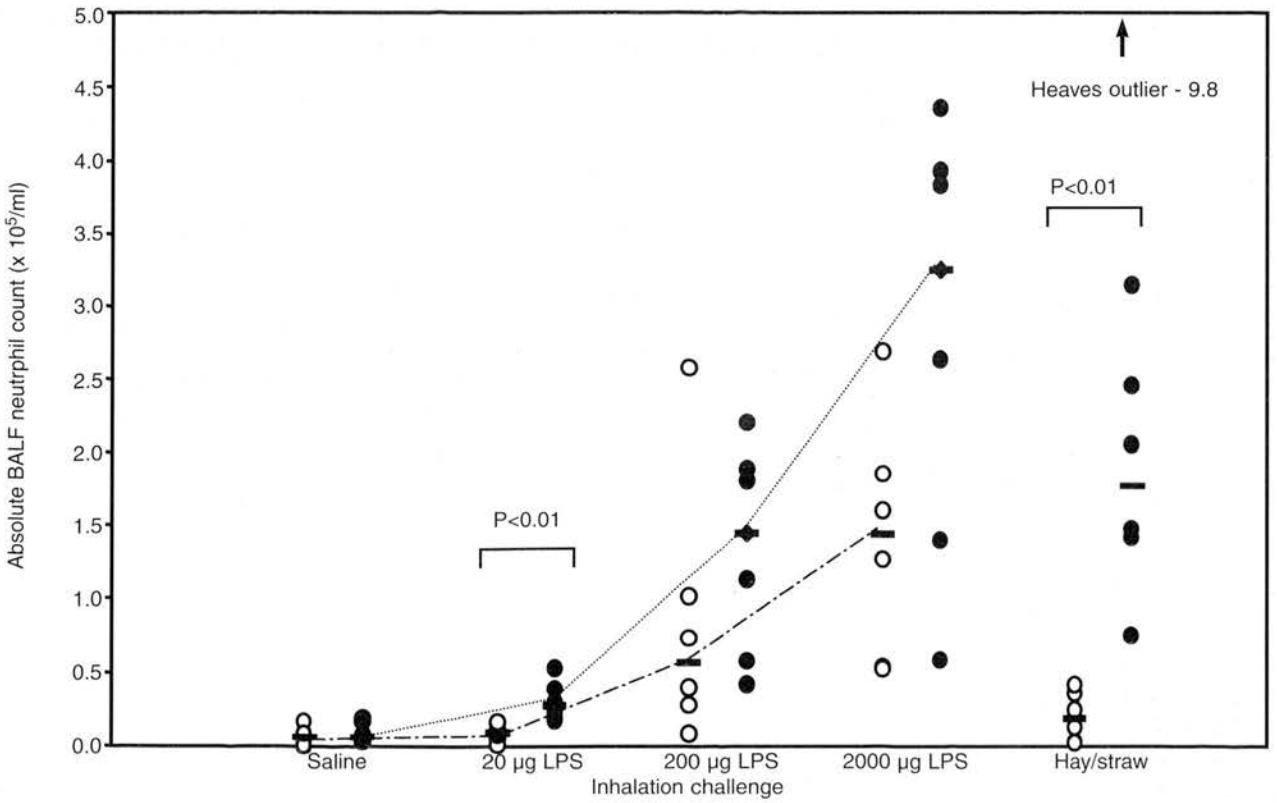

Fig 2: Absolute BALF neutrophil count (x 10^5/ml) in heaves (n = 7) and control (n = 6) horses at 6 h following inhalation challenge (saline, 20, 200 and 2000 µg LPS) and hay/straw challenge. Closed circle = heaves group; open circle = control group; hyphen = median values.

with a combined systemic and pulmonary response. This reduction probably reflects margination and pulmonary recruitment of leucocytes, as occurs following LPS inhalation in guinea pigs (Fogelmark *et al.* 1992).

LPS inhalation challenges had no significant effect on clinical score in either group. This is not surprising, since many of the clinical symptoms reported by human subjects following LPS inhalation, including chest tightness, headaches, joint pains and tiredness (Rylander *et al.* 1989, 1999), are subjective and, therefore, difficult to detect in horses. LPS inhalation had no significant effect on arterial blood gases and only the high dose (2000 µg) induced a significant deterioration in lung mechanics in the heaves group. Interestingly, the study by Michel *et al* (1992b) showed that, although the circulating leucocyte response to inhaled endotoxin was similar in healthy and asthmatic human subjects, only the asthmatic group had significant lung dysfunction. Similarly, in healthy human subjects, only moderate and inconsistent, combined restrictive and obstructive, lung dysfunction occurs following inhalation of high doses (>80–200 µg) of LPS (Cavagna *et al.* 1969; Rylander *et al.* 1989; Michel *et al.* 1995b; Michel 1997). While the increased $RL_{E50\%}$ and $RL_{E75\%}$ noted in the heaves group are consistent with an obstructive component, the relative insensitivity of pulmonary mechanics testing in the horse may have prevented the detection of mild restrictive dysfunction. As in man, where inhaled LPS induces more pronounced lung dysfunction in atopics and asthmatics (Michel *et al.* 1989; Rylander 1996), the control horses in the present study had no significant lung dysfunction, even after inhalation of 2000 µg LPS. Since the effects of endotoxin are inflammatory in nature and not IgE-mediated (Michel 1997), the exaggerated lung dysfunction in the heaves group may reflect a degree of undetected pre existing airway inflammation, despite their being maintained in a low dust environment for several weeks prior to the challenges. No alteration in airway reactivity was detected in either group, in contrast to the increased airway reactivity noted at 6 h after endotoxin inhalation in asthmatics (Michel *et al.* 1989, 1992a). Failure to detect increased airway reactivity may be due to insufficient LPS, or to attenuation of the methacholine-induced bronchconstriction by the bronchodilatory effects of the α2-agonist drug (Broadstone *et al.* 1992) used to sedate horses for this procedure.

LPS challenges did not significantly increase the tracheal secretion score in either group, although 2 heaves horses had increased scores after inhalation of 200 and 2000 µg LPS. In other species, inhaled endotoxin induces (Gordon and Harkema 1994; Gordon *et al.* 1996), or is correlated with, airway mucus hypersecretion (Rylander *et al.* 1999).

The role of inhaled endotoxin in human occupational respiratory diseases is well documented (Douwes and Heederik 1997; Jacobs 1997a) and the necessity for dose-response experiments as a prerequisite for the establishment of a no-response safety threshold has been recognised (Michel *et al.* 1997). For the first time, comparisons can be made between the levels of airborne endotoxin detected in equine environments and the minimal threshold doses of inhaled LPS required for induction of lung inflammation and dysfunction in the horse. The response threshold of LPS for inducing airway inflammation was lower in the heaves (≤ 20 µg) than control (20–200 µg) group, and the magnitude of BALF neutrophilia was, albeit insignificantly, more marked in heaves horses than controls. The response thresholds for lung dysfunction in the heaves (200–2000 µg LPS)

TABLE 4: Airborne respirable and total dust and endotoxin concentrations during hay/straw challenges (median and range)

	Total dust	Respirable dust
Airborne dust concentration (mg/m^3)	2.83 (0.83–6.83)	0.5 (0.17–0.83)
Endotoxin content of dust (ng/mg)	56.00 (31.40–163.92)	11.86 (4.53–98.22)
Airborne endotoxin concentration (ng/m^3)	160.00 (86.88–580.56)	3.95 (1.75–61.39)
5 h endotoxin exposure[a] (µg)	2.48 (1.35–9.00)	0.06 (0.03–0.95)
5 h biologically active endotoxin exposure[b] (µg)	7.44 (4.04–27.00)	0.18 (0.08–2.85)

[a] = airborne endotoxin concentration (ng/m^3) x average hourly ventilation of 13 ponies immediately following hay/straw challenge (m^3/h) x duration of challenge (h);
• average hourly ventilation of 13 ponies immediately following hay/straw challenge = 3.1 m^3.
• duration of challenge = 5 h.
[b] = [a] x correction factor of 3 (Rylander *et al.* 1989) for underestimation of biologically active endotoxin in dust samples using limulus amoebocyte lysate assay.

and control (>2000 µg LPS) groups were higher than the response thresholds for inflammation. Consistent with this finding, markers of inflammation were more sensitive indices of the effects of inhaled endotoxin than lung dysfunction in other species (Gordon 1992; Michel *et al.* 1997).

In this study, the 5 h duration hay/straw challenge exposed horses to a biologically active respirable dust endotoxin dose of 0.18 (0.08–2.85) µg and a biologically active total dust endotoxin dose of 7.44 (4.04–27.00) µg. These doses are generally lower than the thresholds for lung inflammation and dysfunction in both groups. Similarly, while the role of endotoxin in occupational organic dust-induced lung disease is well recognised in man, the threshold dose of soluble LPS in inhalation studies which causes clinical symptoms (Michel *et al.* 1989, 1995a) greatly exceeds the level of endotoxin exposure present in organic dust under certain occupational settings, which also result in clinical symptoms (Larsson *et al.* 1994). This apparent discrepancy may be explained by several factors that limit direct comparison of the threshold doses for inhaled endotoxin in acute experimental LPS inhalation challenges and in natural organic dust exposure. Firstly, other agents present in stable dust, such as moulds and glucans, may potentiate the response to endotoxin (Fogelmark *et al.* 1994; Hunt *et al.* 1994), thereby exacerbating the response in the hay/straw challenge. Since pre-exposure of human asthmatics to allergen potentiates their response to LPS (Martin *et al.* 1992), concomitant mould allergen exposure could increase LPS responsiveness to a greater extent in heaves horses than in controls. Secondly, the biologically active endotoxin content of the stable dust may have been underestimated by the *limulus* method used in this study, since this method detects mainly soluble endotoxin and underestimates the biologically active particulate endotoxin (Rylander *et al.* 1989). While the recommended correction factor of 3 (Rylander *et al.* 1989) was applied when calculating the biologically active endotoxin content of stable dust, this correction factor may be incorrect given the probable variation in the proportions of soluble and particulate endotoxin in dusts from different sources. Thirdly, short duration challenges, as used for soluble LPS inhalation in this study, may produce less effect than longer duration exposure. Fourthly, the acute LPS and hay/straw challenges probably differed with respect to the efficacy of delivery and deposition of aerosol and dust particles, the anatomical site in which they were deposited and the mechanisms and rate by which they were cleared. While approximately 7% of the LPS aerosol generated by the jet nebuliser may have been deposited in the lung during the LPS inhalation (Votion *et al.* 1997), the proportion of respirable and total airborne stable dust reaching the lungs during the hay/straw challenge could not be determined. Finally, it is unclear whether the endotoxin concentration in respirable or total airborne stable dust should be considered when making a comparison with the threshold dose of soluble LPS. While the majority of endotoxin in respirable stable dust probably reached the lower airways in the hay/straw challenge, as endotoxin in the nonrespirable fraction may cause inflammation and dysfunction of the larger airways (Jacobs 1997b), it may also have contributed to the response to hay/straw challenge.

Despite the aforementioned problems in comparing endotoxin levels in the hay/straw challenge and the threshold dose for soluble LPS inhalations, several observations suggest that inhaled endotoxin was not the sole cause of lung inflammation and dysfunction in the heaves group following hay/straw challenge. Firstly, the dose of endotoxin encountered in the hay/straw challenge was markedly lower than the threshold dose of soluble LPS (200–2000 µg) required to induce a similar degree of BALF neutrophilia. Secondly, the hay/straw challenge did not induce BALF neutrophilia in controls, while inhalation of ≥ 200 µg LPS induced BALF neutrophilia in both groups. Thirdly, in contrast to LPS inhalation, hay/straw challenge significantly increased tracheal mucus score in the heaves group. Therefore, it is probable that other pro-inflammatory agents in stable dust (e.g. moulds) contribute to the aetiopathogenesis of heaves. However, it is likely that endotoxin *per se* causes airway inflammation in horses housed in stables with very poor air hygiene, since respirable endotoxin concentrations may be as high as 3437 ng/m^3 (Dutkiewicz *et al.* 1994). A 5 h exposure to this concentration equates to a dose (160 µg) which exceeds the threshold dose of LPS which causes inflammation in horses with asymptomatic heaves (20 µg), and may exceed that which causes inflammation in control horses (between 20 and 200 µg).

In this study, as in human studies (Michel *et al.* 1997), all horses were given LPS in increasing rather than randomised doses. This order was selected because of safety reasons, given

the absence of data on the effects of acute LPS inhalation in horses, and given the potential for significant individual-dependent variability in LPS responsiveness (Michel 1997; Kline *et al.* 1999). It could be argued that randomisation of challenge order may have minimised potential carry-over effects from prior challenges. Carry-over effects could include potentiation due to persistence of inflammation, early-phase tolerance and late-phase (occurring after several weeks) tolerance due to production of anti-endotoxin antibodies (Johnston and Greisman 1985; Ulmer 1997). However, the good repeatibility of inflammatory (BALF neutrophilia) and functional (PCCDyn70) changes following repeated 200 μg LPS challenge, suggests that carry-over effects were insignificant. Further, since early-phase tolerance to inhaled endotoxin lasts no more than 2 days (Johnston and Greisman 1985), it was unlikely to have influenced the response to subsequent challenges that were separated by at least 2 weeks.

There are 2 main advantages in using LPS from the *Salmonella* R60 mutant in the current study. Firstly, the LPS is of Ra chemotype (i.e. complete core oligosaccharide plus lipid A). It is of homogeneous molecular mass, in comparison to smooth-form LPS which has extremely heterogeneous chain lengths due to the O-polysaccharide, and thus heterogeneous biological activity. It should, therefore, give reproducible results in experimental challenge studies. Secondly, it represents a structure shared by many of the Enterobacteriaceae, including all *Escherichia coli* and *Salmonella* species, and this common structure is responsible for a major part of the biological activity of LPS (I. R. Poxton, personal communication). It is probable that this structure is present in large concentrations in a deep litter management system, as was used for the hay/straw challenges.

In conclusion, inhaled endotoxin induced neutrophilic airway inflammation and dysfunction in horses. While this study suggests that inhaled endotoxin is not the sole cause of heaves, we hypothesise that it contributes to its aetiopathogenesis. Furthermore, it is likely to result in lung inflammation in normal horses when housed in environments with high airborne endotoxin concentrations. The dose-response data described is a prerequisite to the development of acceptable endotoxin exposure levels for horse accommodation. Healthy or heaves-affected horses housed in stables with poor air hygiene may be exposed to airborne endotoxin levels exceeding the threshold dose levels that induce airway inflammation. These potentially detrimental effects of inhaled endotoxin may be minimised by optimising air hygiene in stables.

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Manufacturers' addresses

[1]Ivex Pharmaceuticals, Larne, Co. Antrim, UK.
[2]Boehringer Ingelheim Ltd., Bracknell, Berkshire, UK.
[3]Fort Dodge Animal Health, Hedge End, Southampton, Hampshire, UK.
[4]PARI Medical Ltd., West Byfleet, Surrey, UK.
[5]Medic-Aid Ltd., Bognor Regis, West Sussex, UK.
[6]Casella Ltd., Kempston, Bedfordshire, UK.
[7]Seikagaku Co, Tokyo, Japan.
[8]National Instruments Co., Austin, Texas, USA.
[9]Sigma-Aldrich Co. Ltd., Poole, Dorset, UK.
[10]AVL Medical Instruments UK Ltd., Stone, Staffordshire, UK.

References

Anon (1979) Precision of test methods I: Guide for the determination and reproducibility for a standard test method. In: *British Standards Institution 5497, Part 1*, BSI, London.

Bland, J.M. and Altman, D.G. (1986) Statistical-methods for assessing agreement between 2 methods of clinical measurement. *Lancet* **1**, 307-310.

Brazil, T.J. (2000) *Pulmonary Neutrophil Recruitment, Activation and Clearance in Equine COPD.* PhD Thesis, University of Edinburgh.

Broadstone, R.V., Gray, P.R., Robinson, N.E. and Derksen, F.J. (1992) Effects of xylazine on airway function in ponies with recurrent airway-obstruction. *Am. J. vet. Res.* **53**, 1813-1817.

Campbell, M.J. and Gardner, M.J. (1994) Calculating confidence intervals for some non-parametric analyses. In: *Statistics with Confidence – Confidence Intervals and Statistical Guidelines*, Eds: M.J. Gardner and D.G. Altman, British Medical Journal, London. pp 71-79.

Cavagna, G., Foa, V. and Vigliani, E.C. (1969) Effects in man and rabbits of inhalation of cotton dusts or extracts and purified endotoxins. *Br. J. Ind. Med.* **26**, 314-321.

Dixon, P.M., Railton, D.I. and McGorum, B.C. (1995) Equine pulmonary disease: a case control study of 300 referred cases. 1: Examination techniques, diagnostic criteria and diagnoses. *Equine vet. J.* **27**, 416-421.

Douwes, J. and Heederik, D. (1997) Epidemiologic investigations of endotoxins. *Int. J. Occup. env. Health* **3**, S26-S31.

Dutkiewicz, J., Pomorski, Z.J.H., Sitkowska, J., Krysinskatraczyk, E., Skorska, C., Prazmo, Z., Cholewa, G. and Wojtowicz, H. (1994) Airborne microorganisms and endotoxin in animal houses. *Granta* **33**, 85-90.

Fogelmark, B., Sjostrand, M. and Rylander, R. (1994) Pulmonary inflammation-induced by repeated inhalations of beta (1, 3)- D-glucan and endotoxin. *Int. J. expt. Path.* **75**, 85-90.

Fogelmark, B., Goto, H., Yuasa, K., Marchat, B. and Rylander, R. (1992) Acute pulmonary toxicity of inhaled beta-1,3-glucan and endotoxin. *Agents Actions* **35**, 50-56.

Gordon, T. (1992) Dose-dependent pulmonary effects of inhaled endotoxin in guinea-pigs. *Env. Res.* **59**, 416-426.

Gordon, T. and Harkema, J.R. (1994) Effect of inhaled endotoxin on intraepithelial mucosubstances in f344 rat nasal and tracheobronchial airways. *Am. J. Respir. cell mol. Biol.* **10**, 177-183.

Gordon, T., Nadziejko, C., Plant, M., Rodger, I.W. and Pon, D.J. (1996) One-month exposure to inhaled endotoxin produces a dose-dependent increase in stored mucosubstances in rat intrapulmonary airways. *Expt. Lung Res.* **22**, 509-523.

Hunt, L.W., Gleich, G.J., Ohnishi, T., Weiler, D.A., Mansfield, E.S., Kita, H. and Sur, S. (1994) Endotoxin contamination causes neutrophilia following pulmonary allergen challenge. *Am. J. Respir. crit. care Med.* **149**, 1471-1475.

Jacobs, R.R. (1997a) Endotoxins in the environment. *Int J. Occup. env. Health* **3**, S3-S5.

Jacobs, R.R. (1997b) Environmental monitoring of endotoxins. *Int. J. Occup. env. Health* **3**, S37-S41.

Johnston, C.A. and Greisman, S.E. (1985) Mechanisms of endotoxin tolerance. In: *Handbook of Endotoxin*, Ed: L.B. Hinshaw, Amsterdam, Elsevier. p 359.

Kline, J.N., Cowden, D.C., Hunninghake, G.W., Schutte, B.C., Watt, J.L., Wohlford,-Lenane, C.L., Powers, L.S., Jones, M.P. and Schwartz, D.A. (1999) Variable airway responsiveness to inhaled lipopolysaccharide. *Am. J. respir. crit. care Med.* **160**, 297-303.

Larsson, K.A., Eklund, A.G., Hansson, L.O., Isaksson, B.N. and Malmberg, P.O. (1994) Swine dust causes intense airways inflammation in healthy-subjects. *Am. J. respir. crit. care Med.* **150**, 973-977.

Martin, T.R., Mathison, J.C., Tobias, P.S., Leturcq, D.J., Moriarty, A.M., Maunder, R.J. and Ulevitch, R.J. (1992) Lipopolysaccharide binding-protein enhances the responsiveness of alveolar macrophages to bacterial lipopolysaccharide - implications for cytokine production in normal and injured lungs. *J. clin. Invest.* **90**, 2209-2219.

McGorum, B.C. and Dixon, P.M. (1992) Preliminary-observations on inhalation and intradermal challenges of horses with oil seed rape. *Vet. Rec.* **131**, 163-167.

McGorum, B.C., Dixon, P.M. and Halliwell, R.E. (1993) Responses of horses affected with chronic obstructive pulmonary disease to inhalation challenges with mould antigens. *Equine vet. J.* **25**, 261-267.

McGorum, B.C., Ellison, J. and Cullen, R.T. (1998) Total and respirable airborne dust endotoxin concentrations in three equine management systems. *Equine vet. J.* **30**, 430-434.

Michel, O. (1997) Human challenge studies with endotoxins. *Int J. Occup. env. Health* **3**, S18-S25.

Michel, O., Duchateau, J. and Sergysels, R. (1989) Effect of inhaled endotoxin on bronchial reactivity in asthmatic and normal subjects. *J. appl. Physiol.* **66**, 1059-1064.

Michel, O., Ginanni, R. and Sergysels, R. (1992b) Relation between the bronchial obstructive response to inhaled lipopolysaccharide and bronchial responsiveness to histamine. *Thorax* **47**, 288-291.

Michel, O., Ginanni, R. and Sergysels, R. (1995b) Protective effect of sodium cromoglycate on lipopolysaccharide-induced bronchial obstruction in asthmatics. *Int. Arch. Allergy Immunol.* **108**, 298-302.

Michel, O., Ginanni, R., Duchateau, J., Vertongen, F., Lebon, B. and Sergysels, R. (1991) Domestic endotoxin exposure and clinical severity of asthma. *Clin. expt. Allergy* **21**, 441-448.

Michel, O., Ginanni, R., Lebon, B., Content, J., Duchateau, J. and Sergysels, R. (1992a) Inflammatory response to acute inhalation of endotoxin in asthmatic-patients. *Am. Rev. resp. Dis.* **146**, 352-357.

Michel, O., Duchateau, J., Plat, G., Cantinieaux, B., Hotimsky, A., Gerain, J. and Sergysels, R. (1995a) Blood inflammatory response to inhaled endotoxin in normal subjects. *Clin. expt. Allergy* **25**, 73-79.

Michel, O., Nagy, A.M., Schroeven, M., Duchateau, J., Neve, J., Fondu, P. and Sergysels, R. (1997) Dose-response relationship to inhaled endotoxin in normal subjects. *Am. J. Respir. crit. care Med.* **156**, 1157-1164.

Michel, O., Kips, J., Duchateau, J., Vertongen, F., Robert, L., Collet, H., Pauwels, R. and Sergysels, R. (1996) Severity of asthma is related to endotoxin in house dust. *Am. J. respir. crit. care Med.* **154**, 1641-1646.

Olenchock, S.A., Murphy, S.A., Mull, J.C. and Lewis, D.M. (1992) Endotoxin and complement activation in an analysis of environmental dusts from a horse barn. *Scand. J. Work env. Health* **18**, *Suppl.* **2**, 58-59.

Rizzo, M.C., Naspitz, C.K., FernandezCaldas, E., Lockey, R.F., Mimica, I. and Sole, D. (1997) Endotoxin exposure and symptoms in asthmatic children. *Pediat. Allergy Immunol.* **8**, 121-126.

Rylander, R. (1996) Airway responsiveness and chest symptoms after inhalation of endotoxin or (1-3)-beta-D-Glucan. *Indoor Built Env.* **5**, 106-111.

Rylander, R. (1997) Evaluation of the risks of endotoxin exposures. *Int. J. Occup. env. Health* **3**, S32-S36.

Rylander, R. and Bergstrom, R. (1993) Bronchial reactivity among cotton workers in relation to dust and endotoxin exposure. *Ann. Occup. Hygiene* **37**, 57-63.

Rylander, R., Thorn, J. and Attefors, R. (1999) Airways inflammation among workers in a paper industry. *Eur. Resp. J.* **13**, 1151-1157.

Rylander, R., Bake, B., Fischer, J.J. and Helander, I.M. (1989) Pulmonary-function and symptoms after inhalation of endotoxin. *Am. Rev. resp. Dis.* **140**, 981-986.

Schwartz, D.A., Thorne, P.S., Jagielo, P.J., White, G.E., Bleuer, S.A. and Frees, K.L. (1994) Endotoxin responsiveness and grain dust-induced inflammation in the lower respiratory-tract. *Am. J. Physiol. - Lung cell. mol. Physiol.* **11**, L 609-L 617.

Schwartz, D.A., Thorne, P.S., Yagla, S.J., Burmeister, L.F., Olenchock, S.A., Watt, J.L. and Quinn, T.J. (1995) The role of endotoxin in grain dust-induced lung-disease. *Am. J. Respir. crit. care Med.* **152**, 603-608.

Smid, T., Heederik, D., Houba, R. and Quanjer, P.H. (1994) Dust-related and endotoxin-related acute lung-function changes and work-related symptoms in workers in the animal feed-industry. *Am. J. indust. Med.* **25**, 877-888.

Tanner, M.K., Swinker, A.M., Beard, M.L., Cosma, G.N., Traub-Dargatz, J.L., Martinez, A.B. and Olenchock, S.A. (1998) Effect of phone book paper versus sawdust and straw bedding on the presence of airborne gram-negative bacteria, fungi and endotoxin in horse stalls. *J. equine vet. Sci.* **18**, 457-461.

Thorn, J. (1999) *Organic Dusts and Airways Inflammation with Reference to the Specific Agents Endotoxin and (1-3)-beta-D-glucan*. PhD Thesis, University of Gothenburg.

Ulmer, A.J. (1997) Biochemistry and cell biology of endotoxins. *Int. J. Occup. env. Health* **3**, S8-S17.

Urbain, B., Prouvost, J.F., Beerens, D., Ansay, M. and Gustin, P. (1996) Acute effects of endotoxin inhalation on the respiratory tract in pigs: interaction with ammonia. *Inhal. Toxicol.* **8**, 947-968.

Vogelzang, P.F.J., van der Gulden, J.W.J., Folgering, H., Kolk, J.J., Heederik, D., Preller, L., Tielen, M.J.M. and van Schayck, C.P. (1998) Endotoxin exposure as a major determinant of lung function decline in pig farmers. *Am. J. Respir. crit. care Med.* **157**, 15-18.

Votion, D., Ghafir, Y., Munsters, K., Duvivier, D.H., Art, T. and Lekeux, P. (1997) Aerosol deposition in equine lungs following ultrasonic nebulisation vs. jet aerosol delivery system. *Equine vet. J.* **29**, 388-393.

Zejda, J.E., Barber, E., Dosman, J.A., Olenchock, S.A., McDuffie, H.H., Rhodes, C. and Hurst, T. (1994) Respiratory health-status in swine producers relates to endotoxin exposure in the presence of low dust levels. *J. Occup. Med.* **36**, 49-56.

Erratum

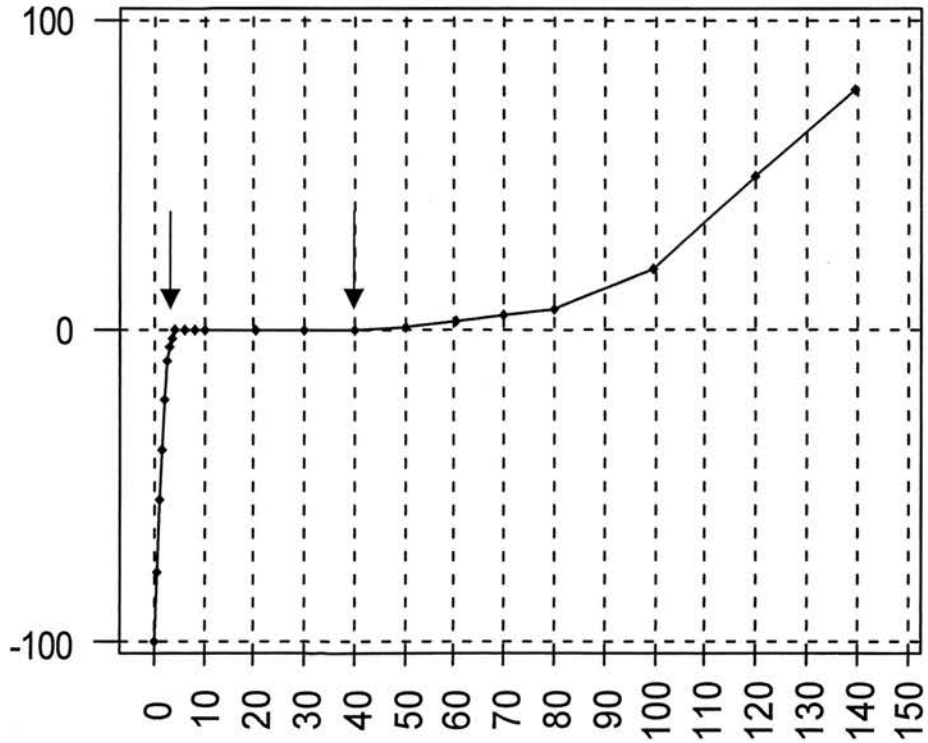
The following table, which appeared in the article *Relationships of age and shape of the navicular bone to the development of navicular disease: a radiological study* by K. J. Dik, A. J. M. van den Belt and J. van den Broek, was misprinted in *Equine Veterinary Journal* Volume 33, Number 3 (March 2001), for which we apologise. The correct version of Table 5 appears below.

TABLE 5: Association navicular bone shape - character of simple *grades 3* and *4* features in clinically affected horses

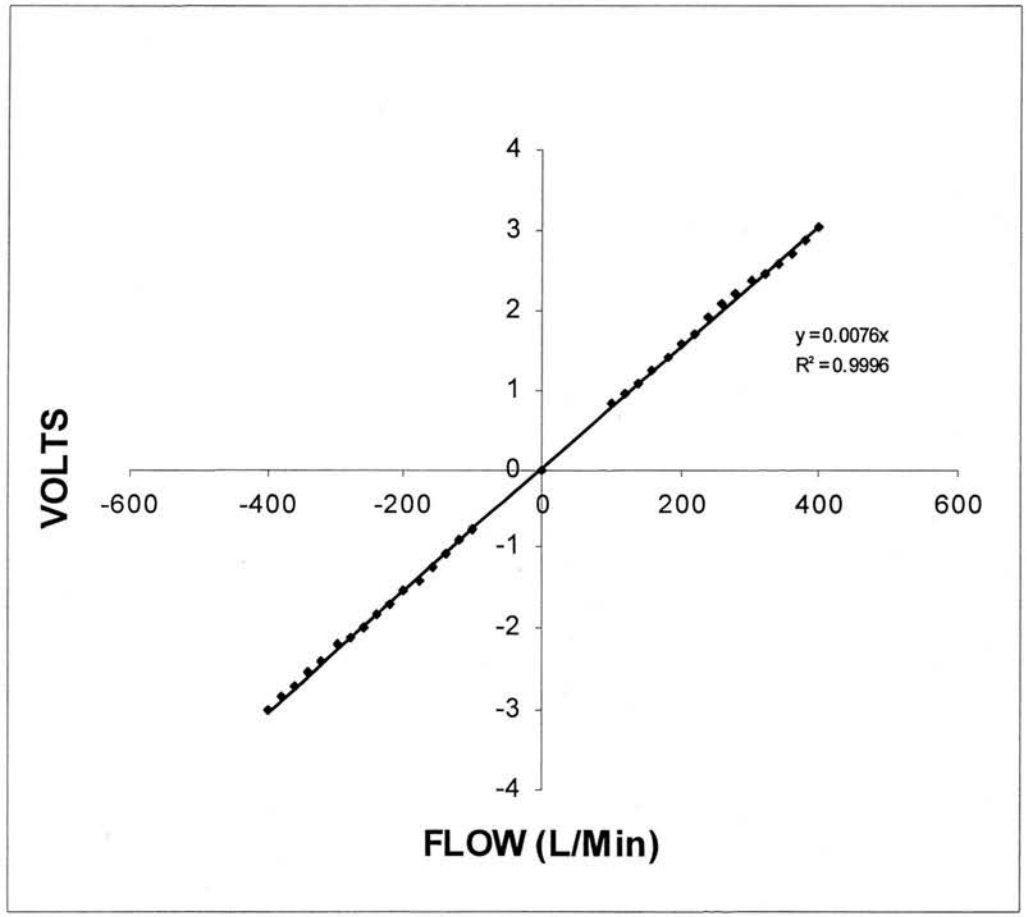
		Inverted flask-shaped channels					Enthesophytes					Cystic lesions				
			Age (years)					Age (years)					Age (years)			
Shape	No.	No.	3–4	5–9	10–14	15–19	No.	3–4	5–9	10–14	15–19	No.	3–4	5–9	10–14	15–19
1	27	14 (52%)	0	8	4	2	11 (41%)	0	2	8	1	2 (7%)	0	0	2	0
2	42	23 (55%)	2	12	7	2	16 (38%)	2	3	10	1	3 (7%)	0	2	1	0
3	16	4 (25%)	0	0	3	1	9 (56%)	0	2	6	1	3 (19%)	0	2	1	0
4	4	1 (25%)	0	0	1	0	2 (50%)	0	0	2	0	1 (25%)	0	0	1	0
Total		42	2 (5%)	20 (49%)	15 (35%)	5 (11%)	38	2 (5%)	7 (19%)	26 (68%)	3 (8%)	9	0 (0%)	4 (44%)	5 56%)	0 (0%)

APPENDICES

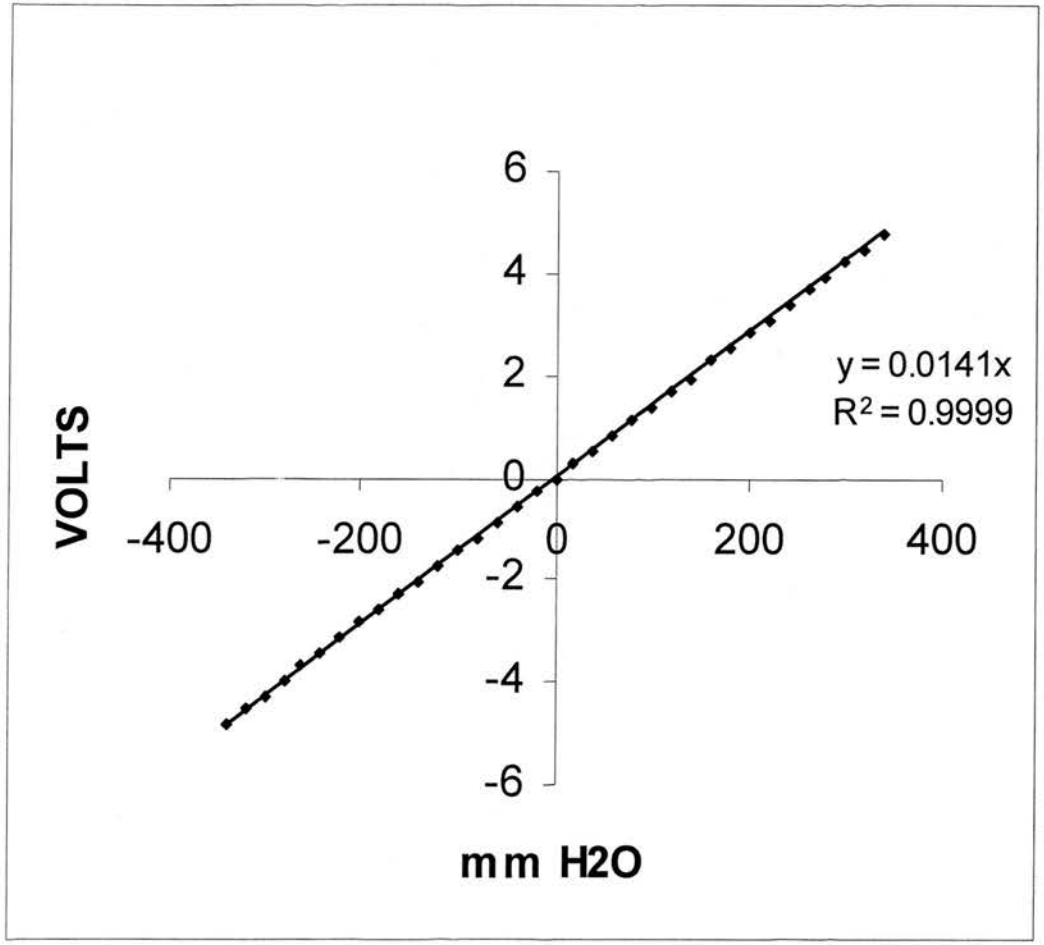
Appendix 2.1: Pressure (mmH_2O) within the oesophageal balloon plotted against volume (ml) within the oesophageal balloon, indicating the range of high compliance of the balloon (between arrows).



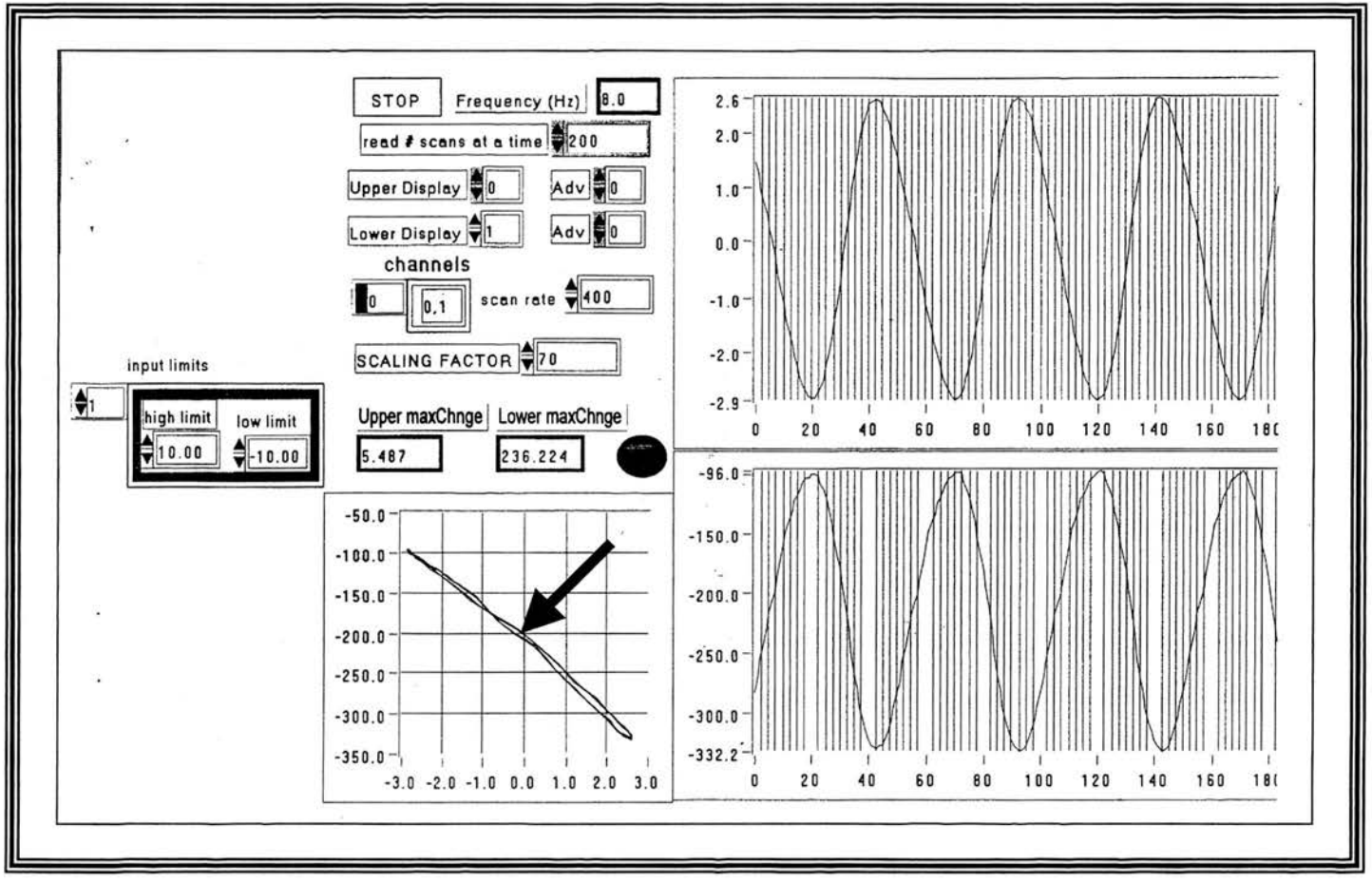
Appendix 2.2: Flow (l/min) plotted against voltage indicating linearity of flow readings over working range.



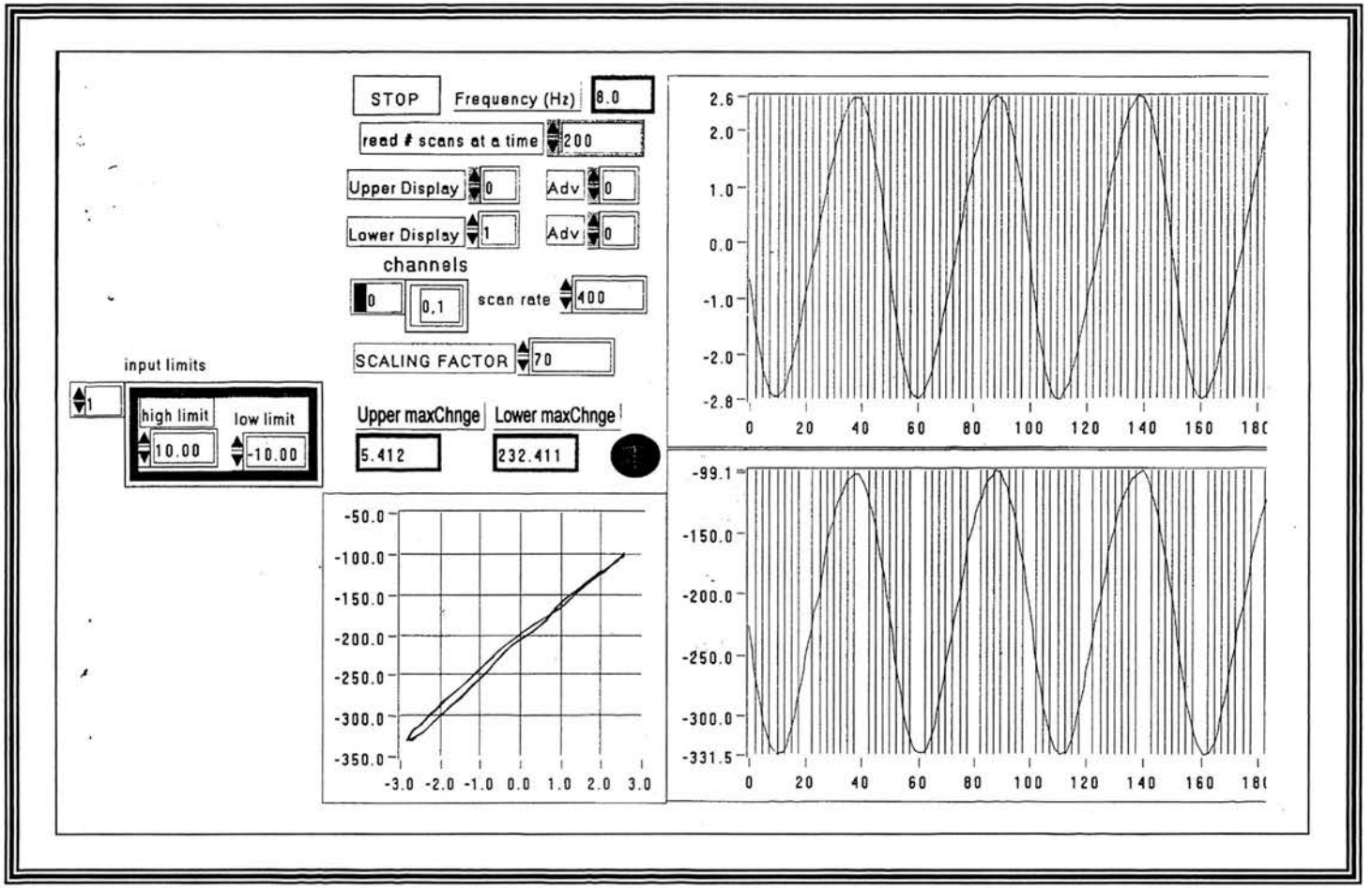
Appendix 2.3: Pressure (mm H_2O) plotted against voltage indicating linearity of pressure readings over working range.



Appendix 2.4a: Example of phase matching of sinusoidal pressure traces at 8Hz. One pressure trace reflects the pressure at one side of the pneumotachograph and the other pressure trace reflects pressure within the balloon catheter. Pressure waves through the catheter attached to the pneumotachograph were delayed by reduction of the diameter of the catheter until the plot of the sinusoidal pressure traces against each other resulted in a straight line (arrow). The same procedure was repeated for phase matching of the pressure waves within the balloon catheter with those from the opposite side of the pneumotachograph (Appendix 2.4b).



Appendix 2.4b



Appendix 2.5: Clinical scores in control (C1-6) and heaves (H1-7) horses prior to (0) and at 90, 240 and 1440min following inhalation challenge with saline, 20, 200 and 2000μg LPS, and 5h hay straw challenge (H/S). Score based on: a (tracheal auscultation), b (thoracic auscultation), c (rectal temperature), d (dyspnoea) and e (respiratory rate).

	SALINE				20μg LPS				200μg LPS				2000μg LPS				H/S			
TIME PT (min)	0	90	240	1440	0	90	240	1440	0	90	240	1440	0	90	240	1440	0	90	240	1440
C1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
C6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1 c	0	0	0	1	0
H1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H4	0	0	0	0	0	0	0	0	0	0	0	0	0	1 b	1 b	1 b	0	1 d	0	0
H5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1 a	0	1 d	2 d,e	0
H6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2 d,e	1 a

Appendix 2.6a: Arterial blood pH in control (C1-6) and heaves (H1-7) horses prior to (0) and at 90, 240 and 1440min following inhalation challenge with saline, 20, 200 and 2000µg LPS, and 5h hay straw challenge (H/S). MED. = median value, MIN. = minimum value, MAX. = maximum value.

	SALINE				20µg LPS				200µg LPS				2000µg LPS				H/S			
TIME PT.	0	90	240	1440	0	90	240	1440	0	90	240	1440	0	90	240	1440	0	90	240	1440
C1	7.40	7.41	7.39	7.39	7.36	7.37	7.39	7.37	7.38	7.34	7.42	7.38	7.42	7.37	7.34	7.42	7.45	7.47	7.44	7.38
C2	7.36	7.42	7.35	7.37	7.35	7.35	7.35	7.33	7.40	7.38	7.42	7.37	7.41	7.38	7.39	7.36	7.34	7.38	7.37	7.33
C3	7.38	7.37	7.38	7.38	7.44	7.38	7.39	7.41	7.42	7.36	7.36	7.33	7.39	7.40	7.39	7.41	7.38	7.38	7.41	7.37
C4	7.40	7.39	7.42	7.43	7.34	7.35	7.40	7.42	7.44	7.39	7.40	7.38	7.45	7.38	7.39	7.39	7.45	7.48	7.43	7.43
C5	7.36	7.35	7.36	7.41	7.35	7.33	7.36	7.34	7.38	7.37	7.42	7.38	7.36	7.39	7.37	7.35	7.40	7.38	7.39	7.40
C6	7.39	7.39	7.39	7.38	7.39	7.37	7.36	7.38	7.42	7.41	7.39	7.39	7.38	7.39	7.41	7.36	7.42	7.42	7.42	7.40
MED.	*7.39*	*7.39*	*7.39*	*7.39*	*7.36*	*7.36*	*7.37*	*7.38*	*7.41*	*7.38*	*7.41*	*7.38*	*7.40*	*7.38*	*7.39*	*7.38*	*7.41*	*7.40*	*7.42*	*7.39*
MIN.	*7.36*	*7.35*	*7.35*	*7.37*	*7.34*	*7.33*	*7.35*	*7.33*	*7.38*	*7.34*	*7.36*	*7.33*	*7.36*	*7.37*	*7.34*	*7.35*	*7.34*	*7.38*	*7.37*	*7.33*
MAX.	*7.40*	*7.42*	*7.42*	*7.43*	*7.44*	*7.38*	*7.40*	*7.42*	*7.44*	*7.41*	*7.42*	*7.39*	*7.45*	*7.40*	*7.41*	*7.42*	*7.45*	*7.48*	*7.44*	*7.43*
H1	7.36	7.38	7.38	7.42	7.34	7.37	7.38	7.37	7.42	7.38	7.38	7.39	7.39	7.40	7.39	7.37	7.45	7.47	7.45	7.40
H2	7.35	7.36	7.36	7.32	7.37	7.38	7.36	7.36	7.35	7.34	7.36	7.38	7.38	7.38	7.34	7.40	7.41	7.43	7.42	7.35
H3	7.40	7.34	7.41	7.37	7.36	7.36	7.37	7.37	7.37	7.37	7.37	7.44	7.37	7.36	7.37	7.41	7.38	7.42	7.39	7.38
H4	7.39	7.34	7.35	7.36	7.39	7.33	7.35	7.35	7.35	7.36	7.36	7.33	7.35	7.37	7.38	7.41	7.38	7.42	7.39	7.40
H5	7.37	7.37	7.37	7.33	7.37	7.35	7.37	7.36	7.31	7.33	7.35	7.35	7.35	7.35	7.38	7.38	7.39	7.37	7.43	7.37
H6	7.42	7.38	7.36	7.39	7.34	7.35	7.36	7.39	7.42	7.39	7.40	7.34	7.36	7.37	7.40	7.36	7.40	7.47	7.46	7.39
H7	7.41	7.40	7.43	7.39	7.73	7.41	7.39	7.41	7.39	7.38	7.38	7.41	7.35	7.38	7.36	7.39	7.37	7.41	7.39	7.41
MED.	*7.39*	*7.37*	*7.37*	*7.37*	*7.37*	*7.36*	*7.37*	*7.37*	*7.37*	*7.37*	*7.37*	*7.38*	*7.36*	*7.37*	*7.38*	*7.39*	*7.39*	*7.42*	*7.42*	*7.39*
MIN.	*7.35*	*7.34*	*7.35*	*7.32*	*7.34*	*7.33*	*7.35*	*7.35*	*7.31*	*7.33*	*7.35*	*7.33*	*7.35*	*7.35*	*7.34*	*7.36*	*7.37*	*7.37*	*7.39*	*7.35*
MAX.	*7.42*	*7.40*	*7.43*	*7.42*	*7.73*	*7.41*	*7.39*	*7.41*	*7.42*	*7.39*	*7.40*	*7.44*	*7.39*	*7.40*	*7.40*	*7.41*	*7.45*	*7.47*	*7.46*	*7.41*

Appendix 2.6b: Arterial blood pCO_2 (mmHg) in control (C1-6) and heaves (H1-7) horses prior to (0) and at 90, 240 and 1440min following inhalation challenge with saline, 20, 200 and 2000µg LPS, and 5h hay straw challenge (H/S). MED. = median value, MIN. = minimum value, MAX. = maximum value.

	SALINE				20µg LPS				200µg LPS				2000µg LPS				H/S			
TIME PT.	0	90	240	1440	0	90	240	1440	0	90	240	1440	0	90	240	1440	0	90	240	1440.0
C1	46.0	46.0	49.0	45.0	44.0	45.0	44.0	44.0	48.0	48.0	43.0	44.0	44.4	46.9	49.2	47.9	40.0	42.0	42.0	39.0
C2	45.0	50.0	48.0	46.0	45.0	46.0	44.0	43.0	41.0	44.0	43.0	40.0	42.0	47.0	47.0	46.0	45.0	45.0	45.0	41.0
C3	46.5	50.1	46.6	47.5	52.1	53.5	52.3	51.9	46.1	53.3	52.2	53.1	50.8	50.3	53.9	48.3	59.8	59.8	53.3	51.0
C4	50.1	48.6	44.7	44.9	50.1	51.1	48.0	48.6	43.8	51.0	48.1	46.0	42.0	45.1	44.1	43.4	45.5	46.1	45.1	41.0
C5	41.0	40.0	39.0	38.0	40.0	45.0	42.0	47.0	45.5	47.6	44.1	49.1	50.6	49.5	49.1	41.6	45.0	40.0	41.0	39.0
C6	40.0	45.0	41.0	44.0	44.0	42.0	44.0	45.0	48.8	48.2	47.2	47.7	48.0	46.6	42.9	46.2	41.0	41.0	39.0	38.0
MED.	*45.5*	*47.3*	*45.7*	*45.0*	*44.5*	*45.5*	*44.0*	*46.0*	*45.8*	*48.1*	*45.7*	*46.9*	*46.2*	*47.0*	*48.1*	*46.1*	*45.0*	*43.5*	*43.5*	*40.0*
MIN.	*40.0*	*40.0*	*39.0*	*38.0*	*40.0*	*42.0*	*42.0*	*43.0*	*41.0*	*44.0*	*43.0*	*40.0*	*42.0*	*45.1*	*42.9*	*41.6*	*40.0*	*40.0*	*39.0*	*38.0*
MAX.	*50.1*	*50.1*	*49.0*	*47.5*	*52.1*	*53.5*	*52.3*	*51.9*	*48.8*	*53.3*	*52.2*	*53.1*	*50.8*	*50.3*	*53.9*	*48.3*	*59.8*	*59.8*	*53.3*	*51.0*
H1	45.0	40.0	40.0	40.0	41.0	39.0	39.0	42.0	39.0	42.0	39.0	36.0	37.0	43.0	40.0	36.0	35.0	32.0	36.0	32.0
H2	47.0	46.0	46.0	46.0	46.0	43.0	46.0	43.0	45.0	48.0	43.0	43.0	50.0	47.0	50.0	39.0	41.0	46.0	43.0	44.0
H3	42.0	46.0	37.0	40.0	45.0	44.0	42.0	41.0	42.0	43.0	43.0	41.0	44.0	43.0	47.0	42.0	42.0	39.0	40.0	39.0
H4	43.0	45.0	45.0	43.0	45.0	48.0	41.0	45.0	43.0	40.0	44.0	41.0	40.0	39.0	43.0	38.0	41.0	41.0	45.0	39.0
H5	53.0	44.0	43.0	45.0	47.0	47.0	46.0	47.0	39.0	38.0	43.0	45.0	43.0	46.0	46.0	44.0	47.0	48.0	40.0	42.0
H6	48.4	48.7	47.6	52.9	54.2	57.3	54.1	49.5	52.0	60.5	56.8	52.0	54.7	56.7	47.2	54.6	49.7	51.0	47.1	45.9
H7	46.0	43.0	45.0	43.0	44.0	40.0	41.0	46.0	42.0	46.0	45.0	41.0	41.0	47.0	40.0	35.0	47.0	43.0	44.0	40.0
MED.	*46.0*	*45.0*	*45.0*	*43.0*	*45.0*	*44.0*	*42.0*	*45.0*	*42.0*	*43.0*	*43.0*	*41.0*	*43.0*	*46.0*	*46.0*	*39.0*	*42.0*	*43.0*	*43.0*	*40.0*
MIN.	*42.0*	*40.0*	*37.0*	*40.0*	*41.0*	*39.0*	*39.0*	*41.0*	*39.0*	*38.0*	*39.0*	*36.0*	*37.0*	*39.0*	*40.0*	*35.0*	*35.0*	*32.0*	*36.0*	*32.0*
MAX.	*53.0*	*48.7*	*47.6*	*52.9*	*54.2*	*57.3*	*54.1*	*49.5*	*52.0*	*60.5*	*56.8*	*52.0*	*54.7*	*56.7*	*50.0*	*54.6*	*49.7*	*51.0*	*47.1*	*45.9*

Appendix 2.6c: Arterial blood pO_2 (mmHg) in control (C1-6) and heaves (H1-7) horses prior to (0) and at 90, 240 and 1440min following inhalation challenge with saline, 20, 200 and 2000μg LPS, and 5h hay straw challenge (H/S). MED. = median value, MIN. = minimum value, MAX. = maximum value.

	SALINE				20μg LPS				200μg LPS				2000μg LPS				H/S			
TIME PT.	0	90	240	1440	0	90	240	1440	0	90	240	1440	0	90	240	1440	0	90	240	1440
C1	100.0	103.0	114.0	105.0	101.0	94.0	93.0	110.0	96.0	100.0	110.0	95.0	90.8	110.4	98.7	91.9	94.0	106.0	98.0	106.0
C2	103.0	100.0	115.0	104.0	91.0	97.0	94.0	94.0	92.0	93.0	86.0	100.0	109.0	111.0	101.0	102.0	88.0	81.0	86.0	88.0
C3	106.5	97.5	104.6	100.7	94.6	103.3	110.9	93.8	93.0	105.5	113.8	123.0	108.3	115.9	109.9	97.2	82.9	96.0	107.7	119.6
C4	90.6	106.2	88.0	93.8	86.4	97.5	93.5	85.4	95.0	100.8	84.8	100.7	89.9	99.8	100.5	94.8	84.2	100.0	100.0	95.4
C5	105.0	99.0	110.0	99.0	98.0	94.0	115.0	93.0	128.0	99.8	104.1	104.2	101.0	109.3	111.0	110.1	105.0	95.0	96.0	106.0
C6	93.0	92.0	104.0	94.0	92.0	104.0	94.0	93.0	96.0	100.9	103.1	105.6	87.7	105.3	79.3	83.5	105.0	93.0	101.0	110.0
MED.	*101.5*	*99.5*	*107.3*	*99.9*	*93.3*	*97.3*	*94.0*	*93.4*	*95.5*	*100.4*	*103.6*	*102.5*	*95.9*	*109.9*	*100.8*	*96.0*	*91.0*	*95.5*	*99.0*	*106.0*
MIN.	*90.6*	*92.0*	*88.0*	*93.8*	*86.4*	*93.0*	*93.0*	*85.4*	*92.0*	*93.0*	*84.8*	*95.0*	*87.7*	*99.8*	*79.3*	*83.5*	*82.9*	*81.0*	*86.0*	*88.0*
MAX.	*106.5*	*106.2*	*115.0*	*105.0*	*101.0*	*104.0*	*115.0*	*110.0*	*128.0*	*105.5*	*113.8*	*123.0*	*109.0*	*115.9*	*111.0*	*110.1*	*105.0*	*106.0*	*107.7*	*119.6*
H1	99.0	99.0	98.0	107.0	99.0	101.0	106.0	104.0	102.0	102.0	106.0	114.0	94.0	96.0	92.0	92.0	114.0	106.0	107.0	111.0
H2	116.0	99.0	102.0	106.0	82.0	98.0	85.0	99.0	93.0	99.0	99.0	101.0	92.0	92.0	95.0	94.0	94.0	93.0	86.0	89.0
H3	94.0	98.0	98.0	98.0	110.0	108.0	100.0	95.0	101.0	92.0	102.0	107.0	98.0	113.0	93.0	89.0	98.0	97.0	90.0	98.0
H4	102.0	101.0	97.0	97.0	98.0	100.0	102.0	96.0	92.0	96.0	94.0	96.0	87.0	111.0	93.0	99.0	103.0	100.0	89.0	92.0
H5	94.0	93.0	83.0	114.0	95.0	99.0	88.0	102.0	89.0	100.0	93.0	98.0	93.0	93.0	100.0	96.0	105.0	85.0	106.0	103.0
H6	90.5	107.3	94.5	80.3	75.6	73.7	78.8	89.8	80.6	107.6	83.4	85.7	109.1	97.3	110.8	104.6	103.5	86.1	104.0	98.0
H7	100.0	109.0	109.0	111.0	94.0	103.0	117.0	102.0	123.0	106.0	115.0	114.0	99.0	104.0	105.0	129.0	97.0	94.0	94.0	107.0
MED.	*99.0*	*99.0*	*98.0*	*106.0*	*95.0*	*100.0*	*100.0*	*99.0*	*93.0*	*100.0*	*99.0*	*101.0*	*94.0*	*97.3*	*95.0*	*96.0*	*103.0*	*94.0*	*94.0*	*98.0*
MIN.	*90.5*	*93.0*	*83.0*	*80.3*	*75.6*	*73.7*	*78.8*	*89.8*	*80.6*	*92.0*	*83.4*	*85.7*	*87.0*	*92.0*	*92.0*	*89.0*	*94.0*	*85.0*	*86.0*	*89.0*
MAX.	*116.0*	*109.0*	*109.0*	*114.0*	*110.0*	*108.0*	*117.0*	*104.0*	*123.0*	*107.6*	*115.0*	*114.0*	*109.1*	*113.0*	*110.8*	*129.0*	*114.0*	*106.0*	*107.0*	*111.0*

Appendix 2.7a (i and ii): Lung function measurements in control (C1-6) and heaves (H1-7) horses prior to (0h) and at 5h following inhalation challenge with saline. TP = time point, MED. = median value, MIN. = minimum value, MAX. = maximum value.

(i)

	Cdyn (l/kPa)		dPpl (kPa)		RL_{iso} (kPa/l/sec)		RR (breaths/min.)		VT (l)		Wb' (J/min)		$RL_{E25\%}$ (kPa/l/sec)		$RL_{E50\%}$ (kPa/l/sec)		$RL_{E75\%}$ (kPa/l/sec)		$RL_{I25\%}$ (kPa/l/sec)		$RL_{I50\%}$ (kPa/l/sec)		$RL_{I75\%}$ (kPa/l/sec)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
C1	9.26	6.10	0.85	1.38	0.07	0.06	10.55	9.85	5.18	5.72	13.62	17.55	0.01	0.01	0.02	0.01	0.04	0.02	0.21	0.28	0.10	0.14	0.09	0.09
C2	28.68	13.50	0.58	0.72	0.03	0.06	10.05	10.70	6.42	6.27	11.55	16.39	0.01	0.05	0.01	0.01	0.03	0.03	0.06	0.09	0.05	0.09	0.05	0.08
C3	12.50	8.87	0.54	0.79	0.06	0.06	11.15	10.00	4.19	4.61	9.93	11.70	0.06	0.05	0.04	0.01	0.04	0.02	0.10	0.18	0.08	0.16	0.08	0.12
C4	6.72	8.29	1.02	1.06	0.11	0.19	11.60	9.65	3.12	3.89	16.14	19.76	0.12	0.16	0.10	0.14	0.09	0.16	0.20	0.28	0.19	0.22	0.17	0.19
C5	17.51	27.62	0.31	0.41	0.03	0.04	18.75	10.20	2.71	4.48	7.43	8.62	0.03	0.04	0.03	0.02	0.05	0.06	0.03	0.06	0.04	0.04	0.05	0.05
C6	10.32	6.72	0.80	1.07	0.11	0.14	9.00	8.75	4.44	4.40	13.97	14.83	0.09	0.08	0.08	0.02	0.09	0.07	0.15	0.33	0.16	0.25	0.13	0.17
MED.	*11.41*	*8.58*	*0.69*	*0.93*	*0.06*	*0.06*	*10.85*	*9.93*	*4.31*	*4.54*	*12.59*	*15.61*	*0.05*	*0.05*	*0.04*	*0.02*	*0.04*	*0.05*	*0.13*	*0.23*	*0.09*	*0.15*	*0.09*	*0.11*
MIN.	*6.72*	*6.10*	*0.31*	*0.41*	*0.03*	*0.04*	*9.00*	*8.75*	*2.71*	*3.89*	*7.43*	*8.62*	*0.01*	*0.01*	*0.01*	*0.01*	*0.03*	*0.02*	*0.03*	*0.06*	*0.04*	*0.04*	*0.05*	*0.05*
MAX.	*28.68*	*27.62*	*1.02*	*1.38*	*0.11*	*0.19*	*18.75*	*10.70*	*6.42*	*6.27*	*16.14*	*19.76*	*0.12*	*0.16*	*0.10*	*0.14*	*0.09*	*0.16*	*0.21*	*0.33*	*0.19*	*0.25*	*0.17*	*0.19*
H1	15.09	11.44	0.62	0.82	0.04	0.03	10.40	11.50	6.35	6.40	16.27	16.14	0.05	0.03	0.05	0.03	0.08	0.04	0.07	0.08	0.06	0.06	0.06	0.05
H2	21.95	23.02	0.67	0.75	0.07	0.07	8.80	8.50	6.28	7.71	19.71	22.90	0.04	0.06	0.06	0.06	0.07	0.07	0.08	0.06	0.08	0.06	0.08	0.07
H3	11.44	9.12	0.50	0.95	0.06	0.11	8.50	5.30	3.96	6.57	9.90	14.50	0.09	0.03	0.08	0.00	0.08	0.05	0.09	0.23	0.10	0.33	0.08	0.25
H4	32.99	31.80	0.53	0.49	0.06	0.05	7.10	6.40	7.72	8.74	15.70	13.38	0.07	0.03	0.06	0.04	0.08	0.05	0.08	0.06	0.06	0.06	0.07	0.06
H5	20.73	20.17	1.09	0.66	0.03	0.05	14.95	10.15	11.19	7.52	76.09	22.03	0.04	0.05	0.05	0.06	0.06	0.07	0.01	0.04	0.02	0.05	0.03	0.05
H6	22.73	13.17	0.31	0.80	0.03	0.05	14.20	6.90	2.85	5.71	4.37	10.18	0.04	0.05	0.11	0.08	0.15	0.06	0.03	0.18	0.03	0.19	0.03	0.14
H7	8.56	8.45	0.84	0.87	0.08	0.06	13.40	12.85	4.93	5.40	22.731	21.01	0.06	0.03	0.07	0.03	0.08	0.05	0.10	0.13	0.10	0.10	0.09	0.08
MED.	*21.34*	*15.80*	*0.58*	*0.79*	*0.05*	*0.05*	*10.40*	*9.33*	*6.32*	*7.04*	*15.98*	*18.58*	*0.05*	*0.03*	*0.06*	*0.04*	*0.08*	*0.05*	*0.07*	*0.07*	*0.06*	*0.06*	*0.07*	*0.06*
MIN.	*11.44*	*8.45*	*0.31*	*0.49*	*0.03*	*0.03*	*7.10*	*5.30*	*2.85*	*5.40*	*4.37*	*13.38*	*0.04*	*0.03*	*0.05*	*0.00*	*0.06*	*0.04*	*0.01*	*0.04*	*0.02*	*0.05*	*0.03*	*0.05*
MAX.	*32.99*	*31.80*	*1.09*	*0.95*	*0.07*	*0.11*	*14.95*	*12.85*	*11.19*	*8.74*	*76.09*	*22.90*	*0.09*	*0.06*	*0.11*	*0.06*	*0.15*	*0.07*	*0.09*	*0.23*	*0.10*	*0.33*	*0.08*	*0.25*

(ii)

	T_E (sec)		T_I (sec)		T_I:T_E		V'_E (l/min)		V'_{Emax} (l/sec)		V'_{Imax} (l/sec)		Wb_{el} (J)		Wb_{res} (J)		Wb_{Eres} (J)		Wb_{Ires} (J)		Wb_{Itot} (J)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
C1	3.01	2.88	3.30	3.04	1.14	1.12	54.71	56.16	2.86	3.33	3.17	3.43	1.50	2.92	1.41	1.73	0.40	0.03	1.02	1.70	2.52	4.62
C2	3.30	3.45	2.67	2.60	0.82	0.76	64.53	66.64	3.29	2.95	3.49	3.47	0.78	1.47	1.13	1.65	0.33	0.41	0.80	1.23	1.58	2.71
C3	2.85	3.02	2.59	2.97	0.93	1.01	46.70	46.15	2.78	2.90	2.44	2.85	0.72	1.32	0.89	1.15	0.28	0.11	0.60	1.04	1.33	2.35
C4	2.44	3.24	2.33	2.95	2.29	0.92	36.56	37.65	2.12	2.07	2.50	2.26	0.79	0.95	1.26	2.05	0.35	0.77	0.92	1.28	1.71	2.23
C5	1.83	3.21	1.41	2.54	0.77	0.79	50.69	45.52	3.35	2.74	3.05	2.79	0.23	0.37	0.41	0.83	0.15	0.32	0.26	0.51	0.48	0.89
C6	3.50	3.17	3.42	3.43	1.02	2.59	40.07	38.38	2.84	2.26	2.37	2.11	1.03	1.45	1.61	1.77	0.61	0.43	1.00	1.34	2.03	2.79
MED.	*2.93*	*3.19*	*2.63*	*2.96*	*0.98*	*0.96*	*48.69*	*45.83*	*2.85*	*2.82*	*2.78*	*2.82*	*0.79*	*1.38*	*1.20*	*1.69*	*0.34*	*0.36*	*0.86*	*1.26*	*1.65*	*2.53*
MIN.	*1.83*	*2.88*	*1.41*	*2.54*	*0.77*	*0.76*	*36.56*	*37.65*	*2.12*	*2.07*	*2.37*	*2.11*	*0.23*	*0.37*	*0.41*	*0.83*	*0.15*	*0.03*	*0.26*	*0.51*	*0.48*	*0.89*
MAX.	*3.50*	*3.45*	*3.42*	*3.43*	*2.29*	*2.59*	*64.53*	*66.64*	*3.35*	*3.33*	*3.49*	*3.47*	*1.50*	*2.92*	*1.61*	*2.05*	*0.61*	*0.77*	*1.02*	*1.70*	*2.52*	*4.62*
H1	2.78	2.31	3.10	2.68	1.14	1.17	65.79	73.34	4.55	6.27	3.04	3.46	1.36	1.68	1.59	1.36	0.77	0.51	0.82	0.86	2.18	2.54
H2	3.84	3.97	2.91	3.12	0.78	0.79	55.72	65.68	3.46	3.46	3.55	3.59	0.90	1.33	2.19	2.70	1.05	1.21	1.14	1.50	2.04	2.83
H3	2.99	6.57	3.02	4.56	1.20	0.70	39.83	34.77	2.13	2.57	3.55	3.39	1.00	2.30	0.96	2.66	0.27	0.60	0.69	2.06	1.69	4.37
H4	5.06	5.03	3.72	4.16	0.94	1.02	53.25	55.75	2.73	3.21	2.97	4.17	1.01	1.33	2.32	2.06	1.10	0.95	1.22	1.11	2.23	2.45
H5	2.14	3.27	2.06	2.77	0.99	1.00	169.96	74.37	8.95	3.87	7.92	4.44	3.25	1.57	4.74	2.28	2.95	1.24	1.78	1.04	5.04	2.61
H6	1.96	3.78	2.09	5.50	3.52	1.45	40.26	39.51	2.86	3.32	3.49	3.21	0.20	1.28	0.31	1.56	0.12	0.51	0.19	1.05	0.39	2.32
H7	2.29	2.24	2.23	2.42	0.98	1.09	65.99	69.32	3.32	3.79	3.10	3.19	1.42	1.75	1.72	1.64	0.63	0.42	1.08	1.21	2.51	2.96
MED.	*2.88*	*3.62*	*2.97*	*2.94*	*1.06*	*1.01*	*55.72*	*67.50*	*3.16*	*3.63*	*3.52*	*3.53*	*1.00*	*1.62*	*1.89*	*2.17*	*0.91*	*0.77*	*0.98*	*1.16*	*2.11*	*2.72*
MIN.	*1.96*	*2.24*	*2.06*	*2.42*	*0.78*	*0.70*	*40.26*	*34.77*	*2.13*	*2.57*	*2.97*	*3.19*	*0.20*	*1.33*	*0.31*	*1.36*	*0.12*	*0.42*	*0.19*	*0.86*	*0.39*	*2.45*
MAX.	*5.06*	*6.57*	*3.72*	*4.56*	*3.52*	*1.17*	*169.96*	*74.37*	*8.95*	*6.27*	*7.92*	*4.44*	*3.25*	*2.30*	*4.74*	*2.70*	*2.95*	*1.24*	*1.78*	*2.06*	*5.04*	*4.37*

Appendix 2.7b (i and ii): Lung function measurements in control (C1-6) and heaves (H1-7) horses prior to (0h) and at 5h following inhalation challenge with 20μg LPS. TP = time point, MED. = median value, MIN. = minimum value, MAX. = maximum value.

(i)

	Cdyn (l/kPa)		dPpl (kPa)		RL_{iso} (kPa/l/sec)		RR (breaths/min.)		VT (l)		Wb' (J/min)		$RL_{E25\%}$ (kPa/l/sec)		$RL_{E50\%}$ (kPa/l/sec)		$RL_{E75\%}$ (kPa/l/sec)		$RL_{I25\%}$ (kPa/l/sec)		$RL_{I50\%}$ (kPa/l/sec)		$RL_{I75\%}$ (kPa/l/sec)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
C1	5.03	6.48	1.43	1.00	0.10	0.09	7.10	9.40	5.61	4.71	11.02	16.20	0.00	0.03	0.00	0.03	0.00	0.06	0.63	0.24	0.29	0.20	0.19	0.15
C2	37.57	17.38	0.50	0.80	0.04	0.03	8.85	8.3	5.84	8.57	13.16	17.80	0.03	0.002	0.04	0	0.05	0.01	0.04	0.14	0.05	0.14	0.06	0.08
C3	20.80	12.55	0.41	0.65	0.04	0.08	10.30	7.90	4.42	4.82	7.83	9.42	0.06	0.09	0.08	0.04	0.06	0.06	0.04	0.11	0.04	0.10	0.05	0.07
C4	5.25	4.98	1.20	1.32	0.22	0.24	9.00	9.05	3.56	3.81	16.87	19.68	0.19	0.22	0.11	0.19	0.16	0.18	0.35	0.34	0.29	0.26	0.25	0.25
C5	20.76	18.69	0.30	0.44	0.04	0.06	20.90	15.70	2.68	3.08	9.82	12.34	0.04	0.06	0.04	0.06	0.05	0.06	0.03	0.05	0.04	0.06	0.05	0.08
C6	16.64	10.56	0.61	0.78	0.06	0.10	11.00	8.70	4.30	4.88	12.03	13.08	0.07	0.05	0.06	0.03	0.06	0.06	0.06	0.12	0.08	0.13	0.08	0.11
MED.	*18.70*	*10.56*	*0.55*	*0.78*	*0.05*	*0.09*	*9.65*	*9.05*	*4.36*	*4.71*	*11.52*	*13.08*	*0.05*	*0.06*	*0.05*	*0.04*	*0.06*	*0.06*	*0.05*	*0.12*	*0.06*	*0.13*	*0.07*	*0.11*
MIN.	*5.03*	*4.98*	*0.30*	*0.44*	*0.04*	*0.06*	*7.10*	*7.90*	*2.68*	*3.08*	*7.83*	*9.42*	*0.00*	*0.03*	*0.00*	*0.03*	*0.00*	*0.06*	*0.03*	*0.05*	*0.04*	*0.06*	*0.05*	*0.07*
MAX.	*37.57*	*18.69*	*1.43*	*1.32*	*0.22*	*0.24*	*20.90*	*15.70*	*5.84*	*4.88*	*16.87*	*19.68*	*0.19*	*0.22*	*0.11*	*0.19*	*0.16*	*0.18*	*0.63*	*0.34*	*0.29*	*0.26*	*0.25*	*0.25*
H1	20.82	12.57	0.60	0.83	0.04	0.06	7.00	7.10	8.37	8.19	14.87	18.58	0.07	0.09	0.06	0.11	0.05	0.10	0.08	0.10	0.06	0.08	0.06	0.06
H2	26.68	20.33	0.81	0.73	0.12	0.07	5.55	6.75	7.50	6.93	17.67	15.13	0.11	0.09	0.11	0.05	0.14	0.09	0.12	0.08	0.10	0.08	0.09	0.07
H3	17.04	9.39	0.54	0.92	0.06	0.09	12.30	7.65	4.40	6.65	16.45	16.31	0.06	0.05	0.06	0.00	0.07	0.05	0.05	0.14	0.06	0.19	0.06	0.13
H4	18.16	14.90	0.72	0.98	0.07	0.08	7.00	7.05	7.40	8.88	14.53	22.45	0.06	0.04	0.05	0.02	0.07	0.05	0.08	0.11	0.07	0.11	0.06	0.07
H5	16.91	17.02	0.75	0.87	0.07	0.06	5.45	5.35	9.75	9.17	15.78	18.74	0.04	0.07	0.07	0.10	0.09	0.11	0.12	0.07	0.10	0.07	0.09	0.07
H6	17.08	8.76	0.54	0.70	0.09	0.10	6.50	5.5	4.69	4.12	6.83	5.352	0.08	0.12	0.05	0.04	0.07	0.13	0.03	0.21	0.06	0.24	0.03	0.11
H7	8.32	5.57	1.14	1.31	0.11	0.11	8.95	11.30	6.19	5.65	24.96	26.42	0.07	0.03	0.06	0.04	0.10	0.07	0.18	0.18	0.16	0.19	0.14	0.14
MED.	*17.08*	*13.73*	*0.72*	*0.89*	*0.07*	*0.08*	*7.00*	*7.08*	*7.40*	*7.56*	*15.78*	*18.66*	*0.07*	*0.06*	*0.06*	*0.05*	*0.07*	*0.08*	*0.08*	*0.10*	*0.07*	*0.09*	*0.06*	*0.07*
MIN.	*8.32*	*5.57*	*0.54*	*0.73*	*0.04*	*0.06*	*5.45*	*5.35*	*4.40*	*5.65*	*6.83*	*15.13*	*0.04*	*0.03*	*0.05*	*0.00*	*0.05*	*0.05*	*0.03*	*0.07*	*0.06*	*0.07*	*0.03*	*0.06*
MAX.	*26.68*	*20.33*	*1.14*	*1.31*	*0.12*	*0.11*	*12.30*	*11.30*	*9.75*	*9.17*	*24.96*	*26.42*	*0.11*	*0.09*	*0.11*	*0.11*	*0.14*	*0.11*	*0.18*	*0.18*	*0.16*	*0.19*	*0.14*	*0.14*

(ii)

	T_E (sec)		T_I (sec)		$T_I:T_E$		V'_E (l/min)		V'_{Emax} (l/sec)		V'_{Imax} (l/sec)		Wb_{el} (J)		Wb_{res} (J)		Wb_{Eres} (J)		Wb_{Ires} (J)		Wb_{tot} (J)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
C1	3.88	3.11	4.49	3.26	1.16	1.10	39.70	44.31	2.50	2.99	2.52	2.75	3.14	1.85	1.49	1.70	0.80	0.34	2.28	1.35	5.42	3.21
C2	3.34	4.27	2.87	3.28	1.21	0.80	51.77	70.67	2.77	3.49	3.21	4.92	0.53	2.30	1.29	2.16	0.58	0.20	0.71	2.36	1.24	4.66
C3	3.27	3.97	2.56	3.58	0.79	0.91	45.16	38.22	2.59	2.60	2.69	2.60	0.49	0.92	0.76	1.19	0.38	0.53	0.38	0.66	0.87	1.58
C4	3.66	3.67	2.97	3.18	0.82	0.88	32.11	34.56	1.68	1.66	1.91	1.86	1.22	1.52	1.86	2.25	0.59	0.73	1.27	1.51	2.49	3.03
C5	1.66	2.17	1.30	1.70	0.80	0.79	54.92	48.20	3.15	2.92	3.50	3.02	0.18	0.28	0.48	0.78	0.17	0.30	0.30	0.48	0.49	0.76
C6	3.33	3.95	2.39	2.97	0.75	0.76	47.43	42.54	2.70	2.29	3.28	2.89	0.66	1.15	1.12	1.49	0.44	0.37	0.68	1.12	1.34	2.27
MED.	*3.33*	*3.67*	*2.71*	*3.18*	*0.81*	*0.88*	*46.29*	*42.54*	*2.65*	*2.60*	*2.95*	*2.75*	*0.59*	*1.15*	*1.20*	*1.49*	*0.41*	*0.37*	*0.70*	*1.12*	*1.29*	*2.27*
MIN.	*1.66*	*2.17*	*1.30*	*1.70*	*0.75*	*0.76*	*32.11*	*34.56*	*1.68*	*1.66*	*1.91*	*1.86*	*0.18*	*0.28*	*0.48*	*0.78*	*0.17*	*0.30*	*0.30*	*0.48*	*0.49*	*0.76*
MAX.	*3.88*	*3.97*	*4.49*	*3.58*	*1.21*	*1.10*	*54.92*	*48.20*	*3.15*	*2.99*	*3.50*	*3.02*	*3.14*	*1.85*	*1.86*	*2.25*	*0.80*	*0.73*	*2.28*	*1.51*	*5.42*	*3.21*
H1	4.08	4.18	4.53	4.28	1.14	1.04	58.50	58.06	4.46	5.02	3.37	3.30	1.67	2.70	2.10	2.61	1.11	1.41	0.99	1.20	2.66	3.90
H2	5.67	4.89	5.04	4.04	0.89	0.83	41.70	46.68	2.78	3.12	2.69	3.78	1.06	1.20	3.15	2.26	1.95	1.31	1.20	0.95	2.26	2.15
H3	2.36	4.19	2.44	3.74	1.06	0.91	54.68	50.36	3.52	3.31	3.30	3.69	0.59	2.37	1.25	2.16	0.74	0.59	0.52	1.57	1.11	3.94
H4	5.26	5.15	3.74	3.49	0.72	0.69	51.38	62.65	2.92	3.63	3.88	4.93	1.65	2.70	2.17	3.24	1.12	1.08	1.06	2.16	2.71	4.86
H5	5.96	6.09	4.97	3.92	0.83	0.63	53.12	49.04	2.99	3.34	3.90	3.81	2.72	2.47	2.87	3.03	1.16	1.74	1.72	1.29	4.44	3.76
H6	3.52	4.72	2.67	6.44	0.82	1.36	27.00	22.71	3.10	1.520	2.31	1.71	0.39	1.01	0.94	0.99	0.63	0.546	0.30	0.44	0.70	1.45
H7	2.99	2.41	3.62	2.78	1.22	1.15	55.30	63.74	2.92	3.48	2.59	3.35	2.30	2.87	2.74	2.29	1.14	0.64	1.60	1.65	3.89	4.52
MED.	*4.08*	*4.54*	*3.74*	*3.83*	*0.89*	*0.87*	*53.12*	*54.21*	*2.99*	*3.41*	*3.30*	*3.73*	*1.65*	*2.58*	*2.17*	*2.45*	*1.12*	*1.19*	*1.06*	*1.43*	*2.66*	*3.92*
MIN.	*2.36*	*2.41*	*2.44*	*2.78*	*0.72*	*0.63*	*27.00*	*46.68*	*2.78*	*3.12*	*2.31*	*3.30*	*0.39*	*1.20*	*0.94*	*2.16*	*0.63*	*0.59*	*0.30*	*0.95*	*0.70*	*2.15*
MAX.	*5.96*	*6.09*	*5.04*	*4.28*	*1.22*	*1.15*	*58.50*	*63.74*	*4.46*	*5.02*	*3.90*	*4.93*	*2.72*	*2.87*	*3.15*	*3.24*	*1.95*	*1.74*	*1.72*	*2.16*	*4.44*	*4.86*

Appendix 2.7c (i and ii): Lung function measurements in control (C1-6) and heaves (H1-7) horses prior to (0h) and at 5h following inhalation challenge with 200μg LPS. TP = time point, MED. = median value, MIN. = minimum value, MAX. = maximum value.

(i)

	Cdyn (l/kPa)		dPpl (kPa)		RL_{iso} (kPa/l/sec)		RR (breaths/min.)		VT (l)		Wb' (J/min)		$RL_{E25\%}$ (kPa/l/sec)		$RL_{E50\%}$ (kPa/l/sec)		$RL_{E75\%}$ (kPa/l/sec)		$RL_{I25\%}$ (kPa/l/sec)		$RL_{I50\%}$ (kPa/l/sec)		$RL_{I75\%}$ (kPa/l/sec)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
C1	5.98	7.65	1.07	1.11	0.09	0.10	11.05	7.40	4.02	5.48	12.58	12.80	0.00	0.04	0.01	0.00	0.03	0.03	0.25	0.20	0.16	0.19	0.12	0.13
C2	9.91	14.37	1.00	0.85	0.08	0.07	9.25	8.45	6.37	7.53	23.51	23.10	0.04	0.04	0.02	0.02	0.04	0.04	0.15	0.11	0.14	0.10	0.12	0.09
C3	18.95	14.93	0.54	0.55	0.05	0.07	8.35	7.45	5.60	4.67	10.56	8.77	0.06	0.09	0.05	0.05	0.05	0.07	0.07	0.11	0.06	0.09	0.06	0.09
C4	4.06	5.26	0.93	1.05	0.15	0.23	9.75	10.55	2.98	3.05	8.99	17.24	0.13	0.20	0.10	0.24	0.08	0.23	0.25	0.25	0.23	0.23	0.20	0.22
C5	17.27	22.32	0.44	0.43	0.05	0.05	17.45	11.20	3.08	3.89	13.98	9.10	0.05	0.05	0.05	0.06	0.06	0.07	0.05	0.04	0.06	0.06	0.07	0.06
C6	11.89	9.68	0.66	0.78	0.13	0.12	5.85	6.05	4.83	5.33	7.14	8.60	0.03	0.04	0.02	0.01	0.08	0.07	0.17	0.21	0.16	0.19	0.14	0.14
MED.	*10.90*	*12.02*	*0.80*	*0.81*	*0.08*	*0.09*	*9.50*	*7.95*	*4.42*	*5.00*	*11.57*	*10.95*	*0.05*	*0.05*	*0.04*	*0.04*	*0.05*	*0.07*	*0.16*	*0.16*	*0.15*	*0.14*	*0.12*	*0.11*
MIN.	*4.06*	*5.26*	*0.44*	*0.43*	*0.05*	*0.05*	*5.85*	*6.05*	*2.98*	*3.05*	*7.14*	*8.60*	*0.00*	*0.04*	*0.01*	*0.00*	*0.03*	*0.03*	*0.05*	*0.04*	*0.06*	*0.06*	*0.06*	*0.06*
MAX.	*18.95*	*22.32*	*1.07*	*1.11*	*0.15*	*0.23*	*17.45*	*11.20*	*6.37*	*7.53*	*23.51*	*23.10*	*0.13*	*0.20*	*0.10*	*0.24*	*0.08*	*0.23*	*0.25*	*0.25*	*0.23*	*0.23*	*0.20*	*0.22*
H1	24.10	22.59	0.54	0.58	0.03	0.03	8.35	8.45	9.87	10.64	18.82	21.85	0.05	0.05	0.06	0.05	0.05	0.05	0.03	0.03	0.02	0.02	0.02	0.02
H2	31.32	15.42	0.95	0.88	0.09	0.12	6.25	6.15	7.74	7.27	23.41	20.26	0.05	0.17	0.10	0.10	0.16	0.12	0.13	0.14	0.10	0.12	0.09	0.10
H3	12.50	12.65	0.53	0.65	0.06	0.11	6.25	5.30	4.33	4.63	6.34	9.11	0.08	0.12	0.04	0.06	0.08	0.10	0.13	0.15	0.23	0.27	0.20	0.12
H4	18.86	18.83	0.54	0.69	0.08	0.07	7.45	6.40	5.67	8.16	12.10	16.05	0.03	0.02	0.07	0.04	0.08	0.07	0.09	0.07	0.09	0.07	0.08	0.07
H5	17.70	20.31	0.62	0.58	0.06	0.06	5.75	5.75	8.39	7.95	10.78	10.43	0.04	0.05	0.07	0.07	0.08	0.08	0.05	0.05	0.05	0.07	0.07	0.07
H6	15.98	13.62	0.56	0.66	0.07	0.08	5.25	6.10	4.47	5.01	3.90	6.29	0.09	0.08	0.07	0.09	0.10	0.12	0.16	0.05	0.06	0.08	0.07	0.04
H7	8.76	7.38	1.06	0.83	0.09	0.06	15.15	18.95	5.49	4.29	46.69	30.46	0.08	0.05	0.10	0.07	0.11	0.08	0.10	0.08	0.10	0.07	0.10	0.07
MED.	*17.70*	*15.42*	*0.56*	*0.66*	*0.07*	*0.07*	*6.25*	*6.15*	*5.67*	*7.27*	*12.10*	*16.05*	*0.05*	*0.05*	*0.07*	*0.07*	*0.08*	*0.08*	*0.10*	*0.07*	*0.09*	*0.07*	*0.08*	*0.07*
MIN.	*8.76*	*7.38*	*0.53*	*0.58*	*0.03*	*0.03*	*5.25*	*5.30*	*4.33*	*4.29*	*3.90*	*6.29*	*0.03*	*0.02*	*0.04*	*0.04*	*0.05*	*0.05*	*0.03*	*0.03*	*0.02*	*0.02*	*0.02*	*0.02*
MAX.	*31.32*	*22.59*	*1.06*	*0.88*	*0.09*	*0.12*	*15.15*	*18.95*	*9.87*	*10.64*	*46.69*	*30.46*	*0.09*	*0.17*	*0.10*	*0.10*	*0.16*	*0.12*	*0.16*	*0.15*	*0.23*	*0.27*	*0.20*	*0.12*

(ii)

	T_E (sec)		T_I (sec)		T_I:T_E		V'_E (l/min)		V'_{Emax} (l/sec)		V'_{Imax} (l/sec)		Wb_{el} (J)		Wb_{res} (J)		Wb_{Eres} (J)		Wb_{Ires} (J)		Wb_{Itot} (J)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
C1	2.88	4.19	2.74	4.07	0.95	0.97	43.26	40.47	1.96	2.23	2.99	3.07	1.46	2.01	1.22	1.75	0.03	0.34	1.19	1.40	2.65	3.41
C2	3.56	4.21	2.81	3.01	0.80	0.73	58.90	63.62	3.08	3.22	3.16	3.48	2.05	1.99	2.46	2.69	0.48	0.72	1.98	1.97	4.04	3.96
C3	3.44	4.10	3.41	3.89	1.00	0.95	46.61	34.87	3.35	2.33	2.68	2.39	0.80	0.76	1.21	1.07	0.64	0.49	0.57	0.58	1.37	1.34
C4	3.38	3.13	3.00	2.59	0.90	0.84	29.01	32.24	1.45	1.59	1.58	1.80	1.08	0.89	0.93	1.62	0.27	0.69	0.66	0.93	1.74	1.82
C5	1.90	2.84	1.51	2.51	0.81	0.89	53.74	43.80	3.31	2.80	3.25	2.62	0.30	0.37	0.77	0.76	0.30	0.36	0.47	0.41	0.76	0.78
C6	5.76	5.40	4.48	4.89	0.80	0.93	28.27	32.29	1.45	1.70	1.84	2.23	1.02	1.52	1.21	1.47	0.34	0.46	0.87	1.01	1.89	2.53
MED.	*3.41*	*4.14*	*2.91*	*3.45*	*0.85*	*0.91*	*44.94*	*37.67*	*2.52*	*2.28*	*2.83*	*2.50*	*1.05*	*1.20*	*1.21*	*1.55*	*0.32*	*0.48*	*0.76*	*0.97*	*1.81*	*2.18*
MIN.	*1.90*	*2.84*	*1.51*	*2.51*	*0.80*	*0.73*	*28.27*	*32.24*	*1.45*	*1.59*	*1.58*	*1.80*	*0.30*	*0.37*	*0.77*	*0.76*	*0.03*	*0.34*	*0.47*	*0.41*	*0.76*	*0.78*
MAX.	*5.76*	*5.40*	*4.48*	*4.89*	*1.00*	*0.97*	*58.90*	*63.62*	*3.35*	*3.22*	*3.25*	*3.48*	*2.05*	*2.01*	*2.46*	*2.69*	*0.64*	*0.72*	*1.98*	*1.97*	*4.04*	*3.96*
H1	3.33	3.44	3.80	3.71	1.17	1.09	81.99	89.74	6.07	6.91	3.70	4.18	2.14	2.55	2.20	2.58	1.37	1.50	0.84	1.08	2.97	3.63
H2	5.31	5.32	4.40	4.40	0.83	0.83	48.51	44.96	3.20	2.29	2.59	2.96	0.88	1.74	3.78	3.27	2.46	1.74	1.33	1.52	2.21	3.26
H3	4.87	4.18	4.82	5.47	1.08	1.81	26.94	24.40	2.03	2.62	2.64	2.94	0.85	1.13	0.99	1.45	0.41	0.55	0.58	0.91	1.44	2.04
H4	4.69	5.64	3.29	3.57	0.72	0.64	42.21	52.43	2.49	3.07	3.02	3.68	0.88	1.79	1.63	2.46	0.87	1.22	0.76	1.24	1.64	3.03
H5	5.99	6.00	4.26	4.48	0.72	0.76	47.85	45.59	2.41	2.31	3.46	3.37	2.00	1.60	1.84	1.82	0.90	0.85	0.94	0.97	2.94	2.57
H6	4.73	4.91	6.50	4.85	1.40	0.99	23.51	30.42	1.52	1.77	1.70	1.95	0.66	0.93	0.73	1.02	0.42	0.75	0.31	0.27	0.97	1.19
H7	1.79	1.46	2.17	1.72	1.22	1.18	83.35	81.18	4.06	4.31	3.81	3.96	1.81	1.30	3.07	1.62	1.65	0.81	1.42	0.80	3.23	2.11
MED.	*4.73*	*4.91*	*4.26*	*4.40*	*1.08*	*0.99*	*47.85*	*45.59*	*2.49*	*2.62*	*3.02*	*3.37*	*0.88*	*1.60*	*1.84*	*1.82*	*0.90*	*0.85*	*0.84*	*0.97*	*2.21*	*2.57*
MIN.	*1.79*	*1.46*	*2.17*	*1.72*	*0.72*	*0.64*	*23.51*	*24.40*	*1.52*	*1.77*	*1.70*	*1.95*	*0.66*	*0.93*	*0.73*	*1.02*	*0.41*	*0.55*	*0.31*	*0.27*	*0.97*	*1.19*
MAX.	*5.99*	*6.00*	*6.50*	*5.47*	*1.40*	*1.81*	*83.35*	*89.74*	*6.07*	*6.91*	*3.81*	*4.18*	*2.14*	*2.55*	*3.78*	*3.27*	*2.46*	*1.74*	*1.42*	*1.52*	*3.23*	*3.63*

Appendix 2.7c (i and ii): Lung function measurements in control (C1-6) and heaves (H1-7) horses prior to (0h) and at 5h following inhalation challenge with 2000μg LPS. TP = time point, MED. = median value, MIN. = minimum value, MAX. = maximum value.

(i)

	Cdyn (l/kPa)		dPpl (kPa)		RL_{iso} (kPa/l/sec)		RR (breaths/min.)		VT (l)		Wb' (J/min)		$RL_{E25\%}$ (kPa/l/sec)		$RL_{E50\%}$ (kPa/l/sec)		$RL_{E75\%}$ (kPa/l/sec)		$RL_{I25\%}$ (kPa/l/sec)		$RL_{I50\%}$ (kPa/l/sec)		$RL_{I75\%}$ (kPa/l/sec)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
C1	13.13	9.56	0.62	0.89	0.05	0.08	8.40	8.60	5.09	5.05	8.47	12.60	0.01	0.02	0.00	0.01	0.02	0.02	0.16	0.22	0.15	0.14	0.09	0.11
C2	18.13	12.04	0.62	0.76	0.08	0.09	6.25	6.10	6.41	6.13	11.52	10.72	0.01	0.00	0.00	0.00	0.05	0.02	0.17	0.22	0.17	0.21	0.11	0.15
C3	13.58	13.64	0.52	0.55	0.06	0.06	17.20	11.75	3.53	4.42	16.45	11.84	0.06	0.06	0.06	0.07	0.06	0.06	0.06	0.06	0.07	0.06	0.08	0.07
C4	5.14	4.99	1.06	1.19	0.22	0.25	8.40	10.2	2.80	2.80	10.27	15.32	0.09	0.19	0.03	0.15	0.13	0.18	0.42	0.50	0.35	0.38	0.25	0.34
C5	19.77	18.99	0.29	0.36	0.03	0.07	17.90	9.15	2.69	2.84	6.63	4.14	0.04	0.03	0.05	0.04	0.05	0.08	0.02	0.09	0.03	0.09	0.04	0.08
C6	11.64	6.36	0.71	1.25	0.09	0.18	8.05	8.60	4.60	4.47	10.00	18.52	0.05	0.08	0.01	0.03	0.04	0.08	0.21	0.31	0.15	0.25	0.13	0.20
MED.	*13.58*	*11.60*	*0.62*	*0.72*	*0.08*	*0.07*	*8.40*	*8.88*	*3.53*	*4.44*	*10.27*	*12.22*	*0.05*	*0.05*	*0.02*	*0.03*	*0.05*	*0.07*	*0.17*	*0.16*	*0.15*	*0.12*	*0.11*	*0.09*
MIN.	*5.14*	*6.36*	*0.29*	*0.36*	*0.03*	*0.06*	*6.25*	*8.60*	*2.69*	*2.84*	*6.63*	*4.14*	*0.01*	*0.02*	*0.00*	*0.01*	*0.04*	*0.02*	*0.02*	*0.06*	*0.03*	*0.06*	*0.04*	*0.07*
MAX.	*19.77*	*18.99*	*1.06*	*1.25*	*0.22*	*0.18*	*17.90*	*11.75*	*6.41*	*5.05*	*16.45*	*18.52*	*0.09*	*0.08*	*0.06*	*0.07*	*0.13*	*0.08*	*0.42*	*0.31*	*0.35*	*0.25*	*0.25*	*0.20*
H1	23.17	25.91	0.61	0.73	0.05	0.08	4.90	5.70	9.53	9.81	12.86	15.42	0.09	0.12	0.05	0.10	0.05	0.10	0.13	0.10	0.12	0.07	0.07	0.07
H2	38.44	26.89	0.30	1.07	0.02	0.16	7.15	6.55	7.57	6.68	5.90	25.90	0.00	0.16	0.00	0.16	0.02	0.20	0.03	0.13	0.04	0.13	0.02	0.12
H3	12.76	11.93	0.66	0.68	0.10	0.11	7.05	4.50	5.31	5.20	9.96	9.83	0.10	0.09	0.05	0.05	0.09	0.09	0.11	0.19	0.11	0.25	0.10	0.17
H4	24.51	30.49	0.49	0.52	0.06	0.06	7.15	10.30	6.66	5.23	10.03	14.59	0.00	0.03	0.04	0.06	0.06	0.08	0.06	0.06	0.05	0.06	0.05	0.06
H5	21.41	20.95	0.62	0.78	0.05	0.10	5.00	4.60	10.12	11.15	13.18	19.76	0.09	0.07	0.08	0.12	0.09	0.13	0.06	0.11	0.05	0.09	0.05	0.09
H6	8.80	9.84	0.64	0.93	0.09	0.12	9.50	10.60	3.59	4.58	9.24	22.25	0.10	0.14	0.14	0.12	0.12	0.12	0.09	0.09	0.07	0.10	0.08	0.12
H7	7.67	9.20	1.07	1.21	0.08	0.11	8.90	9.00	6.24	6.68	18.06	29.20	0.03	0.06	0.01	0.03	0.04	0.09	0.23	0.19	0.20	0.15	0.14	0.13
MED.	*21.41*	*20.95*	*0.62*	*0.78*	*0.06*	*0.11*	*7.15*	*6.55*	*6.66*	*6.68*	*10.03*	*19.76*	*0.09*	*0.09*	*0.05*	*0.10*	*0.06*	*0.10*	*0.09*	*0.11*	*0.07*	*0.10*	*0.07*	*0.12*
MIN.	*7.67*	*9.20*	*0.30*	*0.52*	*0.02*	*0.06*	*4.90*	*4.50*	*3.59*	*4.58*	*5.90*	*9.83*	*0.00*	*0.03*	*0.00*	*0.03*	*0.02*	*0.08*	*0.03*	*0.06*	*0.04*	*0.06*	*0.02*	*0.06*
MAX.	*38.44*	*30.49*	*1.07*	*1.21*	*0.10*	*0.16*	*9.50*	*10.60*	*10.12*	*11.15*	*18.06*	*29.20*	*0.10*	*0.16*	*0.14*	*0.16*	*0.12*	*0.20*	*0.23*	*0.19*	*0.20*	*0.25*	*0.14*	*0.17*

(ii)

	T_E (sec)		T_I (sec)		T_I:T_E		V'_E (l/min)		V'_{Emax} (l/sec)		V'_{Imax} (l/sec)		Wb_{el} (J)		Wb_{res} (J)		Wb_{Eres} (J)		Wb_{Ires} (J)		Wb_{tot} (J)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
C1	3.78	3.76	3.42	3.30	0.91	0.89	42.81	43.20	2.33	2.29	2.14	2.30	0.99	1.41	1.02	1.47	0.14	0.26	0.89	1.21	1.87	2.62
C2	5.91	5.89	3.81	3.92	0.66	0.67	40.12	37.20	2.13	2.24	2.67	2.48	1.21	1.58	1.86	1.75	0.35	0.07	1.52	1.67	2.73	3.25
C3	1.84	2.81	1.71	2.46	0.95	0.92	60.68	52.02	3.57	3.07	3.05	3.02	0.50	0.76	0.97	1.02	0.42	0.43	0.55	0.59	1.05	1.35
C4	4.11	3.33	2.99	2.70	0.74	0.81	23.42	28.65	1.24	1.28	1.62	1.39	0.90	0.78	1.19	1.54	0.31	0.52	0.88	1.02	1.78	1.80
C5	1.97	3.69	1.45	2.74	0.75	0.74	47.23	26.04	2.92	1.54	2.93	1.77	0.21	0.25	0.37	0.44	0.16	0.15	0.21	0.29	0.42	0.55
C6	3.73	3.63	3.63	3.42	1.00	0.96	37.10	38.31	2.02	2.02	2.46	2.05	0.90	1.56	1.22	2.18	0.23	0.65	1.00	1.53	1.90	3.09
MED.	*3.73*	*3.66*	*2.99*	*3.02*	*0.75*	*0.90*	*40.12*	*40.76*	*2.13*	*2.15*	*2.67*	*2.18*	*0.90*	*1.08*	*1.19*	*1.25*	*0.31*	*0.35*	*0.88*	*0.90*	*1.78*	*1.99*
MIN.	*1.84*	*2.81*	*1.45*	*2.46*	*0.66*	*0.74*	*23.42*	*26.04*	*1.24*	*1.54*	*1.62*	*1.77*	*0.21*	*0.25*	*0.37*	*0.44*	*0.16*	*0.15*	*0.21*	*0.29*	*0.42*	*0.55*
MAX.	*5.91*	*3.76*	*3.81*	*3.42*	*1.00*	*0.96*	*60.68*	*52.02*	*3.57*	*3.07*	*3.05*	*3.02*	*1.21*	*1.56*	*1.86*	*2.18*	*0.42*	*0.65*	*1.52*	*1.53*	*2.73*	*3.09*
H1	5.47	5.58	6.75	6.73	1.27	1.23	46.77	55.94	3.43	2.74	3.58	2.62	2.10	2.01	2.59	3.16	1.32	1.92	1.27	1.24	3.36	3.24
H2	4.92	4.94	3.57	4.20	0.73	0.85	54.03	43.86	3.58	2.75	3.77	2.34	0.78	1.09	0.83	3.95	0.34	2.25	0.48	1.70	1.26	2.79
H3	4.98	5.15	4.16	5.18	0.84	1.33	37.64	23.43	2.38	1.98	2.83	2.73	1.13	1.51	1.52	1.66	0.75	0.41	0.77	1.25	1.90	2.76
H4	4.81	3.15	3.73	2.66	0.78	0.85	47.71	53.82	2.82	3.01	3.09	3.00	0.93	0.46	1.43	1.41	0.77	0.80	0.66	0.61	1.58	1.07
H5	6.88	8.07	4.63	4.94	0.71	0.62	50.64	51.09	2.68	2.40	4.00	3.47	2.45	3.01	2.48	4.27	1.29	1.93	1.19	2.34	3.64	5.35
H6	2.96	2.77	3.42	2.92	1.18	1.06	33.93	48.58	2.89	2.58	2.42	2.78	0.75	1.07	0.97	2.11	0.49	0.98	0.48	1.14	1.23	2.20
H7	3.26	3.13	3.85	3.54	1.19	1.15	55.31	60.57	2.96	2.97	2.73	2.69	2.60	2.45	2.15	3.18	0.46	1.17	1.69	2.01	4.29	4.46
MED.	*4.92*	*4.94*	*3.85*	*4.20*	*0.84*	*1.06*	*47.71*	*51.09*	*2.89*	*2.74*	*3.09*	*2.73*	*1.13*	*1.51*	*1.52*	*3.16*	*0.75*	*1.17*	*0.77*	*1.25*	*1.90*	*2.79*
MIN.	*2.96*	*2.77*	*3.42*	*2.66*	*0.71*	*0.62*	*33.93*	*23.43*	*2.38*	*1.98*	*2.42*	*2.34*	*0.75*	*0.46*	*0.83*	*1.41*	*0.34*	*0.41*	*0.48*	*0.61*	*1.23*	*1.07*
MAX.	*6.88*	*8.07*	*6.75*	*6.73*	*1.27*	*1.33*	*55.31*	*60.57*	*3.58*	*3.01*	*4.00*	*3.47*	*2.60*	*3.01*	*2.59*	*4.27*	*1.32*	*2.25*	*1.69*	*2.34*	*4.29*	*5.35*

Appendix 2.7c (i and ii): Lung function measurements in control (C1-6) and heaves (H1-7) horses prior to (0h) and at 5h following initiation of 5h hay/straw challenge. TP = time point, MED. = median value, MIN. = minimum value, MAX. = maximum value.

(i)

	Cdyn (l/kPa)		dPpl (kPa)		RL_{iso} (kPa/l/sec)		RR (breaths/min.)		VT (l)		Wb' (J/min)		$RL_{E25\%}$ (kPa/l/sec)		$RL_{E50\%}$ (kPa/l/sec)		$RL_{E75\%}$ (kPa/l/sec)		$RL_{I25\%}$ (kPa/l/sec)		$RL_{I50\%}$ (kPa/l/sec)		$RL_{I75\%}$ (kPa/l/sec)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
C1	5.31	8.81	1.38	0.86	0.06	0.09	7.00	8.45	5.85	4.53	11.68	9.68	0.00	0.01	0.00	0.02	0.00	0.05	0.39	0.13	0.12	0.12	0.11	0.11
C2	12.37	14.75	0.82	0.82	0.11	0.11	5.50	5.30	6.45	6.65	10.63	12.17	0.04	0.10	0.00	0.08	0.05	0.13	0.26	0.13	0.23	0.12	0.16	0.12
C3	31.49	21.02	0.61	0.55	0.04	0.04	6.65	5.55	10.97	7.93	18.12	6.59	0.04	0.05	0.03	0.05	0.04	0.06	0.04	0.05	0.03	0.04	0.05	0.04
C4	4.05	6.23	1.30	0.73	0.20	0.15	16.60	13.85	2.81	2.29	35.25	11.07	0.18	0.07	0.21	0.08	0.26	0.12	0.22	0.18	0.23	0.20	0.22	0.16
C5	36.57	13.34	0.25	0.45	0.02	0.02	33.00	26.10	2.59	3.15	13.63	14.79	0.02	0.03	0.03	0.05	0.04	0.06	0.02	0.02	0.02	0.02	0.03	0.03
C6	11.25	9.99	0.68	0.49	0.05	0.05	13.95	20.65	3.89	2.65	11.22	13.62	0.05	0.03	0.04	0.05	0.05	0.10	0.10	0.07	0.09	0.09	0.08	0.06
MED.	*11.81*	*11.67*	*0.75*	*0.64*	*0.06*	*0.07*	*10.48*	*11.15*	*4.87*	*3.84*	*12.66*	*11.62*	*0.04*	*0.04*	*0.03*	*0.05*	*0.04*	*0.08*	*0.16*	*0.10*	*0.10*	*0.10*	*0.10*	*0.09*
MIN.	*4.05*	*6.23*	*0.25*	*0.45*	*0.02*	*0.02*	*5.50*	*5.30*	*2.59*	*2.29*	*10.63*	*6.59*	*0.00*	*0.01*	*0.00*	*0.02*	*0.00*	*0.05*	*0.02*	*0.02*	*0.02*	*0.02*	*0.03*	*0.03*
MAX.	*36.57*	*21.02*	*1.38*	*0.86*	*0.20*	*0.15*	*33.00*	*26.10*	*10.97*	*7.93*	*35.25*	*14.79*	*0.18*	*0.10*	*0.21*	*0.08*	*0.26*	*0.13*	*0.39*	*0.18*	*0.23*	*0.20*	*0.22*	*0.16*
H1	27.12	26.47	1.02	0.66	0.16	0.06	4.95	7.55	8.14	7.50	22.64	19.24	0.17	0.08	0.13	0.07	0.19	0.08	0.15	0.06	0.15	0.06	0.21	0.08
H2	46.49	20.52	0.43	0.76	0.05	0.09	7.60	9.45	6.03	5.66	9.70	18.75	0.03	0.05	0.05	0.06	0.06	0.09	0.05	0.07	0.05	0.08	0.06	0.11
H3	10.91	14.23	0.69	0.57	0.10	0.07	8.70	5.85	4.98	4.68	11.06	6.15	0.03	0.00	0.03	0.01	0.07	0.06	0.17	0.12	0.22	0.13	0.13	0.03
H4	25.75	31.19	0.43	0.38	0.05	0.03	7.20	9.50	5.70	5.84	8.08	8.58	0.02	0.03	0.04	0.03	0.07	0.05	0.06	0.04	0.06	0.04	0.06	0.07
H5	21.88	20.48	0.51	0.55	0.06	0.07	8.10	6.85	6.84	7.13	13.96	11.91	0.02	0.04	0.04	0.07	0.07	0.07	0.07	0.03	0.08	0.06	0.08	0.05
H6	9.60	14.87	0.81	0.50	0.10	0.06	8.50	10.10	4.82	4.07	15.68	6.40	0.08	0.04	0.12	0.04	0.07	0.05	0.12	0.04	0.07	0.04	0.07	0.16
H7	33.40	21.49	0.55	0.91	0.06	0.14	19.55	14.50	4.17	4.25	28.45	35.06	0.05	0.11	0.06	0.13	0.07	0.14	0.05	0.12	0.06	0.14	0.07	0.07
MED.	*26.44*	*20.52*	*0.53*	*0.57*	*0.06*	*0.07*	*8.10*	*9.45*	*5.86*	*5.66*	*12.51*	*11.91*	*0.03*	*0.04*	*0.05*	*0.06*	*0.07*	*0.07*	*0.06*	*0.06*	*0.07*	*0.06*	*0.08*	*0.03*
MIN.	*10.91*	*14.23*	*0.43*	*0.38*	*0.05*	*0.03*	*4.95*	*5.85*	*4.17*	*4.07*	*8.08*	*6.15*	*0.02*	*0.00*	*0.03*	*0.01*	*0.06*	*0.05*	*0.05*	*0.03*	*0.05*	*0.04*	*0.06*	*0.16*
MAX.	*46.49*	*31.19*	*1.02*	*0.91*	*0.16*	*0.14*	*19.55*	*14.50*	*8.14*	*7.50*	*28.45*	*35.06*	*0.17*	*0.11*	*0.13*	*0.13*	*0.19*	*0.14*	*0.17*	*0.12*	*0.22*	*0.14*	*0.21*	

(ii)

	T_E (sec)		T_I (sec)		T_I:T_E		V'_E (l/min)		V'_{Emax} (l/sec)		V'_{Imax} (l/sec)		Wb_{el} (J)		Wb_{res} (J)		Wb_{Eres} (J)		Wb_{Ires} (J)		Wb_{tot} (J)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
C1	4.35	3.80	4.37	3.52	1.01	0.94	39.00	38.17	2.21	1.88	2.27	2.59	3.53	1.17	1.67	1.19	0.24	0.28	1.91	0.91	5.44	2.08
C2	5.72	6.07	5.28	5.10	0.93	0.86	35.49	35.20	1.98	2.00	2.08	2.18	1.66	1.47	1.94	2.24	0.41	1.15	1.53	1.09	3.19	2.56
C3	5.36	5.78	4.18	5.11	0.78	0.89	74.86	43.93	3.31	1.98	4.18	2.33	1.99	1.52	2.56	1.19	1.17	0.67	1.39	0.53	3.38	2.05
C4	2.11	2.70	1.52	1.73	0.75	0.64	46.50	31.59	2.34	1.25	2.73	2.29	1.05	0.42	2.09	0.81	0.83	0.20	1.26	0.61	2.31	1.03
C5	1.07	1.32	0.79	1.02	0.78	0.80	85.36	81.88	4.11	4.39	5.00	4.67	0.10	0.44	0.42	0.58	0.19	0.34	0.23	0.24	0.33	0.68
C6	2.36	1.60	2.10	1.35	0.90	0.85	53.81	54.12	3.93	3.84	3.17	3.52	0.87	0.42	0.80	0.69	0.27	0.23	0.53	0.46	1.40	0.88
MED.	*3.35*	*3.25*	*3.14*	*2.63*	*0.84*	*0.85*	*50.16*	*41.05*	*2.83*	*1.99*	*2.95*	*2.46*	*1.35*	*0.81*	*1.80*	*1.00*	*0.34*	*0.31*	*1.33*	*0.57*	*2.75*	*1.54*
MIN.	*1.07*	*1.32*	*0.79*	*1.02*	*0.75*	*0.64*	*35.49*	*31.59*	*1.98*	*1.25*	*2.08*	*2.18*	*0.10*	*0.42*	*0.42*	*0.58*	*0.194*	*0.20*	*0.23*	*0.24*	*0.33*	*0.68*
MAX.	*5.72*	*6.07*	*5.28*	*5.11*	*1.01*	*0.94*	*85.36*	*81.88*	*4.11*	*4.39*	*5.00*	*4.67*	*3.53*	*1.52*	*2.56*	*2.24*	*1.17*	*1.15*	*1.91*	*1.09*	*5.44*	*2.56*
H1	5.67	4.00	6.18	3.82	1.10	0.99	40.19	56.47	2.13	3.41	1.76	3.00	1.25	1.05	4.38	2.44	2.18	1.26	2.20	1.17	3.45	2.22
H2	4.72	3.72	3.11	2.70	0.66	0.73	45.65	53.25	2.55	3.12	3.01	2.84	0.39	0.79	1.28	2.01	0.61	1.00	0.66	1.01	1.06	1.81
H3	3.88	6.66	3.98	3.49	1.03	0.55	43.03	27.33	2.53	2.00	2.37	2.13	1.18	0.87	1.46	1.02	0.48	0.33	0.98	0.69	2.15	1.56
H4	4.55	3.32	3.65	3.05	0.81	0.92	41.02	55.47	2.52	3.09	2.67	3.36	0.64	0.52	1.10	0.91	0.59	0.56	0.51	0.36	1.16	0.88
H5	4.09	5.19	3.34	3.87	0.82	0.75	55.61	48.86	2.85	2.68	3.52	3.16	1.28	1.37	1.71	1.81	0.60	1.00	1.11	0.80	2.39	2.17
H6	3.02	3.02	2.87	3.06	0.95	1.02	30.00	41.09	2.91	2.38	3.65	2.70	1.31	0.58	1.55	0.64	0.428	0.30	1.12	0.35	2.42	0.93
H7	1.57	2.23	1.56	1.93	1.01	0.88	81.68	61.07	3.36	2.31	4.06	2.93	0.32	0.47	1.45	2.46	0.73	1.05	0.72	1.41	1.04	1.88
MED.	*4.32*	*3.72*	*3.49*	*3.06*	*0.91*	*0.88*	*43.03*	*53.25*	*2.54*	*2.68*	*2.84*	*2.93*	*0.91*	*0.79*	*1.45*	*1.81*	*0.61*	*1.00*	*0.85*	*0.80*	*1.65*	*1.81*
MIN.	*1.57*	*2.23*	*1.56*	*1.93*	*0.66*	*0.55*	*30.00*	*27.33*	*2.13*	*2.00*	*1.76*	*2.13*	*0.32*	*0.47*	*1.10*	*0.64*	*0.48*	*0.30*	*0.51*	*0.35*	*1.04*	*0.88*
MAX.	*5.67*	*6.66*	*6.18*	*3.87*	*1.10*	*1.02*	*81,68*	*61.07*	*3.36*	*3.41*	*4.06*	*3.36*	*1.28*	*1.37*	*4.38*	*2.46*	*2.18*	*1.26*	*2.20*	*1.41*	*3.45*	*2.22*

Appendix 2.8: PCCdyn70 values (mg/ml) in control (C1-6) and heaves (H1-7) horses at 5h following inhalation challenge with saline 20, 200 and 2000μg LPS and 5h hay/straw challenge (H/S). MED. = median value, MIN. = minimum value, MAX. = maximum value.

	SALINE	20μg LPS	200μg LPS	2000μg LPS	H/S
C1	3.82	2.47	3.39	2.93	4.44
C2	9.91	6.60	8.61	5.89	8.38
C3	3.46	24.53	10.18	11.14	11.02
C4	2.82	8.04	3.63	3.84	2.82
C5	3.87	8.53	7.58	4.71	4.71
C6	2.29	2.19	1.47	36.38	2.73
MED.	*3.64*	*7.32*	*5.60*	*5.30*	*4.57*
MIN.	*2.29*	*2.19*	*1.47*	*2.93*	*2.73*
MAX.	*9.91*	*24.53*	*10.18*	*36.38*	*11.02*
H1	7.86	2.93	6.17	11.81	4.98
H2	3.06	10.12	3.80	1.24	2.15
H3	5.64	8.93	9.32	8.33	4.66
H4	10.53	10.82	19.40	6.18	14.04
H5	6.40	6.39	2.20	8.90	9.02
H6	2.46	2.56	1.79	2.29	2.41
H7	5.63	5.34	7.69	2.10	6.10
MED.	*5.64*	*6.39*	*6.17*	*6.18*	*4.98*
MIN.	*2.46*	*2.56*	*1.79*	*1.24*	*2.15*
MAX.	*10.53*	*10.82*	*19.40*	*11.81*	*14.04*

Appendix 2.9 (a) BALF total nucleated cell counts (x10^5/ml) in control (C1-6) and heaves (H1-7) at 6h and 24h following challenge with saline, 20, 200 and 2000μg LPS, and 5h hay straw challenge (H/S). MED. = median value, MIN. = minimum value, MAX. = maximum value.

	SALINE	SALINE	20μg	20μg	200μg	200μg	2000μg	2000μg	200μgR	200μgR	H/S	H/S
TIME PT	6h	24h	6h	24h	6h	24h	6h	24h	6h	24h	6h	24h
C1	5.10	4.90	3.50	4.30	4.30	8.30	7.30	5.00	6.30	NP	2.20	5.20
C2	3.90	3.70	3.50	3.70	2.70	4.80	4.90	5.90	6.20	NP	2.90	4.60
C3	2.00	3.00	1.90	1.90	2.90	3.30	1.50	4.80	NP	NP	2.10	2.90
C4	2.90	1.40	2.00	4.50	3.60	2.50	3.30	4.80	NP	NP	3.20	1.80
C5	9.60	3.40	6.40	4.30	5.20	3.40	3.90	4.20	4.00	NP	4.60	2.00
C6	3.80	2.90	11.10	7.00	9.40	3.00	4.20	5.30	3.00	NP	2.20	1.70
MED.	*3.85*	*3.20*	*3.50*	*4.30*	*3.95*	*3.35*	*4.05*	*4.90*			*2.55*	*2.45*
MIN	*2.00*	*1.40*	*1.90*	*1.90*	*2.70*	*2.50*	*1.50*	*4.20*			*2.10*	*1.70*
MAX	*9.60*	*4.90*	*11.10*	*7.00*	*9.40*	*8.30*	*7.30*	*5.90*			*4.60*	*5.20*
H1	5.60	9.00	6.30	5.60	4.90	6.20	4.10	4.60	NP	NP	6.20	3.40
H2	3.40	2.90	3.20	4.40	5.70	2.50	7.20	3.40	3.50	NP	4.20	3.00
H3	3.80	3.90	4.10	4.50	5.30	4.20	5.20	3.30	4.30	NP	2.80	6.30
H4	5.50	3.90	5.40	5.10	8.20	8.60	12.40	5.60	3.80	NP	7.00	5.00
H5	3.20	4.80	4.60	4.90	4.50	8.20	6.60	16.00	2.30	NP	16.20	3.80
H6	1.30	0.60	2.90	2.90	4.00	4.20	1.70	4.50	NP	NP	5.70	6.70
H7	5.20	3.85	7.40	4.85	4.20	5.60	9.30	7.20	6.40	NP	6.50	2.60
MED.	*3.80*	*3.90*	*4.60*	*4.85*	*4.90*	*5.60*	*6.60*	*4.60*			*6.20*	*3.80*
MIN	*1.30*	*0.60*	*2.90*	*2.90*	*4.00*	*2.50*	*1.70*	*3.30*			*2.80*	*2.60*
MAX	*5.60*	*9.00*	*7.40*	*5.60*	*8.20*	*8.60*	*12.40*	*16.00*			*16.20*	*6.70*

Appendix 2.9 (b) BALF lymphocyte counts (x10^5/ml) in control (C1-6) and heaves (H1-7) at 6h and 24h following challenge with saline, 20, 200 and 2000μg LPS, and 5h hay straw challenge (H/S). MED. = median value, MIN. = minimum value, MAX. = maximum value.

	SALINE	SALINE	20μg	20μg	200μg	200μg	2000μg	2000μg	200μgR	200μgR	H/S	H/S
TIME PT	6h	24h	6h	24h	6h	24h	6h	24h	6h	24h	6h	24h
C1	1.89	1.95	0.99	1.74	2.03	2.78	3.69	1.22	2.55	NP	0.61	3.28
C2	1.28	1.17	1.44	1.52	1.37	2.20	2.45	2.70	2.59	NP	0.99	1.97
C3	0.99	1.53	1.30	1.21	1.71	2.16	0.71	1.88	NP	NP	1.07	1.67
C4	0.99	0.47	0.64	2.07	1.17	0.95	0.68	1.16	NP	NP	0.76	0.53
C5	6.22	2.06	5.24	1.74	3.02	1.41	1.35	1.45	1.94	NP	3.09	0.90
C6	2.41	1.68	6.22	3.26	2.40	0.84	0.87	1.45	1.32	NP	0.79	0.54
MED.	*1.59*	*1.61*	*1.37*	*1.74*	*1.87*	*1.78*	*1.11*	*1.45*	*2.24*		*0.89*	*1.29*
MIN	*0.99*	*0.47*	*0.64*	*1.21*	*1.17*	*0.84*	*0.68*	*1.16*	*1.32*		*0.61*	*0.53*
MAX	*6.22*	*2.06*	*6.22*	*3.26*	*3.02*	*2.78*	*3.69*	*2.70*	*2.59*		*3.09*	*3.28*
H1	3.33	4.64	3.45	2.97	2.60	2.60	1.78	1.93	2.07	NP	1.44	1.48
H2	1.54	1.15	2.04	2.36	1.94	1.15	2.55	1.44	1.52	NP	0.71	0.99
H3	2.09	1.25	1.88	2.35	1.26	1.27	0.68	0.81	1.20	NP	0.60	2.63
H4	2.36	1.59	2.45	2.64	3.83	4.51	4.56	2.02	1.50	NP	2.30	2.13
H5	1.64	2.39	2.93	2.76	2.37	3.80	1.47	4.85	0.64	NP	4.26	1.73
H6	0.80	0.24	1.93	2.04	2.12	2.28	0.73	1.56	NP	NP	2.46	3.34
H7	2.57	1.90	4.83	3.19	2.40	3.48	5.39	4.41	4.28	NP	2.32	1.15
MED.	*2.09*	*1.59*	*2.45*	*2.64*	*2.37*	*2.60*	*1.78*	*1.93*	*1.51*		*2.30*	*1.73*
MIN	*0.80*	*0.24*	*1.88*	*2.04*	*1.26*	*1.15*	*0.68*	*0.81*	*0.64*		*0.60*	*0.99*
MAX	*3.33*	*4.64*	*4.83*	*3.19*	*3.83*	*4.51*	*5.39*	*4.85*	*4.28*		*4.26*	*3.34*

Appendix 2.9 (c) BALF macrophage counts (x10^5/ml) in control (C1-6) and heaves (H1-7) at 6h and 24h following challenge with saline, 20, 200 and 2000μg LPS, and 5h hay straw challenge (H/S). MED. = median value, MIN. = minimum value, MAX. = maximum value.

	SALINE	SALINE	20μg	20μg	200μg	200μg	2000μg	2000μg	200μgR	200μgR	H/S	H/S
TIME PT	6h	24h	6h	24h	6h	24h	6h	24h	6h	24h	6h	24h
C1	2.80	2.56	2.01	2.15	1.65	1.95	1.40	1.35	2.76	NP	1.35	1.46
C2	2.25	2.30	1.89	1.91	1.12	2.15	1.76	1.91	3.25	NP	1.89	2.32
C3	0.73	0.91	0.33	0.44	0.51	0.48	0.17	0.82	NP	NP	0.66	0.76
C4	1.71	0.86	1.19	2.16	1.25	1.21	1.21	2.28	NP	NP	1.89	1.12
C5	2.57	0.58	0.92	2.15	1.12	1.41	0.50	0.86	1.18	NP	0.98	0.87
C6	1.14	1.07	3.94	2.84	4.18	1.37	0.61	1.11	0.83	NP	1.17	0.98
MED.	*1.98*	*0.99*	*1.54*	*2.15*	*1.19*	*1.39*	*0.91*	*1.23*	*1.97*		*1.26*	*1.05*
MIN	*0.73*	*0.58*	*0.33*	*0.44*	*0.51*	*0.48*	*0.17*	*0.82*	*0.83*		*0.66*	*0.76*
MAX	*2.80*	*2.56*	*3.94*	*2.84*	*4.18*	*2.15*	*1.76*	*2.28*	*3.25*		*1.89*	*2.32*
H1	1.96	3.46	2.26	2.14	1.06	2.44	0.77	1.21	1.56	NP	1.44	1.07
H2	1.58	1.42	0.88	0.90	1.69	0.72	0.68	1.06	0.44	NP	0.90	1.02
H3	1.24	2.10	1.68	1.65	1.58	1.97	1.15	1.30	1.12	NP	1.35	2.41
H4	2.96	2.13	2.57	2.05	2.48	2.69	3.94	1.31	1.10	NP	3.16	2.43
H5	1.27	2.26	1.15	1.39	0.61	2.90	0.72	4.61	0.53	NP	2.03	1.30
H6	0.34	0.24	0.70	0.54	1.02	1.20	0.23	1.07	NP	NP	1.04	1.53
H7	2.40	1.77	1.94	1.43	1.06	1.64	1.23	1.43	1.31	NP	2.57	0.95
MED.	*1.58*	*2.10*	*1.68*	*1.43*	*1.06*	*1.97*	*0.77*	*1.30*	*1.11*		*1.44*	*1.30*
MIN	*0.34*	*0.24*	*0.70*	*0.54*	*0.61*	*0.72*	*0.23*	*1.06*	*0.44*		*0.90*	*0.95*
MAX	*2.96*	*3.46*	*2.57*	*2.14*	*2.48*	*2.90*	*3.94*	*4.61*	*1.56*		*3.16*	*2.43*

Appendix 2.9 (d) BALF neutrophil counts (x10^5/ml) in control (C1-6) and heaves (H1-7) at 6h and 24h following challenge with saline, 20, 200 and 2000μg LPS, and 5h hay straw challenge (H/S). MED. = median value, MIN. = minimum value, MAX. = maximum value.

	SALINE	SALINE	20μg	20μg	200μg	200μg	2000μg	2000μg	200μgR	200μgR	H/S	H/S
TIME PT	6h	24h	6h	24h	6h	24h	6h	24h	6h	24h	6h	24h
C1	0.04	0.19	0.06	0.03	0.28	3.43	1.61	2.25	0.64	NP	0.02	0.22
C2	0.01	0.02	0.01	0.02	0.08	0.27	0.53	1.12	0.18	NP	0.01	0.09
C3	0.02	0.02	0.12	0.03	0.40	0.35	0.52	1.50	NP	NP	0.24	0.21
C4	0.09	0.03	0.08	0.12	1.02	0.31	1.27	1.22	NP	NP	0.35	0.10
C5	0.17	0.60	0.10	0.03	0.73	0.42	1.86	1.87	0.69	NP	0.40	0.06
C6	0.09	0.05	0.17	0.18	2.59	0.73	2.70	2.70	0.79	NP	0.11	0.07
MED.	*0.06*	*0.04*	*0.09*	*0.03*	*0.57*	*0.39*	*1.44*	*1.69*	*0.67*		*0.17*	*0.09*
MIN	*0.01*	*0.02*	*0.01*	*0.02*	*0.08*	*0.27*	*0.52*	*1.12*	*0.18*		*0.01*	*0.06*
MAX	*0.17*	*0.60*	*0.17*	*0.18*	*2.59*	*3.43*	*2.70*	*2.70*	*0.79*		*0.40*	*0.22*
H1	0.20	0.12	0.39	0.13	1.14	0.77	1.40	1.17	1.18	NP	3.14	0.54
H2	0.08	0.08	0.20	1.04	1.90	0.55	3.92	0.76	1.49	NP	2.47	0.95
H3	0.17	0.16	0.23	0.26	2.22	0.86	3.25	1.10	1.86	NP	0.74	1.12
H4	0.03	0.04	0.30	0.20	1.82	1.18	3.83	2.15	1.17	NP	1.47	0.27
H5	0.06	0.08	0.28	0.67	1.45	1.26	4.34	6.24	1.08	NP	9.83	0.67
H6	0.06	0.07	0.18	0.14	0.42	0.26	0.57	1.78	NP	NP	2.05	1.41
H7	0.06	0.04	0.53	0.14	0.58	0.35	2.64	1.28	0.74	NP	1.41	0.39
MED.	*0.06*	*0.08*	*0.28*	*0.20*	*1.45*	*0.77*	*3.25*	*1.28*	*1.17*		*2.05*	*0.67*
MIN	*0.03*	*0.04*	*0.18*	*0.13*	*0.42*	*0.26*	*0.57*	*0.76*	*0.74*		*0.74*	*0.27*
MAX	*0.20*	*0.16*	*0.53*	*1.04*	*2.22*	*1.26*	*4.34*	*6.24*	*1.86*		*9.83*	*1.41*

Appendix 2.9 (e) BALF mast cell counts ($x10^5$/ml) in control (C1-6) and heaves (H1-7) at 6h AND 24h following challenge with saline, 20, 200 and 2000μg LPS, and 5h hay straw challenge (H/S). MED. = median value, MIN. = minimum value, MAX. = maximum value.

	SALINE	SALINE	20μg	20μg	200μg	200μg	2000μg	2000μg	200μgR	200μgR	H/S	H/S
TIME PT	6h	24h	6h	24h	6h	24h	6h	24h	6h	24h	6h	24h
C1	0.23	0.19	0.32	0.33	0.28	0.12	0.23	0.14	0.20	NP	0.11	0.20
C2	0.30	0.19	0.12	0.21	0.10	0.11	0.12	0.11	0.16	NP	0.01	0.20
C3	0.26	0.50	0.12	0.16	0.26	0.24	0.08	0.30	NP	NP	0.11	0.20
C4	0.06	0.04	0.05	0.05	0.04	0.03	0.04	0.07	NP	NP	0.07	0.05
C5	0.39	0.15	0.10	0.19	0.09	0.10	0.09	0.00	0.16	NP	0.12	0.17
C6	0.09	0.08	0.20	0.19	0.06	0.05	0.00	0.04	0.04	NP	0.11	0.10
MED.	*0.25*	*0.17*	*0.12*	*0.19*	*0.10*	*0.10*	*0.08*	*0.09*	*0.16*		*0.11*	*0.18*
MIN	*0.06*	*0.04*	*0.05*	*0.05*	*0.04*	*0.03*	*0.00*	*0.00*	*0.04*		*0.01*	*0.05*
MAX	*0.39*	*0.50*	*0.32*	*0.33*	*0.28*	*0.24*	*0.23*	*0.30*	*0.20*		*0.12*	*0.20*
H1	0.11	0.75	0.18	0.34	0.10	0.39	0.05	0.24	0.09	NP	0.12	0.32
H2	0.15	0.25	0.07	0.04	0.06	0.08	0.03	0.13	0.03	NP	0.11	0.05
H3	0.19	0.38	0.30	0.24	0.24	0.10	0.10	0.09	0.10	NP	0.10	0.14
H4	0.13	0.12	0.07	0.12	0.07	0.19	0.05	0.05	0.03	NP	0.08	0.17
H5	0.09	0.07	0.08	0.08	0.05	0.22	0.01	0.27	0.01	NP	0.08	0.10
H6	0.10	0.04	0.07	0.12	0.40	0.45	0.17	0.07	NP	NP	0.10	0.31
H7	0.17	0.12	0.06	0.07	0.02	0.13	0.03	0.07	0.03	NP	0.10	0.09
MED.	*0.13*	*0.12*	*0.07*	*0.12*	*0.07*	*0.19*	*0.05*	*0.09*	*0.03*		*0.10*	*0.14*
MIN	*0.09*	*0.04*	*0.06*	*0.04*	*0.02*	*0.08*	*0.01*	*0.05*	*0.01*		*0.08*	*0.05*
MAX	*0.19*	*0.75*	*0.30*	*0.34*	*0.40*	*0.45*	*0.17*	*0.27*	*0.10*		*0.12*	*0.32*

Appendix 2.9 (f) BALF basophiloid cell counts ($x10^5$/ml) in control (C1-6) and heaves (H1-7) at 6h and 24h following challenge with saline, 20, 200 and 2000μg LPS, and 5h hay straw challenge (H/S). MED. = median value, MIN. = minimum value, MAX. = maximum value.

	SALINE	SALINE	20μg	20μg	200μg	200μg	2000μg	2000μg	200μgR	200μgR	H/S	H/S
TIME PT	6h	24h	6h	24h	6h	24h	6h	24h	6h	24h	6h	24h
C1	0.07	0.00	0.09	0.03	0.06	0.01	0.04	0.04	0.03	NP	0.09	0.02
C2	0.01	0.00	0.00	0.00	0.01	0.00	0.00	0.01	0.02	NP	0.00	0.01
C3	0.00	0.04	0.03	0.05	0.02	0.03	0.01	0.09	NP	NP	0.03	0.05
C4	0.03	0.00	0.03	0.02	0.10	0.01	0.07	0.05	NP	NP	0.12	0.01
C5	0.22	0.00	0.02	0.02	0.03	0.02	0.02	0.00	0.03	NP	0.00	0.00
C6	0.01	0.00	0.00	0.00	0.01	0.01	0.00	0.00	0.01	NP	0.00	0.00
MED.	*0.02*	*0.00*	*0.03*	*0.02*	*0.02*	*0.01*	*0.02*	*0.03*	*0.02*		*0.02*	*0.01*
MIN	*0.00*	*0.00*	*0.00*	*0.00*	*0.01*	*0.00*	*0.00*	*0.00*	*0.01*		*0.00*	*0.00*
MAX	*0.22*	*0.04*	*0.09*	*0.05*	*0.10*	*0.03*	*0.07*	*0.09*	*0.03*		*0.12*	*0.05*
H1	0.01	0.00	0.00	0.02	0.00	0.00	0.00	0.04	0.00	NP	0.04	0.00
H2	0.05	0.00	0.01	0.02	0.09	0.00	0.01	0.00	0.02	NP	0.01	0.00
H3	0.11	0.00	0.01	0.00	0.01	0.00	0.02	0.00	0.02	NP	0.01	0.01
H4	0.01	0.02	0.01	0.01	0.00	0.01	0.01	0.01	0.00	NP	0.00	0.02
H5	0.02	0.00	0.12	0.00	0.00	0.01	0.05	0.00	0.01	NP	0.00	0.00
H6	0.00	0.00	0.02	0.01	0.01	0.00	0.00	0.01	NP	NP	0.03	0.00
H7	0.01	0.01	0.01	0.01	0.15	0.00	0.01	0.01	0.04	NP	0.08	0.00
MED.	*0.01*	*0.00*	*0.01*	*0.01*	*0.01*	*0.00*	*0.01*	*0.01*	*0.01*		*0.01*	*0.00*
MIN	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*		*0.00*	*0.00*
MAX	*0.11*	*0.02*	*0.12*	*0.02*	*0.15*	*0.01*	*0.05*	*0.04*	*0.04*		*0.08*	*0.02*

Appendix 2.9 (g) BALF eosinophil counts ($x10^5$/ml) in control (C1-6) and heaves (H1-7) at 6h AND 24h following challenge with saline, 20, 200 and 2000μg LPS, and 5h hay straw challenge (H/S). MED. = median value, MIN. = minimum value, MAX. = maximum value.

	SALINE	SALINE	20μg	20μg	200μg	200μg	2000μg	2000μg	200μgR	200μgR	H/S	H/S
TIME PT	6h	24h	6h	24h	6h	24h	6h	24h	6h	24h	6h	24h
C1	0.05	0.02	0.00	0.02	0.00	0.02	0.34	0.01	0.12	NP	0.01	0.04
C2	0.06	0.03	0.01	0.04	0.01	0.07	0.02	0.05	0.00	NP	0.00	0.00
C3	0.00	0.01	0.00	0.01	0.00	0.04	0.01	0.20	NP	NP	0.00	0.00
C4	0.00	0.00	0.00	0.08	0.01	0.00	0.03	0.02	NP	NP	0.02	0.00
C5	0.02	0.01	0.02	0.04	0.21	0.03	0.09	0.03	0.00	NP	0.01	0.00
C6	0.06	0.02	0.58	0.55	0.16	0.01	0.02	0.00	0.01	NP	0.00	0.01
MED.	*0.03*	*0.01*	*0.01*	*0.04*	*0.01*	*0.03*	*0.03*	*0.02*	*0.00*		*0.01*	*0.00*
MIN	*0.00*	*0.00*	*0.00*	*0.01*	*0.00*	*0.00*	*0.01*	*0.00*	*0.00*		*0.00*	*0.00*
MAX	*0.06*	*0.03*	*0.58*	*0.55*	*0.21*	*0.07*	*0.34*	*0.20*	*0.12*		*0.02*	*0.04*
H1	0.00	0.04	0.01	0.00	0.00	0.01	0.10	0.00	0.00	NP	0.01	0.00
H2	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.00	0.00	NP	0.00	0.00
H3	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	NP	0.00	0.01
H4	0.01	0.00	0.00	0.09	0.00	0.03	0.00	0.06	0.00	NP	0.00	0.00
H5	0.01	0.00	0.00	0.00	0.01	0.00	0.01	0.03	0.03	NP	0.00	0.01
H6	0.01	0.00	0.00	0.06	0.03	0.02	0.01	0.01	NP	NP	0.02	0.09
H7	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.01	NP	0.02	0.02
MED.	*0.01*	*0.00*	*0.00*	*0.00*	*0.00*	*0.01*	*0.01*	*0.01*	*0.00*		*0.00*	*0.01*
MIN	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*		*0.00*	*0.00*
MAX	*0.01*	*0.04*	*0.01*	*0.09*	*0.03*	*0.03*	*0.10*	*0.06*	*0.03*		*0.02*	*0.09*

Appendix 2.9 (h) BALF epithelial cell counts ($x10^5$/ml) in control (C1-6) and heaves (H1-7) at 6h and 24h following challenge with saline, 20, 200 and 2000μg LPS, and 5h hay straw challenge (H/S). MED. = median value, MIN. = minimum value, MAX. = maximum value.

	SALINE	SALINE	20μg	20μg	200μg	200μg	2000μg	2000μg	200μgR	200μgR	H/S	H/S
TIME PT	6h	24h	6h	24h	6h	24h	6h	24h	6h	24h	6h	24h
C1	0.03	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	NP	0.00	0.00
C2	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.00	NP	0.00	0.00
C3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	NP	NP	0.00	0.00
C4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	NP	NP	0.00	0.00
C5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	NP	0.00	0.00
C6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	NP	0.00	0.00
MED.	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*		*0.00*	*0.00*
MIN	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*		*0.00*	*0.00*
MAX	*0.03*	*0.00*	*0.03*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*		*0.00*	*0.00*
H1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	NP	0.00	0.00
H2	0.00	0.00	0.00	0.05	0.02	0.01	0.00	0.01	0.00	NP	0.00	0.00
H3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	NP	0.00	0.00
H4	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	NP	0.00	0.00
H5	0.10	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00	NP	0.00	0.00
H6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	NP	NP	0.00	0.00
H7	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	NP	0.00	0.00
MED.	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*		*0.00*	*0.00*
MIN	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*		*0.00*	*0.00*
MAX	*0.10*	*0.00*	*0.05*	*0.05*	*0.02*	*0.01*	*0.00*	*0.01*	*0.00*		*0.00*	*0.00*

Appendix 2.10a: Peripheral venous blood total leukocyte concentrations ($x10^9$/l) in control (C1-6) and heaves (H1-7) horses prior to (0) and at 90, 240 and 1440min following inhalation challenge with saline, 20, 200 and 2000μg LPS, and 5h hay straw challenge (H/S). MED. = median value, MIN. = minimum value, MAX. = maximum value.

	SALINE				20μg LPS				200μg LPS				2000μg LPS				H/S			
TIME PT.	0	90	240	1440	0	90	240	1440	0	90	240	1440	0	90	240	1440	0	90	240	1440
C1	8.5	9.2	9.5	12.7	9.4	9.7	10.6	11.5	9.1	9.8	10.6	11.2	7.7	8.3	7.7	5.3	7.2	8.9	7.5	9.9
C2	10.6	10.3	12.1	13.3	8.9	8.5	9.2	13.5	12.9	12.2	12.0	12.7	10.3	9.6	10.5	12.1	8.5	9.4	9.1	10.1
C3	8.1	8.1	9.1	13.5	8.9	7.8	8.8	9.2	10.4	8.6	9.6	11.3	7.6	7.1	7.3	9.1	7.4	8.5	8.9	7.0
C4	8.5	7.6	10.1	8.2	8.8	8.2	9.5	12.4	7.9	7.0	8.2	9.6	8.4	8.0	7.7	10.3	6.4	6.9	6.2	9.5
C5	8.1	7.5	9.0	12.1	9.5	8.1	8.5	11.2	9.2	7.3	8.0	6.9	8.5	7.1	6.6	11.3	6.6	7.7	8.0	8.0
C6	7.2	7.1	7.4	9.8	8.5	7.1	8.0	10.8	7.8	6.5	7.5	8.2	6.1	4.7	4.1	6.5	7.7	7.8	8.9	10.5
MED.	*8.3*	*7.9*	*9.3*	*12.4*	*8.9*	*8.2*	*9.0*	*11.4*	*9.2*	*8.0*	*8.9*	*10.4*	*8.1*	*7.6*	*7.5*	*9.7*	*7.3*	*8.2*	*8.5*	*9.7*
MIN.	*7.2*	*7.1*	*7.4*	*8.2*	*8.5*	*7.1*	*8.0*	*9.2*	*7.8*	*6.5*	*7.5*	*6.9*	*6.1*	*4.7*	*4.1*	*5.3*	*6.4*	*6.9*	*6.2*	*7.0*
MAX.	*10.6*	*10.3*	*12.1*	*13.5*	*9.5*	*9.7*	*10.6*	*13.5*	*12.9*	*12.2*	*12.0*	*12.7*	*10.3*	*9.6*	*10.5*	*12.1*	*8.5*	*9.4*	*9.1*	*10.5*
H1	9.3	9.1	10.7	10.3	11.5	11.8	12.7	10.8	9.1	11.0	11.4	10.0	10.7	10.5	9.1	10.9	8.6	7.3	8.6	10.9
H2	6.0	6.3	6.8	8.2	6.8	6.4	6.6	8.0	5.5	4.5	5.6	8.7	3.6	4.0	3.7	7.3	5.5	6.2	6.2	6.1
H3	9.4	8.8	9.0	11.7	9.5	7.6	8.5	10.2	8.4	6.5	6.7	8.4	8.0	6.3	6.2	7.3	9.1	9.0	9.4	11.4
H4	9.2	7.9	9.3	9.1	7.9	7.9	9.1	8.9	7.7	9.0	9.3	11.8	8.8	9.1	7.4	10.1	6.9	7.3	7.2	9.2
H5	10.6	10.5	12.2	11.9	12.1	11.6	10.5	12.4	14.5	14.0	13.2	13.4	14.2	13.5	13.8	19.2	9.7	8.8	9.1	14.6
H6	7.3	6.3	7.2	10.0	6.8	7.5	7.2	13.4	7.0	6.1	6.5	8.2	8.2	6.7	7.3	8.0	7.0	7.5	7.1	7.4
H7	10.5	10.0	11.0	13.5	10.3	10.7	11.1	12.7	10.4	11.3	13.2	12.7	10.0	10.4	10.5	13.5	9.3	9.5	10.4	7.8
MED.	*9.3*	*8.8*	*9.3*	*10.3*	*9.5*	*7.9*	*9.1*	*10.8*	*8.4*	*9.0*	*9.3*	*10.0*	*8.8*	*9.1*	*7.4*	*10.1*	*8.6*	*7.5*	*8.6*	*9.2*
MIN.	*6.0*	*6.3*	*6.8*	*8.2*	*6.8*	*6.4*	*6.6*	*8.0*	*5.5*	*4.5*	*5.6*	*8.2*	*3.6*	*4.0*	*3.7*	*7.3*	*5.5*	*6.2*	*6.2*	*6.1*
MAX.	*10.6*	*10.5*	*12.2*	*13.5*	*12.1*	*11.8*	*12.7*	*13.4*	*14.5*	*14.0*	*13.2*	*13.4*	*14.2*	*13.5*	*13.8*	*19.2*	*9.7*	*9.5*	*10.4*	*14.6*

Appendix 2.10b: Peripheral venous blood neutrophil concentration ($x10^9$/l) in control (C1-6) and heaves (H1-7) horses prior to (0) and at 90, 240 and 1440min following inhalation challenge with saline, 20, 200 and 2000μg LPS, and 5h hay straw challenge (H/S). MED. = median value, MIN. = minimum value, MAX. = maximum value.

	SALINE				20μg LPS				200μg LPS				2000μg LPS				H/S			
TIME PT.	0	90	240	1440	0	90	240	1440	0	90	240	1440	0	90	240	1440	0	90	240	1440
C1	4.3	4.4	6.0	7.2	5.5	5.5	6.6	7.1	5.0	5.3	7.3	6.7	2.8	4.2	2.9	4.1	4.3	4.5	5.0	6.6
C2	5.3	5.7	7.6	6.0	3.2	4.2	3.3	8.8	7.2	6.5	7.0	7.9	6.3	5.8	6.5	6.9	3.9	4.8	4.3	6.5
C3	2.4	4.0	4.0	7.7	4.0	3.2	4.3	5.2	5.2	5.4	4.8	6.7	3.4	2.6	2.6	4.7	3.6	3.7	4.0	3.1
C4	3.7	4.9	4.1	4.3	3.7	3.9	4.0	9.2	2.8	2.6	3.3	4.3	4.0	3.4	4.3	5.7	2.4	2.0	1.6	5.4
C5	4.6	4.1	3.6	10.2	6.1	4.4	5.0	6.8	5.2	4.2	4.4	4.6	4.7	4.1	3.8	5.8	3.9	4.5	4.3	5.8
C6	3.2	4.3	3.1	6.0	3.8	4.1	4.6	7.6	5.6	3.9	4.2	6.0	4.2	2.9	2.1	3.4	3.2	5.1	4.8	7.6
MED.	*4.0*	*4.3*	*4.1*	*6.6*	*3.9*	*4.1*	*4.5*	*7.4*	*5.2*	*4.7*	*4.6*	*6.3*	*4.1*	*3.8*	*3.4*	*5.2*	*3.8*	*4.5*	*4.3*	*6.1*
MIN.	*2.4*	*4.0*	*3.1*	*4.3*	*3.2*	*3.2*	*3.3*	*5.2*	*2.8*	*2.6*	*3.3*	*4.3*	*2.8*	*2.6*	*2.1*	*3.4*	*2.4*	*2.0*	*1.6*	*3.1*
MAX.	*5.3*	*5.7*	*7.6*	*10.2*	*6.1*	*5.5*	*6.6*	*9.2*	*7.2*	*6.5*	*7.3*	*7.9*	*6.3*	*5.8*	*6.5*	*6.9*	*4.3*	*5.1*	*5.0*	*7.6*
H1	6.1	6.5	8.5	6.6	7.9	8.3	7.6	4.6	5.4	7.4	9.1	5.0	9.4	9.0	7.6	8.0	4.5	4.3	4.1	7.7
H2	4.0	3.5	4.1	6.0	4.0	3.9	4.0	5.8	3.7	2.9	3.9	6.8	2.2	3.2	2.5	5.5	3.1	2.8	3.6	4.1
H3	6.3	6.7	6.3	7.8	5.9	5.4	6.4	6.3	4.5	3.6	4.4	6.2	5.5	3.7	4.3	5.0	6.0	5.8	6.9	7.1
H4	5.1	4.8	6.5	6.2	5.5	5.4	5.9	5.6	4.5	5.9	7.0	8.3	5.6	5.7	5.6	7.5	4.3	4.8	5.3	5.8
H5	7.1	7.5	8.1	9.2	7.1	9.2	7.1	9.4	10.7	10.4	9.1	8.7	9.8	10.0	12.3	14.6	5.5	5.6	5.6	11.8
H6	3.9	4.0	4.9	5.6	2.7	4.1	3.2	9.5	3.2	3.4	3.3	5.3	2.4	2.7	3.0	4.4	3.7	3.5	3.3	4.6
H7	6.5	6.0	6.7	9.5	5.6	6.4	6.2	8.8	6.3	7.8	8.7	7.5	5.5	7.9	6.7	9.6	5.0	5.4	6.1	5.1
MED.	*6.1*	*6.0*	*6.5*	*6.6*	*5.6*	*5.4*	*6.2*	*6.3*	*4.5*	*5.9*	*7.0*	*6.8*	*5.5*	*5.7*	*5.6*	*7.5*	*4.5*	*4.8*	*5.3*	*5.8*
MIN.	*3.9*	*3.5*	*4.1*	*5.6*	*2.7*	*3.9*	*3.2*	*4.6*	*3.2*	*2.9*	*3.3*	*5.0*	*2.2*	*2.7*	*2.5*	*4.4*	*3.1*	*2.8*	*3.3*	*4.1*
MAX.	*7.1*	*7.5*	*8.5*	*9.5*	*7.9*	*9.2*	*7.6*	*9.5*	*10.7*	*10.4*	*9.1*	*8.7*	*9.8*	*10.0*	*12.3*	*14.6*	*6.0*	*5.8*	*6.9*	*11.8*

Appendix 3.1: Clinical scores in heaves (H1-5 and 7) horses prior to (0) and at 90, 240 and 1440min following initiation of 5h hay straw challenges H/S A and H/S B. Score based on: a (tracheal auscultation), d (dyspnoea) and e (respiratory rate).

CHALLENGE	H/S A				H/S B			
TIME PT. (min)	0	90	240	1440	0	90	240	1440
H1	0	0	2 d,e	0	0	0	0	0
H2	0	0	0	0	0	0	0	0
H3	0	0	0	0	0	0	0	0
H4	0	0	1 d	0	0	1 d	0	0
H5	0	0	2 d,e	0	0	1 d	2 d,e	0
H7	0	1 e	1 d	1 d	0	0	2 d,e	1 a

Appendix 3.2a (i and ii): Lung function measurements in heaves (H1-5 and 7) horses prior to (0h) and at 5h following initiation of 5h hay/straw challenge H/S A. TP = time point, MED. = median value, MIN. = minimum value, MAX. = maximum value.

(i)

	Cdyn (l/kPa)		dPpl (kPa)		RL_{iso} (kPa/l/sec)		RR (breaths/min.)		VT (l)		Wb' (J/min)		$RL_{E25\%}$ (kPa/l/sec)		$RL_{E50\%}$ (kPa/l/sec)		$RL_{E75\%}$ (kPa/l/sec)		$RL_{I25\%}$ (kPa/l/sec)		$RL_{I50\%}$ (kPa/l/sec)		$RL_{I75\%}$ (kPa/l/sec)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
H1	27.96	25.85	0.58	0.59	0.04	0.04	6.10	7.45	9.01	8.63	12.59	15.30	0.06	0.05	0.04	0.05	0.06	0.05	0.07	0.02	0.06	0.03	0.05	0.03
H2	31.37	28.53	0.43	0.42	0.06	0.04	6.85	7.75	5.18	5.86	7.59	7.82	0.07	0.01	0.06	0.02	0.07	0.04	0.06	0.05	0.05	0.05	0.05	0.05
H3	14.21	11.46	0.51	0.50	0.03	0.06	7.20	8.00	4.73	3.60	7.68	6.23	0.07	0.02	0.03	0.02	0.03	0.04	0.10	0.10	0.11	0.11	0.08	0.12
H4	24.59	36.76	0.40	0.42	0.05	0.04	10.25	11.80	0.00	0.00	11.45	16.12	0.02	0.02	0.04	0.04	0.05	0.05	0.05	0.04	0.05	0.04	0.05	0.04
H5	25.23	30.14	0.58	0.49	0.04	0.04	6.95	8.10	8.94	8.42	14.18	14.713	0.05	0.02	0.05	0.03	0.06	0.04	0.03	0.03	0.03	0.03	0.04	0.04
H7	15.60	23.38	0.54	0.36	0.04	0.04	22.25	14.00	4.19	4.06	25.06	10.26	0.03	0.02	0.04	0.04	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
MED.	*24.91*	*25.85*	*0.53*	*0.42*	*0.04*	*0.04*	*6.95*	*9.78*	*4.96*	*4.06*	*12.02*	*10.26*	*0.05*	*0.02*	*0.04*	*0.04*	*0.06*	*0.05*	*0.05*	*0.05*	*0.05*	*0.05*	*0.05*	*0.05*
MIN.	*14.21*	*11.46*	*0.40*	*0.36*	*0.03*	*0.04*	*6.10*	*7.45*	*0.00*	*0.00*	*7.59*	*6.23*	*0.02*	*0.01*	*0.03*	*0.02*	*0.03*	*0.04*	*0.03*	*0.02*	*0.03*	*0.03*	*0.04*	*0.03*
MAX.	*31.37*	*36.76*	*0.58*	*0.59*	*0.06*	*0.06*	*22.25*	*14.00*	*9.01*	*8.63*	*25.06*	*16.12*	*0.07*	*0.05*	*0.06*	*0.05*	*0.07*	*0.05*	*0.10*	*0.10*	*0.11*	*0.11*	*0.08*	*0.12*

(ii)

	T_E (sec)		T_I (sec)		T_I:T_E		V'_E (l/min)		V'_{Emax} (l/sec)		V'_{Imax} (l/sec)		Wb_{el} (J)		Wb_{res} (J)		Wb_{Eres} (J)		Wb_{Ires} (J)		Wb_{Itot} (J)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
H1	4.61	3.57	5.52	4.13	1.23	1.20	54.88	64.25	3.80	5.57	3.40	3.69	1.50	1.12	2.12	1.99	1.25	1.46	0.87	0.53	2.37	1.64
H2	4.84	4.80	3.82	2.97	0.79	0.62	35.87	45.52	2.48	2.82	2.86	3.62	0.44	0.60	1.08	1.01	0.60	0.46	0.48	0.54	0.92	1.14
H3	3.16	3.82	4.55	3.06	1.99	0.96	41.25	33.35	1.73	1.87	2.64	2.76	1.21	0.68	0.95	0.74	0.25	0.21	0.70	0.53	1.91	1.21
H4	3.25	2.91	2.46	2.25	0.75	0.79	54.83	67.08	2.94	3.78	3.56	4.05	0.67	0.50	1.10	1.37	0.51	0.71	0.59	0.66	1.26	1.16
H5	5.18	4.38	3.21	3.09	0.64	0.722	62.24	68.29	3.16	3.65	3.86	4.33	1.41	1.31	1.93	1.83	1.00	0.78	0.93	1.05	2.34	2.37
H7	1.38	1.97	1.46	2.28	1.05	1.16	91.76	56.46	4.14	2.90	4.34	2.78	0.63	0.38	1.18	0.74	0.53	0.35	0.65	0.39	1.28	0.76
MED.	*3.93*	*3.57*	*3.52*	*2.97*	*0.92*	*0.96*	*54.88*	*60.35*	*3.05*	*2.90*	*3.48*	*3.62*	*0.94*	*0.60*	*1.14*	*1.01*	*0.57*	*0.46*	*0.67*	*0.53*	*1.59*	*1.16*
MIN.	*1.38*	*1.97*	*1.46*	*2.25*	*0.64*	*0.62*	*35.87*	*45.52*	*1.73*	*1.87*	*2.64*	*2.76*	*0.44*	*0.38*	*0.95*	*0.74*	*0.25*	*0.21*	*0.48*	*0.39*	*0.92*	*0.76*
MAX.	*5.18*	*4.80*	*5.52*	*4.13*	*1.99*	*1.20*	*91.76*	*67.08*	*4.14*	*5.57*	*4.34*	*4.05*	*1.50*	*1.12*	*2.12*	*1.99*	*1.25*	*1.46*	*0.93*	*0.66*	*2.37*	*1.64*

Appendix 3.2 (i and ii): Lung function measurements in heaves (H1-5 and 7) horses prior to (0h) and at 5h following initiation of 5h hay/straw challenge H/S B. TP = time point, MED. = median value, MIN. = minimum value, MAX. = maximum value.

(i)

	Cdyn (l/kPa)		dPpl (kPa)		RL_{iso} (kPa/l/sec)		RR (breaths/min.)		VT (l)		Wb' (J/min)		$RL_{E25\%}$ (kPa/l/sec)		$RL_{E50\%}$ (kPa/l/sec)		$RL_{E75\%}$ (kPa/l/sec)		$RL_{I25\%}$ (kPa/l/sec)		$RL_{I50\%}$ (kPa/l/sec)		$RL_{I75\%}$ (kPa/l/sec)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
H1	27.12	26.47	1.02	0.66	0.16	0.06	4.95	7.55	8.14	7.50	22.64	19.24	0.17	0.08	0.13	0.07	0.19	0.08	0.15	0.06	0.15	0.06	0.21	0.07
H2	46.49	20.52	0.43	0.76	0.05	0.09	7.60	9.45	6.03	5.66	9.70	18.75	0.03	0.05	0.05	0.06	0.06	0.09	0.05	0.07	0.05	0.08	0.06	0.08
H3	10.91	14.23	0.69	0.57	0.10	0.07	8.70	5.85	4.98	4.68	11.06	6.15	0.03	0.00	0.03	0.01	0.07	0.06	0.17	0.12	0.22	0.13	0.13	0.11
H4	25.75	31.19	0.43	0.38	0.05	0.03	7.20	9.50	5.70	5.84	8.08	8.58	0.02	0.03	0.04	0.03	0.07	0.05	0.06	0.04	0.06	0.04	0.06	0.03
H5	21.88	20.48	0.51	0.55	0.06	0.07	8.10	6.85	6.84	7.13	13.96	11.91	0.02	0.04	0.04	0.07	0.07	0.07	0.07	0.03	0.08	0.06	0.08	0.07
H7	33.40	21.49	0.55	0.91	0.06	0.14	19.55	14.50	4.17	4.25	28.45	35.06	0.05	0.11	0.06	0.13	0.07	0.14	0.05	0.12	0.06	0.14	0.07	0.16
MED.	*26.44*	*21.00*	*0.53*	*0.62*	*0.06*	*0.07*	*7.85*	*8.50*	*5.86*	*5.75*	*12.51*	*15.33*	*0.03*	*0.05*	*0.05*	*0.06*	*0.07*	*0.07*	*0.06*	*0.07*	*0.07*	*0.07*	*0.08*	*0.08*
MIN.	*10.91*	*14.23*	*0.43*	*0.38*	*0.05*	*0.03*	*4.95*	*5.85*	*4.17*	*4.25*	*8.08*	*6.15*	*0.02*	*0.00*	*0.03*	*0.01*	*0.06*	*0.05*	*0.05*	*0.03*	*0.05*	*0.04*	*0.06*	*0.03*
MAX.	*46.49*	*31.19*	*1.02*	*0.91*	*0.16*	*0.14*	*19.55*	*14.50*	*8.14*	*7.50*	*28.45*	*35.06*	*0.17*	*0.11*	*0.13*	*0.13*	*0.19*	*0.14*	*0.17*	*0.12*	*0.22*	*0.14*	*0.21*	*0.16*

(ii)

	T_E (sec)		T_I (sec)		$T_I:T_E$		V'_E (l/min)		V'_{Emax} (l/sec)		V'_{Imax} (l/sec)		Wb_{el} (J)		Wb_{res} (J)		Wb_{Eres} (J)		Wb_{Ires} (J)		Wb_{tot} (J)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
H1	5.67	4.00	6.18	3.82	1.10	0.99	40.19	56.47	2.13	3.41	1.76	3.00	1.25	1.05	4.38	2.44	2.18	1.26	2.20	1.17	3.45	2.22
H2	4.72	3.72	3.11	2.70	0.66	0.73	45.65	53.25	2.55	3.12	3.01	2.84	0.39	0.79	1.28	2.01	0.61	1.00	0.66	1.01	1.06	1.81
H3	3.88	6.66	3.98	3.49	1.03	0.55	43.03	27.33	2.53	2.00	2.37	2.13	1.18	0.87	1.46	1.02	0.48	0.33	0.98	0.69	2.15	1.56
H4	4.55	3.32	3.65	3.05	0.81	0.92	41.02	55.47	2.52	3.09	2.67	3.36	0.64	0.52	1.10	0.91	0.59	0.56	0.51	0.36	1.16	0.88
H5	4.09	5.19	3.34	3.87	0.82	0.75	55.61	48.86	2.85	2.68	3.52	3.16	1.28	1.37	1.71	1.81	0.60	1.00	1.11	0.80	2.39	2.17
H7	1.57	2.23	1.56	1.93	1.01	0.88	81.68	61.07	3.36	2.31	4.06	2.93	0.32	0.47	1.45	2.46	0.73	1.05	0.72	1.41	1.04	1.88
MED.	*4.32*	*3.86*	*3.49*	*3.27*	*0.91*	*0.81*	*44.34*	*54.36*	*2.54*	*2.88*	*2.84*	*2.96*	*0.91*	*0.83*	*1.45*	*1.91*	*0.61*	*1.00*	*0.85*	*0.91*	*1.65*	*1.85*
MIN.	*1.57*	*2.23*	*1.56*	*1.93*	*0.66*	*0.55*	*40.19*	*27.33*	*2.13*	*2.00*	*1.76*	*2.13*	*0.32*	*0.47*	*1.10*	*0.91*	*0.48*	*0.33*	*0.51*	*0.36*	*1.04*	*0.88*
MAX.	*5.67*	*6.66*	*6.18*	*3.87*	*1.10*	*0.99*	*81.68*	*61.07*	*3.36*	*3.41*	*4.06*	*3.36*	*1.28*	*1.37*	*4.38*	*2.46*	*2.18*	*1.26*	*2.20*	*1.41*	*3.45*	*2.22*

Appendix 3.3: PCCdyn70 values (mg/ml) in heaves (H1-5 and 7) horses at 5h following initiation of 5h hay/straw challenges H/S A and H/S B. MED. = median value, MIN. = minimum value, MAX. = maximum value.

	H/S A	H/S B
H1	4.11	4.98
H2	10.24	2.15
H3	3.99	4.66
H4	13.07	14.04
H5	5.63	9.02
H7	3.88	6.10
MED.	*4.87*	*5.54*
MIN.	*3.88*	*2.15*
MAX.	*13.07*	*14.04*

Appendix 3.4: BALF total nucleated cell (TCC), lymphocyte (L), macrophage (M), neutrophil (N), mast cell (Ma), basiphiloid cell (B), eosinophil (Eo) and epithelial cell (Ep) counts ($x10^5$/ml) in heaves (H1-5 and 7) at 6h following initiation of 5h hay/straw challenges H/S A and H/S B. MED. = median value, MIN. = minimum value, MAX. = maximum value.

	TCC		L		M		N		Ma		B		Eo		Ep	
	H/S A	H/S B	H/S A	H/S B	H/S A	H/S B	H/S A	H/S B	H/S A	H/S B	H/S A	H/S B	H/S A	H/S B	H/S A	H/S B
H1	5.10	6.20	3.34	1.44	0.98	1.44	0.53	3.14	0.25	0.12	0.00	0.04	0.01	0.01	0.00	0.00
H2	4.00	4.20	1.81	0.71	1.68	0.90	0.37	2.47	0.12	0.11	0.02	0.01	0.00	0.00	0.00	0.00
H3	5.00	2.80	2.02	0.60	1.97	1.35	0.58	0.74	0.38	0.10	0.05	0.01	0.01	0.00	0.00	0.00
H4	8.20	7.00	3.06	2.30	4.49	3.16	0.37	1.47	0.28	0.08	0.00	0.00	0.01	0.00	0.00	0.00
H5	9.80	16.20	4.32	4.26	4.66	2.03	0.70	9.83	0.11	0.08	0.01	0.00	0.01	0.00	0.00	0.00
H7	2.10	6.50	0.86	2.32	0.78	2.57	0.34	1.41	0.08	0.10	0.03	0.08	0.01	0.02	0.00	0.00
MED.	*5.05*	*6.35*	*2.54*	*1.87*	*1.82*	*1.73*	*0.45*	*1.97*	*0.18*	*0.10*	*0.01*	*0.01*	*0.01*	*0.00*	*0.00*	*0.00*
MIN	*2.10*	*2.80*	*0.86*	*0.60*	*0.78*	*0.90*	*0.34*	*0.74*	*0.08*	*0.08*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*
MAX	*9.80*	*16.20*	*4.32*	*4.26*	*4.66*	*3.16*	*0.70*	*9.83*	*0.38*	*0.12*	*0.05*	*0.08*	*0.01*	*0.02*	*0.00*	*0.00*

Appendix 4.1: Clinical scores in heaves (H1-5 and 7) horses prior to (0) and at 240min following inhalation challenge with saline, 0.5, 1.6 and 5.0mg *Aspergillus fumigatus* extract (AFE). Score based on: b (thoracic auscultation).

CHALLENGE	SALINE		0.5 mg AFE		1.6 mg AFE		5.0 mg AFE	
TIME PT. (min)	0	240	0	240	0	240	0	240
H1	0	0	0	0	0	0	0	0
H2	0	0	0	0	0	0	0	0
H3	0	0	0	0	0	0	0	0
H4	0	0	0	0	0	0	0	0
H5	0	0	0	0	0	0	0	0
H7	0	0	0	0	0	0	0	1 b

Appendix 4.2a: Arterial blood pH, pCO_2 (mmHg) and pO_2 (mmHg) in heaves (H1-5 and 7) horses prior to (0) and at 240min following inhalation challenge with saline, 0.5, 1.6 and 5.0mg *Aspergillus fumigatus* extract (AFE). MED. = median value, MIN. = minimum value, MAX. = maximum value.

	pH								pCO_2								pO_2							
	SALINE		0.5mg AFE		1.6mg AFE		5.0mg AFE		SALINE		0.5mg AFE		1.6mg AFE		5.0mg AFE		SALINE		0.5mg AFE		1.6mg AFE		5.0mg AFE	
HORSE	0	240	0	240	0	240	0	240	0	240	0	240	0	240	0	240	0	240	0	240	0	240	0	240
H1	7.36	7.38	7.41	7.40	7.38	7.37	7.36	7.35	45.0	40.0	45.6	46.9	41.6	43.0	43.9	48.5	99.0	98.0	94.3	96.4	113.5	110.3	109.9	98.1
H2	7.35	7.36	7.37	7.40	7.36	7.39	7.33	7.35	47.0	46.0	50.9	53.8	48.7	53.1	52.8	54.4	116.0	102.0	102.6	100.8	116.2	99.3	87.8	87.8
H3	7.40	7.41	7.35	7.40	7.38	7.37	7.39	7.39	42.0	37.0	49.5	48.3	52.4	50.9	50.5	47.6	94.0	98.0	107.1	97.2	111.3	99.6	99.5	108.7
H4	7.39	7.35	7.38	7.41	7.41	7.41	7.38	7.38	43.0	45.0	48.1	46.2	48.5	50.3	45.2	47.1	102.0	97.0	101.8	93.0	102.7	101.5	104.5	102.6
H5	7.37	7.37	7.39	7.41	7.37	7.43	7.37	7.39	53.0	43.0	48.4	50.2	51.0	46.2	48.1	46.5	94.0	83.0	100.4	101.0	114.6	104.3	106.3	102.5
H7	7.41	7.43	7.41	7.42	7.38	7.37	7.37	7.40	46.0	45.0	45.6	46.0	41.6	43.0	48.3	50.0	100.0	109.0	113.6	112.1	113.5	110.3	106.7	101.3
MED.	*7.38*	*7.38*	*7.39*	*7.40*	*7.38*	*7.38*	*7.37*	*7.39*	*45.5*	*44.0*	*48.3*	*47.6*	*48.6*	*48.3*	*48.2*	*48.1*	*99.5*	*98.0*	*102.2*	*99.0*	*113.5*	*102.9*	*105.4*	*101.9*
MIN.	*7.35*	*7.35*	*7.35*	*7.40*	*7.36*	*7.37*	*7.33*	*7.35*	*42.0*	*37.0*	*45.6*	*46.0*	*41.6*	*43.0*	*43.9*	*46.5*	*94.0*	*83.0*	*94.3*	*93.0*	*102.7*	*99.3*	*87.8*	*87.8*
MAX.	*7.41*	*7.43*	*7.41*	*7.42*	*7.41*	*7.43*	*7.39*	*7.40*	*53.0*	*46.0*	*50.9*	*53.8*	*52.4*	*53.1*	*52.8*	*54.4*	*116.0*	*109.0*	*113.6*	*112.1*	*116.2*	*110.3*	*109.9*	*108.7*

Appendix 4.3a (i and ii): Lung function measurements in heaves (H1-5 and 7) horses prior to (0h) and at 5h following inhalation challenge with saline. TP = time point, MED. = median value, MIN. = minimum value, MAX. = maximum value.

(i)

	Cdyn (l/kPa)		dPpl (kPa)		RL_{iso} (kPa/l/sec)		RR (breaths/min.)		VT (l)		Wb' (J/min)		$RL_{E25\%}$ (kPa/l/sec)		$RL_{E50\%}$ (kPa/l/sec)		$RL_{E75\%}$ (kPa/l/sec)		$RL_{I25\%}$ (kPa/l/sec)		$RL_{I50\%}$ (kPa/l/sec)		$RL_{I75\%}$ (kPa/l/sec)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
H1	15.09	11.44	0.62	0.82	0.04	0.03	10.40	11.50	6.35	6.40	16.27	16.14	0.05	0.03	0.05	0.03	0.08	0.04	0.07	0.08	0.06	0.06	0.06	0.05
H2	21.95	23.02	0.67	0.75	0.07	0.07	8.80	8.50	6.28	7.71	19.71	22.90	0.04	0.06	0.06	0.06	0.07	0.07	0.08	0.06	0.08	0.06	0.08	0.07
H3	11.44	9.12	0.50	0.95	0.06	0.11	8.5	5.30	3.96	6.57	9.90	14.50	0.09	0.03	0.08	0.00	0.08	0.05	0.09	0.23	0.10	0.33	0.08	0.25
H4	32.99	31.80	0.53	0.49	0.06	0.05	7.10	6.40	7.72	8.74	15.70	13.38	0.07	0.03	0.06	0.04	0.08	0.05	0.08	0.06	0.06	0.06	0.07	0.06
H5	20.73	20.17	1.09	0.66	0.03	0.05	14.95	10.15	11.19	7.52	76.09	22.03	0.04	0.05	0.05	0.06	0.06	0.07	0.01	0.04	0.02	0.05	0.03	0.05
H7	8.56	8.45	0.835	0.87	0.08	0.06	13.4	12.85	4.93	5.40	22.731	21.01	0.06	0.03	0.07	0.03	0.08	0.05	0.10	0.13	0.10	0.10	0.09	0.08
MED.	*20.73*	*15.80*	*0.62*	*0.79*	*0.06*	*0.05*	*9.60*	*9.33*	*6.35*	*7.04*	*16.27*	*18.58*	*0.05*	*0.03*	*0.06*	*0.04*	*0.08*	*0.05*	*0.08*	*0.07*	*0.06*	*0.06*	*0.07*	*0.06*
MIN.	*11.44*	*8.45*	*0.50*	*0.49*	*0.03*	*0.03*	*7.10*	*5.30*	*3.96*	*5.40*	*9.90*	*13.38*	*0.04*	*0.03*	*0.05*	*0.00*	*0.06*	*0.04*	*0.01*	*0.04*	*0.02*	*0.05*	*0.03*	*0.05*
MAX.	*32.99*	*31.80*	*1.09*	*0.95*	*0.07*	*0.11*	*14.95*	*12.85*	*11.19*	*8.74*	*76.09*	*22.90*	*0.09*	*0.06*	*0.08*	*0.06*	*0.08*	*0.07*	*0.09*	*0.23*	*0.10*	*0.33*	*0.08*	*0.25*

(ii)

	T_E (sec)		T_I (sec)		$T_I:T_E$		V'_E (l/min)		V'_{Emax} (l/sec)		V'_{Imax} (l/sec)		Wb_{el} (J)		Wb_{res} (J)		Wb_{Eres} (J)		Wb_{Ires} (J)		Wb_{tot} (J)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
H1	2.78	2.31	3.10	2.68	1.14	1.17	65.79	73.34	4.55	6.27	3.04	3.46	1.36	1.68	1.59	1.36	0.77	0.51	0.82	0.86	2.18	2.54
H2	3.84	3.97	2.91	3.12	0.78	0.79	55.72	65.68	3.46	3.46	3.55	3.59	0.90	1.33	2.19	2.70	1.05	1.21	1.14	1.50	2.04	2.83
H3	2.99	6.57	3.02	4.56	1.20	0.70	39.83	34.77	2.13	2.57	3.55	3.39	1.00	2.30	0.96	2.66	0.27	0.60	0.69	2.06	1.69	4.37
H4	5.06	5.03	3.72	4.16	0.94	1.02	53.25	55.75	2.73	3.21	2.97	4.17	1.01	1.33	2.32	2.06	1.10	0.95	1.22	1.11	2.23	2.45
H5	2.14	3.27	2.06	2.77	0.99	1.00	169.96	74.37	8.95	3.87	7.92	4.44	3.25	1.57	4.74	2.28	2.95	1.24	1.78	1.04	5.04	2.61
H7	2.29	2.24	2.23	2.42	0.98	1.09	65.99	69.32	3.321	3.79	3.102	3.19	1.422	1.75	1.72	1.64	0.63	0.42	1.08	1.21	2.51	2.96
MED.	*2.99*	*3.62*	*3.02*	*2.94*	*0.99*	*1.01*	*60.76*	*67.50*	*3.46*	*3.63*	*3.55*	*3.53*	*1.01*	*1.62*	*2.19*	*2.17*	*1.05*	*0.77*	*1.14*	*1.16*	*2.18*	*2.72*
MIN.	*2.14*	*2.24*	*2.06*	*2.42*	*0.78*	*0.70*	*53.25*	*34.77*	*2.13*	*2.57*	*2.97*	*3.19*	*0.90*	*1.33*	*0.96*	*1.36*	*0.27*	*0.42*	*0.69*	*0.86*	*1.69*	*2.45*
MAX.	*5.06*	*6.57*	*3.72*	*4.56*	*1.20*	*1.17*	*169.96*	*74.37*	*8.95*	*6.27*	*7.92*	*4.44*	*3.25*	*2.30*	*4.74*	*2.70*	*2.95*	*1.24*	*1.78*	*2.06*	*5.04*	*4.37*

Appendix 4.3b (i and ii): Lung function measurements in heaves (H1-5 and 7) horses prior to (0h) and at 5h following inhalation challenge with 0.5mg *Aspergillus fumigatus* extract (AFE). TP = time point, MED. = median value, MIN. = minimum value, MAX. = maximum value.

(i)

	Cdyn (l/kPa)		dPpl (kPa)		RL_{iso} (kPa/l/sec)		RR (breaths/min.)		VT (l)		Wb' (J/min)		$RL_{E25\%}$ (kPa/l/sec)		$RL_{E50\%}$ (kPa/l/sec)		$RL_{E75\%}$ (kPa/l/sec)		$RL_{I25\%}$ (kPa/l/sec)		$RL_{I50\%}$ (kPa/l/sec)		$RL_{I75\%}$ (kPa/l/sec)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
H1	22.44	17.02	0.52	0.72	0.05	0.06	7.50	5.50	6.01	8.15	11.78	13.25	0.06	0.10	0.07	0.10	0.07	0.07	0.07	0.114	0.06	0.091	0.07	0.09
H2	50.77	38.90	0.58	0.53	0.04	0.05	7.20	8.10	6.97	5.66	12.28	9.58	0.03	0.03	0.05	0.04	0.08	0.05	0.06	0.40	0.06	0.39	0.06	0.45
H3	13.47	14.30	0.73	0.52	0.10	0.04	6.25	3.90	5.91	4.28	10.09	8.75	0.07	0.06	0.03	0.07	0.07	0.14	0.12	0.12	0.16	0.138	0.11	0.13
H4	29.11	37.02	0.44	0.40	0.04	0.03	9.65	10.70	6.27	5.94	12.06	10.84	0.01	0.02	0.03	0.04	0.05	0.05	0.04	0.04	0.04	0.03	0.04	0.04
H5	32.06	37.14	0.39	0.39	0.04	0.04	7.15	6.75	7.48	6.83	8.56	8.24	0.03	0.04	0.05	0.04	0.06	0.05	0.04	0.05	0.05	0.05	0.05	0.05
H7	15.67	30.36	0.61	0.70	0.06	0.07	13.40	22.30	5.23	4.38	19.75	47.29	0.05	0.07	0.05	0.07	0.06	0.08	0.06	0.06	0.06	0.07	0.07	0.08
MED.	*25.78*	*37.08*	*0.55*	*0.47*	*0.05*	*0.05*	*7.35*	*9.40*	*6.14*	*5.80*	*11.92*	*10.21*	*0.04*	*0.03*	*0.05*	*0.04*	*0.06*	*0.05*	*0.06*	*0.06*	*0.06*	*0.06*	*0.06*	*0.06*
MIN.	*13.47*	*30.36*	*0.39*	*0.39*	*0.04*	*0.03*	*6.25*	*6.75*	*5.23*	*4.38*	*8.56*	*8.24*	*0.01*	*0.02*	*0.03*	*0.04*	*0.05*	*0.05*	*0.04*	*0.04*	*0.04*	*0.03*	*0.04*	*0.04*
MAX.	*50.77*	*38.90*	*0.73*	*0.70*	*0.10*	*0.07*	*13.40*	*22.30*	*7.48*	*6.83*	*19.75*	*47.29*	*0.07*	*0.07*	*0.07*	*0.07*	*0.08*	*0.08*	*0.12*	*0.40*	*0.16*	*0.39*	*0.11*	*0.45*

(ii)

	T_E (sec)		T_I (sec)		$T_I:T_E$		V'_E (l/min)		V'_{Emax} (l/sec)		V'_{Imax} (l/sec)		Wb_{el} (J)		Wb_{res} (J)		Wb_{Eres} (J)		Wb_{Ires} (J)		Wb_{tot} (J)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
H1	3.50	5.37	4.15	5.36	1.25	0.10	44.90	44.69	3.49	3.6	2.66	2.97	0.87	1.936	1.48	2.368	0.81	1.258	0.67	1.11	1.54	3.047
H2	4.71	4.40	3.56	3.19	0.76	0.71	50.31	45.63	2.77	2.55	3.07	2.94	0.88	0.44	1.69	1.72	0.84	1.14	0.85	0.58	1.74	1.02
H3	5.53	3.62	4.58	4.52	0.85	1.25	36.89	16.88	2.49	1.79	3.15	3.14	1.35	1.44	1.69	1.19	0.71	0.10	0.98	1.09	2.33	2.53
H4	3.36	3.04	2.83	2.80	0.86	0.94	60.75	63.59	3.52	3.59	3.87	3.51	0.67	0.51	1.22	1.07	0.67	0.55	0.55	0.52	1.22	1.03
H5	5.00	5.53	3.57	3.33	0.72	0.63	53.09	46.10	2.78	2.20	3.27	3.56	0.90	0.68	1.22	1.20	0.46	0.38	0.76	0.82	1.66	1.50
H7	2.27	1.31	2.30	1.43	1.02	1.10	69.74	97.70	3.06	4.24	3.11	3.96	0.92	0.32	1.52	2.16	0.71	1.18	0.81	0.98	1.73	1.30
MED.	*4.11*	*3.72*	*3.56*	*3.00*	*0.85*	*0.82*	*51.70*	*54.84*	*2.92*	*3.07*	*3.13*	*3.54*	*0.89*	*0.47*	*1.50*	*1.46*	*0.71*	*0.84*	*0.79*	*0.70*	*1.70*	*1.16*
MIN.	*2.27*	*1.31*	*2.30*	*1.43*	*0.72*	*0.63*	*36.89*	*45.63*	*2.49*	*2.20*	*2.66*	*2.94*	*0.67*	*0.32*	*1.22*	*1.07*	*0.46*	*0.38*	*0.55*	*0.52*	*1.22*	*1.02*
MAX.	*5.53*	*5.53*	*4.58*	*3.33*	*1.25*	*1.10*	*69.74*	*97.70*	*3.52*	*4.24*	*3.87*	*3.96*	*1.35*	*0.68*	*1.69*	*2.16*	*0.84*	*1.18*	*0.98*	*0.98*	*2.33*	*1.50*

Appendix 4.3c (i and ii): Lung function measurements in heaves (H1-5 and 7) horses prior to (0h) and at 5h following inhalation challenge with 1.6mg *Aspergillus fumigatus* extract (AFE). TP = time point, MED. = median value, MIN. = minimum value, MAX. = maximum value.

(i)

	Cdyn (l/kPa)		dPpl (kPa)		RL_{iso} (kPa/l/sec)		RR (breaths/min.)		VT (l)		Wb' (J/min)		$RL_{E25\%}$ (kPa/l/sec)		$RL_{E50\%}$ (kPa/l/sec)		$RL_{E75\%}$ (kPa/l/sec)		$RL_{I25\%}$ (kPa/l/sec)		$RL_{I50\%}$ (kPa/l/sec)		$RL_{I75\%}$ (kPa/l/sec)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
H1	19.69	17.10	0.68	0.94	0.05	0.10	6.60	5.55	7.87	8.24	14.38	19.36	0.06	0.13	0.02	0.08	0.06	0.13	0.11	0.13	0.11	0.12	0.08	0.12
H2	35.84	33.67	0.56	0.60	0.05	0.08	8.65	7.70	6.81	6.69	14.45	15.89	0.03	0.06	0.04	0.06	0.06	0.08	0.04	0.07	0.04	0.07	0.06	0.08
H3	14.10	8.07	0.62	0.68	0.07	0.15	6.65	3.95	4.88	3.66	10.68	10.62	0.06	0.09	0.03	0.12	0.06	0.11	0.15	0.19	0.17	0.31	0.12	0.25
H4	24.40	23.55	0.60	0.62	0.07	0.06	6.10	9.30	6.98	7.23	12.02	15.52	0.01	0.04	0.06	0.08	0.08	0.08	0.08	0.09	0.09	0.06	0.10	0.08
H5	22.81	31.44	0.53	0.38	0.04	0.03	9.20	8.5	6.97	6.15	12.31	7.75	0.02	0.03	0.04	0.03	0.05	0.05	0.03	0.03	0.04	0.03	0.06	0.05
H7	16.33	15.81	0.61	0.71	0.06	0.08	12.75	10.05	4.98	5.10	17.95	16.66	0.04	0.04	0.03	0.05	0.06	0.09	0.08	0.10	0.08	0.10	0.07	0.09
MED.	*21.25*	*20.32*	*0.61*	*0.65*	*0.05*	*0.08*	*7.65*	*7.70*	*6.89*	*6.42*	*13.34*	*15.71*	*0.03*	*0.05*	*0.03*	*0.07*	*0.06*	*0.09*	*0.08*	*0.09*	*0.08*	*0.09*	*0.08*	*0.09*
MIN.	*14.10*	*8.07*	*0.53*	*0.38*	*0.04*	*0.03*	*6.10*	*3.95*	*4.88*	*3.66*	*10.68*	*7.75*	*0.01*	*0.03*	*0.02*	*0.03*	*0.05*	*0.05*	*0.03*	*0.03*	*0.04*	*0.03*	*0.06*	*0.05*
MAX.	*35.84*	*33.67*	*0.68*	*0.94*	*0.07*	*0.15*	*12.75*	*10.05*	*7.87*	*8.24*	*17.95*	*19.36*	*0.06*	*0.13*	*0.06*	*0.12*	*0.08*	*0.13*	*0.15*	*0.19*	*0.17*	*0.31*	*0.12*	*0.25*

(ii)

	T_E (sec)		T_I (sec)		T_I:T_E		V'_E (l/min)		V'_{Emax} (l/sec)		V'_{Imax} (l/sec)		Wb_{el} (J)		Wb_{res} (J)		Wb_{Eres} (J)		Wb_{Ires} (J)		Wb_{tot} (J)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
H1	4.19	4.92	4.88	5.67	1.17	1.16	51.74	45.82	3.69	2.81	3.22	2.46	1.53	1.98	2.18	3.42	1.09	1.93	1.09	1.49	2.62	3.47
H2	4.01	4.73	2.93	3.24	0.73	0.69	59.13	51.43	3.44	2.88	3.29	2.98	0.67	0.68	1.67	2.09	0.80	0.95	0.87	1.13	1.54	1.81
H3	4.19	2.40	4.10	5.14	1.25	2.15	32.75	14.40	2.38	1.87	3.41	2.65	1.14	1.70	1.47	1.33	0.35	0.12	1.12	1.21	2.26	2.91
H4	5.07	4.26	4.55	4.22	0.90	1.16	42.38	64.20	2.92	3.28	3.40	3.98	0.98	1.44	1.93	2.21	1.06	0.87	0.87	1.33	1.85	2.77
H5	3.76	4.06	2.95	2.79	0.82	0.69	63.72	52.73	3.73	2.83	4.03	3.58	1.15	0.78	1.37	0.92	0.47	0.33	0.91	0.59	2.06	1.37
H7	2.28	2.90	2.33	3.01	1.02	1.05	63.33	51.41	3.19	2.67	3.02	2.63	0.75	0.85	1.38	1.64	0.51	0.77	0.87	0.87	1.62	1.72
MED.	*4.10*	*4.16*	*3.53*	*3.73*	*0.96*	*1.11*	*55.44*	*51.41*	*3.32*	*2.82*	*3.34*	*2.82*	*1.06*	*1.14*	*1.57*	*1.86*	*0.65*	*0.82*	*0.89*	*1.17*	*1.95*	*2.29*
MIN.	*2.28*	*2.40*	*2.33*	*2.79*	*0.73*	*0.69*	*32.75*	*14.40*	*2.38*	*1.87*	*3.02*	*2.46*	*0.67*	*0.68*	*1.37*	*0.92*	*0.35*	*0.12*	*0.87*	*0.59*	*1.54*	*1.37*
MAX.	*5.07*	*4.92*	*4.88*	*5.67*	*1.25*	*2.15*	*63.72*	*64.20*	*3.73*	*3.28*	*4.03*	*3.98*	*1.53*	*1.98*	*2.18*	*3.42*	*1.09*	*1.93*	*1.12*	*1.49*	*2.62*	*3.47*

Appendix 4.3d (i and ii): Lung function measurements in heaves (H1-5 and 7) horses prior to (0h) and at 5h following inhalation challenge with 5.0mg *Aspergillus fumigatus* extract (AFE). TP = time point, MED. = median value, MIN. = minimum value, MAX. = maximum value.

(i)

	Cdyn (l/kPa)		dPpl (kPa)		RL_{iso} (kPa/l/sec)		RR (breaths/min.)		VT (l)		Wb' (J/min)		$RL_{E25\%}$ (kPa/l/sec)		$RL_{E50\%}$ (kPa/l/sec)		$RL_{E75\%}$ (kPa/l/sec)		$RL_{I25\%}$ (kPa/l/sec)		$RL_{I50\%}$ (kPa/l/sec)		$RL_{I75\%}$ (kPa/l/sec)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
H1	17.79	16.53	0.58	0.71	0.05	0.06	7.60	7.40	7.00	8.02	13.38	17.69	0.06	0.07	0.06	0.08	0.04	0.07	0.09	0.09	0.07	0.06	0.07	0.05
H2	33.11	33.32	0.33	0.57	0.04	0.07	10.15	6.80	5.56	7.40	7.21	13.91	0.01	0.07	0.02	0.06	0.03	0.08	0.03	0.05	0.04	0.06	0.04	0.06
H3	11.96	11.95	0.88	0.74	0.07	0.11	6.70	4.55	6.22	6.57	11.10	9.54	0.03	0.05	0.00	0.00	0.05	0.06	0.17	0.21	0.18	0.26	0.12	0.16
H4	44.04	29.11	0.28	0.43	0.03	0.04	13.40	11.85	4.83	5.28	9.29	10.87	0.02	0.02	0.03	0.03	0.04	0.03	0.03	0.04	0.03	0.04	0.03	0.04
H5	26.56	28.02	0.47	0.49	0.05	0.04	8.70	8.40	6.34	6.88	11.01	12.08	0.03	0.04	0.05	0.05	0.06	0.06	0.04	0.03	0.04	0.04	0.06	0.05
H7	14.48	17.56	0.58	0.70	0.06	0.07	14.30	11.50	4.26	5.93	16.70	24.56	0.04	0.07	0.04	0.07	0.06	0.08	0.08	0.07	0.08	0.07	0.07	0.08
MED.	*22.17*	*22.79*	*0.53*	*0.64*	*0.05*	*0.06*	*9.43*	*7.90*	*5.89*	*6.72*	*11.06*	*13.00*	*0.03*	*0.06*	*0.03*	*0.06*	*0.05*	*0.06*	*0.06*	*0.06*	*0.06*	*0.06*	*0.06*	*0.06*
MIN.	*11.96*	*11.95*	*0.28*	*0.43*	*0.03*	*0.04*	*6.70*	*4.55*	*4.26*	*5.28*	*7.21*	*9.54*	*0.01*	*0.02*	*0.00*	*0.00*	*0.03*	*0.03*	*0.03*	*0.03*	*0.03*	*0.04*	*0.03*	*0.04*
MAX.	*44.04*	*33.32*	*0.88*	*0.74*	*0.07*	*0.11*	*14.30*	*11.85*	*7.00*	*8.02*	*16.70*	*24.56*	*0.06*	*0.07*	*0.06*	*0.08*	*0.06*	*0.08*	*0.17*	*0.21*	*0.18*	*0.26*	*0.12*	*0.16*

(ii)

	T_E (sec)		T_I (sec)		$T_I:T_E$		V'_E (l/min)		V'_{Emax} (l/sec)		V'_{Imax} (l/sec)		Wb_{el} (J)		Wb_{res} (J)		Wb_{Eres} (J)		Wb_{Ires} (J)		Wb_{Itot} (J)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
H1	3.94	3.77	3.95	4.50	1.02	1.27	52.88	59.55	4.56	5.23	3.10	3.49	1.38	1.95	1.76	2.36	0.73	1.26	1.03	1.10	2.41	3.04
H2	3.39	5.25	2.56	3.33	0.76	0.74	56.31	50.08	3.04	3.00	3.64	3.20	0.48	0.87	0.71	1.98	0.29	0.91	0.42	1.08	0.90	1.95
H3	5.04	7.88	4.27	5.40	0.94	0.69	42.49	29.78	2.72	2.11	3.22	2.86	1.57	1.82	1.62	2.09	0.61	0.57	1.01	1.52	2.58	3.34
H4	2.50	2.82	2.20	2.32	0.88	0.82	61.84	61.77	2.95	3.03	3.68	3.73	0.29	0.51	0.70	0.91	0.32	0.42	0.38	0.49	0.67	1.00
H5	3.72	4.04	3.17	3.20	0.86	0.80	55.07	57.75	2.78	2.92	3.46	3.81	0.82	0.89	1.28	1.45	0.51	0.53	0.76	0.92	1.58	1.81
H7	2.07	2.48	2.19	2.80	1.06	1.14	60.35	68.06	2.70	3.53	2.90	3.16	0.82	1.06	1.23	2.11	0.41	1.08	0.82	1.03	1.64	2.09
MED.	*3.55*	*3.90*	*2.86*	*3.26*	*0.91*	*0.81*	*55.69*	*58.65*	*2.87*	*3.02*	*3.34*	*3.35*	*0.82*	*0.98*	*1.25*	*2.03*	*0.46*	*0.74*	*0.79*	*1.05*	*1.61*	*2.02*
MIN.	*2.07*	*2.48*	*2.19*	*2.32*	*0.76*	*0.69*	*42.49*	*29.78*	*2.70*	*2.11*	*2.90*	*2.86*	*0.29*	*0.51*	*0.70*	*0.91*	*0.29*	*0.42*	*0.38*	*0.49*	*0.67*	*1.00*
MAX.	*5.04*	*7.88*	*4.27*	*5.40*	*1.06*	*1.27*	*61.84*	*68.06*	*4.56*	*5.23*	*3.68*	*3.81*	*1.57*	*1.95*	*1.76*	*2.36*	*0.73*	*1.26*	*1.03*	*1.52*	*2.58*	*3.34*

Appendix 4.4: PCCdyn70 values (mg/ml) in heaves (H1-5 and 7) horses at 5h following inhalation challenge with saline 0.5, 1.6 and 5.0mg *Aspergillus fumigatus* extract (AFE). MED. = median value, MIN. = minimum value, MAX. = maximum value.

	SALINE	0.5mg AFE	1.6mg AFE	5mg AFE
H1	7.86	7.58	5.97	5.72
H2	3.06	9.88	5.77	5.45
H3	5.64	6.10	0.72	4.18
H4	10.53	24.57	9.57	11.02
H5	6.40	7.01	4.53	5.49
H7	5.63	2.45	3.09	7.19
MED.	*6.02*	*7.30*	*5.15*	*5.60*
MIN.	*3.06*	*2.45*	*0.72*	*4.18*
MAX.	*10.53*	*24.57*	*9.57*	*11.02*

Appendix 4.5: BALF total nucleated cell (TCC), lymphocyte, macrophage, neutrophil, mast cell, basiphiloid cell, eosinophil and epithelial cell counts ($x10^5$/ml) in heaves (H1-5 and 7) at 6h following inhalation challenge with saline, 0.5, 1.6 and 5.0mg *Aspergillus fumigatus* extract (AFE). MED. = median value, MIN. = minimum value, MAX. = maximum value.

TCC

	SALINE	0.5mg AFE	1.6mg AFE	5.0mg AFE
H1	5.60	3.50	2.70	6.60
H2	3.40	2.30	3.90	2.00
H3	3.80	1.50	3.30	4.50
H4	5.50	3.40	6.00	4.40
H5	3.20	5.10	3.80	4.20
H7	5.20	3.60	9.40	4.60
MED.	*4.50*	*3.45*	*3.85*	*4.45*
MIN	*3.20*	*1.50*	*2.70*	*2.00*
MAX	*5.60*	*5.10*	*9.40*	*6.60*

Lymphocytes

	SALINE	0.5mg AFE	1.6mg AFE	5.0mg AFE
H1	3.33	1.68	0.85	2.38
H2	1.54	1.18	1.02	0.74
H3	2.09	0.36	0.56	1.48
H4	2.36	1.38	2.44	2.23
H5	1.64	2.01	1.41	0.89
H7	2.57	1.38	6.04	2.32
MED.	*2.22*	*1.38*	*1.21*	*1.86*
MIN	*1.54*	*0.36*	*0.56*	*0.74*
MAX	*3.33*	*2.01*	*6.04*	*2.38*

Macrophages

	SALINE	0.5mg AFE	1.6mg AFE	5.0mg AFE
H1	1.96	1.55	0.93	2.69
H2	1.58	0.85	0.72	0.33
H3	1.24	0.86	1.63	1.67
H4	2.96	1.90	2.78	1.56
H5	1.27	2.56	1.50	1.40
H7	2.40	2.10	2.21	1.87
MED.	*1.77*	*1.72*	*1.57*	*1.62*
MIN	*1.24*	*0.85*	*0.72*	*0.33*
MAX	*2.96*	*2.56*	*2.78*	*2.69*

Neutrophils

	SALINE	0.5mg AFE	1.6mg AFE	5.0mg AFE
H1	0.20	0.15	0.86	1.39
H2	0.08	0.21	2.10	0.89
H3	0.17	0.24	1.03	1.26
H4	0.03	0.07	0.68	0.55
H5	0.06	0.48	0.85	1.83
H7	0.06	0.08	1.08	0.36
MED.	*0.07*	*0.18*	*0.94*	*1.08*
MIN	*0.03*	*0.07*	*0.68*	*0.36*
MAX	*0.20*	*0.48*	*2.10*	*1.83*

Mast cells

	SALINE	0.5mg AFE	1.6mg AFE	5.0mg AFE
H1	0.11	0.11	0.06	0.14
H2	0.15	0.05	0.04	0.02
H3	0.19	0.04	0.08	0.08
H4	0.13	0.05	0.10	0.04
H5	0.09	0.03	0.04	0.05
H7	0.17	0.03	0.05	0.04
MED.	*0.14*	*0.04*	*0.05*	*0.05*
MIN	*0.09*	*0.03*	*0.04*	*0.02*
MAX	*0.19*	*0.11*	*0.10*	*0.14*

Basiphiloid cells

	SALINE	0.5mg AFE	1.6mg AFE	5.0mg AFE
H1	0.01	0.00	0.00	0.00
H2	0.05	0.00	0.02	0.01
H3	0.11	0.00	0.00	0.00
H4	0.01	0.00	0.00	0.00
H5	0.02	0.01	0.00	0.01
H7	0.01	0.02	0.01	0.00
MED.	*0.01*	*0.00*	*0.00*	*0.00*
MIN	*0.01*	*0.00*	*0.00*	*0.00*
MAX	*0.11*	*0.02*	*0.02*	*0.01*

Eosinophils

	SALINE	0.5mg AFE	1.6mg AFE	5.0mg AFE
H1	0.00	0.00	0.00	0.00
H2	0.00	0.01	0.00	0.00
H3	0.01	0.00	0.00	0.01
H4	0.01	0.00	0.01	0.02
H5	0.01	0.02	0.00	0.02
H7	0.00	0.00	0.01	0.01
MED.	*0.00*	*0.00*	*0.00*	*0.01*
MIN	*0.00*	*0.00*	*0.00*	*0.00*
MAX	*0.01*	*0.02*	*0.01*	*0.02*

Epithelial cells

	SALINE	0.5mg AFE	1.6mg AFE	5.0mg AFE
H1	0.00	0.00	0.00	0.00
H2	0.00	0.00	0.00	0.00
H3	0.00	0.00	0.00	0.00
H4	0.00	0.00	0.00	0.00
H5	0.10	0.00	0.00	0.00
H7	0.00	0.00	0.00	0.00
MED.	*0.00*	*0.00*	*0.00*	*0.00*
MIN	*0.00*	*0.00*	*0.00*	*0.00*
MAX	*0.10*	*0.00*	*0.00*	*0.00*

Appendix 5.1: Clinical scores in heaves (H1-5 and 7) horses prior to (0) and at 240min following inhalation challenge with saline, 1.6mg *Aspergillus fumigatus* extract (AFE) and 1.6mg LPS depleted *Aspergillus fumigatus* extract (AFE-LPS).

CHALLENGE	SALINE		1.6 mg AFE		1.6 mg AFE-LPS	
TIME PT. (min)	0	240	0	240	0	240
H1	0	0	0	0	0	0
H2	0	0	0	0	0	0
H3	0	0	0	0	0	0
H4	0	0	0	0	0	0
H5	0	0	0	0	0	0
H7	0	0	0	0	0	0

Appendix 5.2: Arterial blood pH, pCO_2 (mmHg) and pO_2 (mmHg) in heaves (H1-5 and 7) horses prior to (0) and at 240min following inhalation challenge with saline, 1.6mg *Aspergillus fumigatus* extract (AFE) and 1.6mg LPS-depleted *Aspergillus fumigatus* extract (AFE-LPS). MED. = median value, MIN. = minimum value, MAX. = maximum value.

	pH						pCO_2						pO_2					
PH	SALINE		AFE		AFE-LPS		SALINE		AFE		AFE-LPS		SALINE		FC1.6		FC-LPS	
HORSE	0	240	0	240	0	240	0.0	240.0	0.0	240.0	0.0	240.0	0	240	0	240	0	240
H1	7.36	7.38	7.38	7.37	7.38	7.39	45.0	40.0	41.6	43.0	44.6	48.0	99.0	98.0	113.5	110.3	101.4	102.4
H2	7.35	7.36	7.36	7.39	7.39	7.36	47.0	46.0	48.7	53.1	54.9	51.8	116.0	102.0	116.2	99.3	96.8	100.9
H3	7.40	7.41	7.38	7.37	7.39	7.37	42.0	37.0	52.4	50.9	50.0	49.4	94.0	98.0	111.3	99.6	89.3	103.6
H4	7.39	7.35	7.41	7.41	7.41	7.43	43.0	45.0	48.5	50.3	49.4	47.2	102.0	97.0	102.7	101.5	98.3	103.2
H5	7.37	7.37	7.37	7.43	7.39	7.40	53.0	43.0	51.0	46.2	51.6	49.2	94.0	83.0	114.6	104.3	100.2	101.6
H7	7.41	7.43	7.38	7.37	7.37	7.39	46.0	45.0	41.6	43.0	43.3	43.8	100.0	109.0	113.5	110.3	104.4	111.0
MED.	*7.38*	*7.38*	*7.38*	*7.38*	*7.39*	*7.39*	*45.5*	*44.0*	*48.6*	*48.3*	*49.7*	*48.6*	*99.5*	*98.0*	*113.5*	*102.9*	*99.3*	*102.8*
MIN.	*7.35*	*7.35*	*7.36*	*7.37*	*7.37*	*7.36*	*42.0*	*37.0*	*41.6*	*43.0*	*43.3*	*43.8*	*94.0*	*83.0*	*102.7*	*99.3*	*89.3*	*100.9*
MAX.	*7.41*	*7.43*	*7.41*	*7.43*	*7.41*	*7.43*	*53.0*	*46.0*	*52.4*	*53.1*	*54.9*	*51.8*	*116.0*	*109.0*	*116.2*	*110.3*	*104.4*	*111.0*

Appendix 5.3: Lung function measurements in heaves (H1-5 and 7) horses prior to (0h) and at 5h following inhalation challenge with 1.6mg LPS-depleted *Aspergillus fumigatus* extract (AFE-LPS). MED. = median value, MIN. = minimum value, MAX. = maximum value.

	Cdyn (l/kPa)		dPpl (kPa)		RL_{iso} (kPa/l/sec)		RR (breaths/min.)		VT (l)		Wb' (J/min)		$RL_{E25\%}$ (kPa/l/sec)		$RL_{E50\%}$ (kPa/l/sec)		$RL_{E75\%}$ (kPa/l/sec)		$RL_{I25\%}$ (kPa/l/sec)		$RL_{I50\%}$ (kPa/l/sec)		$RL_{I75\%}$ (kPa/l/sec)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
H1	24.16	18.04	0.65	0.71	0.06	0.08	7.45	5.35	7.54	8.23	16.14	12.57	0.07	0.10	0.06	0.06	0.08	0.08	0.06	0.11	0.05	0.09	0.06	0.09
H2	84.83	28.72	0.48	0.92	0.05	0.12	9.40	6.10	6.28	8.01	15.59	22.98	0.05	0.10	0.07	0.11	0.07	0.14	0.04	0.12	0.04	0.09	0.05	0.12
H3	13.53	14.44	0.84	0.82	0.17	0.16	4.60	4.70	5.84	6.50	11.15	11.76	0.01	0.04	0.09	0.05	0.17	0.15	0.24	0.20	0.31	0.25	0.25	0.18
H4	25.29	31.66	0.57	0.54	0.07	0.07	6.10	7.05	7.03	6.22	14.90	14.84	0.03	0.03	0.06	0.06	0.08	0.08	0.08	0.09	0.09	0.09	0.09	0.10
H5	22.22	26.65	0.70	0.65	0.05	0.05	5.60	6.60	9.58	9.44	17.19	18.84	0.08	0.06	0.10	0.08	0.10	0.10	0.07	0.06	0.09	0.06	0.07	0.06
H7	20.81	33.33	0.54	0.81	0.06	0.09	10.85	10.50	5.40	5.59	15.70	24.77	0.07	0.09	0.06	0.10	0.06	0.13	0.06	0.09	0.05	0.09	0.06	0.10
MED.	*23.19*	*27.69*	*0.61*	*0.76*	*0.06*	*0.08*	*6.78*	*6.35*	*6.66*	*7.25*	*15.64*	*16.84*	*0.06*	*0.07*	*0.06*	*0.07*	*0.08*	*0.11*	*0.07*	*0.10*	*0.07*	*0.09*	*0.07*	*0.10*
MIN.	*13.53*	*14.44*	*0.48*	*0.54*	*0.05*	*0.05*	*4.60*	*4.70*	*5.40*	*5.59*	*11.15*	*11.76*	*0.01*	*0.03*	*0.06*	*0.05*	*0.06*	*0.08*	*0.04*	*0.06*	*0.04*	*0.06*	*0.05*	*0.06*
MAX.	*84.83*	*33.33*	*0.84*	*0.92*	*0.17*	*0.16*	*10.85*	*10.50*	*9.58*	*9.44*	*17.19*	*24.77*	*0.08*	*0.10*	*0.10*	*0.11*	*0.17*	*0.15*	*0.24*	*0.20*	*0.31*	*0.25*	*0.25*	*0.18*

	T_E (sec)		T_I (sec)		T_I:T_E		V'_E (l/min)		V'_{Emax} (l/sec)		V'_{Imax} (l/sec)		Wb_{el} (J)		Wb_{res} (J)		Wb_{Eres} (J)		Wb_{Ires} (J)		Wb_{Itot} (J)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
H1	3.66	5.77	4.54	5.57	1.29	0.97	55.91	43.78	3.92	3.02	3.49	3.08	1.24	1.90	2.18	2.36	1.28	1.26	0.90	1.10	2.14	3.00
H2	3.29	5.43	2.90	4.31	1.02	0.80	59.01	49.02	3.17	2.97	3.46	2.76	0.29	1.17	1.63	3.75	0.86	2.09	0.76	1.65	1.05	2.83
H3	6.64	6.79	6.55	5.96	1.00	0.89	26.88	30.36	1.96	2.17	2.09	2.54	1.25	1.50	2.42	2.50	1.16	1.10	1.27	1.41	2.52	2.91
H4	4.90	4.35	4.22	3.78	1.93	1.09	43.02	43.77	2.70	2.44	3.33	3.60	1.04	0.77	2.21	1.96	1.17	0.86	1.04	1.11	2.08	1.88
H5	6.49	5.55	4.17	3.61	0.66	0.65	53.56	62.67	3.46	3.58	4.06	4.27	2.13	1.80	2.92	2.82	1.08	1.12	1.84	1.70	3.97	3.50
H7	2.73	2.80	2.76	3.04	1.02	1.09	58.65	58.73	3.05	3.16	2.86	2.90	0.71	0.47	1.42	2.40	0.76	1.37	0.66	1.03	1.38	1.50
MED.	*4.28*	*5.49*	*4.19*	*4.04*	*1.02*	*0.93*	*54.73*	*46.40*	*3.11*	*2.99*	*3.40*	*2.99*	*1.14*	*1.34*	*2.20*	*2.45*	*1.12*	*1.19*	*0.97*	*1.26*	*2.11*	*2.87*
MIN.	*2.73*	*2.80*	*2.76*	*3.04*	*0.66*	*0.65*	*26.88*	*30.36*	*1.96*	*2.17*	*2.09*	*2.54*	*0.29*	*0.47*	*1.42*	*1.96*	*0.76*	*0.86*	*0.66*	*1.03*	*1.05*	*1.50*
MAX.	*6.64*	*6.79*	*6.55*	*5.96*	*1.93*	*1.09*	*59.01*	*62.67*	*3.92*	*3.58*	*4.06*	*4.27*	*2.13*	*1.90*	*2.92*	*3.75*	*1.28*	*2.09*	*1.84*	*1.70*	*3.97*	*3.50*

Appendix 5.4: PCCdyn70 values (mg/ml) in heaves (H1-5 and 7) horses at 5h following inhalation challenge with saline, 1.6mg *Aspergillus fumigatus* extract (AFE) and 1.6mg LPS-depleted *Aspergillus fumigatus* extract (AFE-LPS). MED. = median value, MIN. = minimum value, MAX. = maximum value.

	SALINE	AFE	AFE-LPS
H1	7.86	5.97	7.66
H2	3.06	5.77	8.12
H3	5.64	0.72	4.42
H4	10.53	9.57	11.24
H5	6.40	4.53	10.33
H7	5.63	3.09	4.67
MED.	*6.02*	*5.15*	*7.89*
MIN.	*3.06*	*0.72*	*4.42*
MAX.	*10.53*	*9.57*	*11.24*

Appendix 5.5: BALF total nucleated cell (TCC), lymphocyte, macrophage, neutrophil, mast cell, basiphiloid cell, eosinophil and epithelial cell counts ($x10^5$/ml) in heaves (H1-5 and 7) at 6h following inhalation challenge with saline, 1.6mg *Aspergillus fumigatus* extract (AFE) and 1.6mg LPS-depleted *Aspergillus fumigatus* extract (AFE-LPS). MED. = median value, MIN. = minimum value, MAX. = maximum value.

TCC

	SALINE	AFE	AFE-LPS
H1	5.60	2.70	4.20
H2	3.40	3.90	4.00
H3	3.80	3.30	3.10
H4	5.50	6.00	5.60
H5	3.20	3.80	2.70
H7	5.20	9.40	3.70
MED.	*4.50*	*3.85*	*3.85*
MIN	*3.20*	*2.70*	*2.70*
MAX	*5.60*	*9.40*	*5.60*

Lymphocytes

	SALINE	AFE	AFE-LPS
H1	3.33	0.85	1.33
H2	1.54	1.02	1.27
H3	2.09	0.56	1.09
H4	2.36	2.44	2.37
H5	1.64	1.41	1.13
H7	2.57	6.04	1.72
MED.	*2.22*	*1.21*	*1.30*
MIN	*1.54*	*0.56*	*1.09*
MAX	*3.33*	*6.04*	*2.37*

Macrophages

	SALINE	AFE	AFE-LPS
H1	1.96	0.93	2.00
H2	1.58	0.72	0.76
H3	1.24	1.63	1.75
H4	2.96	2.78	2.61
H5	1.27	1.50	1.13
H7	2.40	2.21	1.42
MED.	*1.77*	*1.57*	*1.58*
MIN	*1.24*	*0.72*	*0.76*
MAX	*2.96*	*2.78*	*2.61*

Neutrophils

	SALINE	AFE	AFE-LPS
H1	0.20	0.86	0.76
H2	0.08	2.10	1.90
H3	0.17	1.03	0.16
H4	0.03	0.68	0.46
H5	0.06	0.85	0.38
H7	0.06	1.08	0.43
MED.	*0.07*	*0.94*	*0.44*
MIN	*0.03*	*0.68*	*0.16*
MAX	*0.20*	*2.10*	*1.90*

Mast cells

	SALINE	AFE	AFE-LPS
H1	0.11	0.06	0.11
H2	0.15	0.04	0.06
H3	0.19	0.08	0.11
H4	0.13	0.10	0.15
H5	0.09	0.04	0.05
H7	0.17	0.05	0.07
MED.	*0.14*	*0.05*	*0.09*
MIN	*0.09*	*0.04*	*0.05*
MAX	*0.19*	*0.10*	*0.15*

Basophiloid cells

	SALINE	AFE	AFE-LPS
H1	0.01	0.00	0.00
H2	0.05	0.02	0.00
H3	0.11	0.00	0.00
H4	0.01	0.00	0.02
H5	0.02	0.00	0.01
H7	0.01	0.01	0.06
MED.	*0.01*	*0.00*	*0.01*
MIN	*0.01*	*0.00*	*0.00*
MAX	*0.11*	*0.02*	*0.06*

Eosinophils

	SALINE	AFE	AFE-LPS
H1	0.00	0.01	0.01
H2	0.00	0.00	0.00
H3	0.01	0.00	0.00
H4	0.01	0.01	0.00
H5	0.01	0.00	0.01
H7	0.00	0.01	0.00
MED.	*0.00*	*0.00*	*0.00*
MIN	*0.00*	*0.00*	*0.00*
MAX	*0.01*	*0.01*	*0.01*

Epithelial cells

	SALINE	AFE	AFE-LPS
H1	0.00	0.00	0.00
H2	0.00	0.00	0.00
H3	0.00	0.00	0.00
H4	0.00	0.00	0.00
H5	0.10	0.00	0.00
H7	0.00	0.00	0.00
MED.	*0.00*	*0.00*	*0.00*
MIN	*0.00*	*0.00*	*0.00*
MAX	*0.10*	*0.00*	*0.00*

Appendix 7.1: Clinical scores in control (C1-6) and heaves (H1-7) horses prior to (0) and at 240min following inhalation challenge with saline and 3 separate doses of HDS-1 (HDS-1 [31], HDS-1 [57], HDS-1 [100] and HDS-1 [316]). Score based on: a (tracheal auscultation), d (dyspnoea) and e (respiratory rate). NP = challenge not performed, NM = clinical score not measured.

CHALLENGE	SALINE		HDS-1 [31]		HDS-1 [57]		HDS-1 [100]		HDS-1 [316]	
TIME PT. (min)	0	240	0	240	0	240	0	240	0	240
C1	0	0	NP	NP	0	0	0	0	NM	NM
C2	0	0	NP	NP	0	0	0	0	NM	NM
C3	0	0	NP	NP	1 e	0	1 e	0	NM	NM
C4	0	0	NP	NP	0	0	0	0	NM	NM
C5	0	0	NP	NP	0	0	0	0	NM	NM
C6	0	0	NP	NP	0	0	0	0	NM	NM
H1	0	0	0	0	0	0	0	0	NP	NP
H2	0	0	0	0	0	0	0	1 d	NP	NP
H3	0	0	0	0	1 a	0	0	0	NP	NP
H4	0	0	0	0	0	0	0	0	NP	NP
H5	0	0	0	0	0	0	0	0	NP	NP
H6	0	0	0	0	0	0	0	0	NP	NP
H7	0	0	0	0	0	0	0	0	NP	NP

Appendix 7.2: (a) BALF total nucleated cell counts (x10^5/ml) in control (C1-6) and heaves (H1-7) at 6h following challenge with saline, HDS-1[31], HDS-1[57], HDS-1[100], HDS-1[316], HDS-2, HDS-2[R] and HDS-3. MED. = median value, MIN. = minimum value, MAX. = maximum value.

	SALINE	HDS-1 [31]	HDS-1 [57]	HDS-1 [100]	HDS-1 [316]	HDS-2	HDS-2[R]	HDS-3
C1	5.10	NP	3.00	3.00	4.90	4.30	NP	NP
C2	3.90	NP	3.90	4.30	0.00	2.60	NP	NP
C3	2.00	NP	2.30	1.60	2.20	1.70	NP	NP
C4	2.90	NP	1.80	8.00	4.30	3.20	NP	NP
C5	9.60	NP	4.10	5.00	6.90	3.70	NP	NP
C6	3.80	NP	3.80	5.10	4.40	2.50	NP	NP
MED.	*3.85*		*3.40*	*4.65*	*4.35*	*2.90*		
MIN	*2.00*		*1.80*	*1.60*	*0.00*	*1.70*		
MAX	*9.60*		*4.10*	*8.00*	*6.90*	*4.30*		
H1	5.60	3.80	7.70	4.00	NP	4.30	7.30	9.10
H2	3.40	3.40	2.90	3.70	NP	7.90	8.10	8.90
H3	3.80	3.30	4.90	6.20	NP	8.70	6.30	8.10
H4	5.50	3.00	7.60	4.10	NP	7.30	4.00	15.20
H5	3.20	8.00	8.80	7.00	NP	9.10	21.80	12.60
H6	1.30	3.20	2.00	2.10	NP	3.70	0.00	10.10
H7	5.20	4.50	5.10	4.10	NP	8.40	11.10	19.00
MED.	*3.80*	*3.40*	*5.10*	*4.10*		*7.90*	*7.30*	*10.10*
MIN	*1.30*	*3.00*	*2.00*	*2.10*		*3.70*	*0.00*	*8.10*
MAX	*5.60*	*8.00*	*8.80*	*7.00*		*9.10*	*21.80*	*19.00*

Appendix 7.2: (b) BALF lymphocyte counts (x10^5/ml) in control (C1-6) and heaves (H1-7) at 6h following challenge with saline, HDS-1[31], HDS-1[57], HDS-1[100], HDS-1[316], HDS-2, HDS-2[R] and HDS-3. MED. = median value. MIN. = minimum value. MAX. = maximum value.

	SALINE	HDS-1 [31]	HDS-1 [57]	HDS-1 [100]	HDS-1 [316]	HDS-2	HDS-2[R]	HDS-3
C1	1.89	ND	1.04	0.66	1.32	2.37	NP	NP
C2	1.28	ND	1.15	1.24		0.92	NP	NP
C3	0.99	ND	1.31	0.83	0.85	1.07	NP	NP
C4	0.99	ND	0.61	1.74	1.42	1.13	NP	NP
C5	6.22	ND	2.06	2.29	1.79	1.51	NP	NP
C6	2.41	ND	1.02	2.23	0.62	0.96	NP	NP
MED.	*1.59*		*1.10*	*1.49*	*1.32*	*1.10*		
MIN	*0.99*		*0.61*	*0.66*	*0.62*	*0.92*		
MAX	*6.22*		*2.06*	*2.29*	*1.79*	*2.37*		
H1	3.33	1.80	3.60	1.62	NP	1.64	1.41	0.98
H2	1.54	1.45	0.95	0.61	NP	0.56	0.49	0.63
H3	2.09	1.03	2.12	1.19	NP	0.56	0.59	0.36
H4	2.36	1.23	4.23	1.75	NP	2.34	1.16	2.14
H5	1.64	2.52	2.55	1.86	NP	2.20	2.07	1.17
H6	0.80	2.10	1.04	0.81	NP	0.51	NP	1.19
H7	2.57	2.20	1.71	1.12	NP	1.53	2.24	1.86
MED.	*2.09*	*1.80*	*2.12*	*1.19*		*1.53*	*1.28*	*1.17*
MIN	*0.80*	*1.03*	*0.95*	*0.61*		*0.51*	*0.49*	*0.36*
MAX	*3.33*	*2.52*	*4.23*	*1.86*		*2.34*	*2.24*	*2.14*

Appendix 7.2: (c) BALF macrophage counts (x10^5/ml) in control (C1-6) and heaves (H1-7) at 6h following challenge with saline, HDS-1[31], HDS-1[57], HDS-1[100], HDS-1[316], HDS-2, HDS-2[R] and HDS-3. MED. = median value, MIN. = minimum value, MAX. = maximum value.

	SALINE	HDS-1 [31]	HDS-1 [57]	HDS-1 [100]	HDS-1 [316]	HDS-2	HDS-2[R]	HDS-3
C1	2.80	NP	1.76	1.72	1.43	1.43	NP	NP
C2	2.25	NP	2.55	2.75		1.46	NP	NP
C3	0.73	NP	0.78	0.51	0.37	0.30	NP	NP
C4	1.71	NP	0.77	4.19	0.94	1.62	NP	NP
C5	2.57	NP	1.80	1.91	0.76	1.39	NP	NP
C6	1.14	NP	2.33	2.46	0.63	1.20	NP	NP
MED.	*1.98*		*1.78*	*2.18*	*0.76*	*1.41*		
MIN	*0.73*		*0.77*	*0.51*	*0.37*	*0.30*		
MAX	*2.80*		*2.55*	*4.19*	*1.43*	*1.62*		
H1	1.96	1.77	2.80	1.16	NP	0.84	1.90	0.66
H2	1.58	1.09	0.65	0.41	NP	0.71	0.83	0.53
H3	1.24	1.15	1.68	1.75	NP	1.22	0.88	0.72
H4	2.96	1.63	2.55	1.48	NP	1.78	1.59	1.93
H5	1.27	3.06	2.80	1.18	NP	1.66	2.27	0.92
H6	0.34	0.90	0.33	0.50	NP	0.39		0.24
H7	2.40	1.90	1.26	0.77	NP	1.19	1.78	0.78
MED.	*1.58*	*1.63*	*1.68*	*1.16*		*1.19*	*1.68*	*0.72*
MIN	*0.34*	*0.90*	*0.33*	*0.41*		*0.39*	*0.83*	*0.24*
MAX	*2.96*	*3.06*	*2.80*	*1.75*		*1.78*	*2.27*	*1.93*

Appendix 7.2: (d) BALF neutrophil counts (x10^5/ml) in control (C1-6) and heaves (H1-7) at 6h following challenge with saline, HDS-1[31], HDS-1[57], HDS-1[100], HDS-1[316], HDS-2, HDS-2[R] and HDS-3. MED. = median value, MIN. = minimum value, MAX. = maximum value.

	SALINE	HDS-1 [31]	HDS-1 [57]	HDS-1 [100]	HDS-1 [316]	HDS-2	HDS-2[R]	HDS-3
C1	0.04	NP	0.01	0.29	1.70	0.14	NP	NP
C2	0.01	NP	0.06	0.12		0.02	NP	NP
C3	0.02	NP	0.02	0.08	0.90	0.17	NP	NP
C4	0.09	NP	0.05	0.56	1.72	0.16	NP	NP
C5	0.17	NP	0.05	0.66	4.28	0.41	NP	NP
C6	0.09	NP	0.24	0.28	3.12	0.19	NP	NP
MED.	*0.06*		*0.05*	*0.28*	*1.72*	*0.16*		
MIN	*0.01*		*0.01*	*0.08*	*0.90*	*0.02*		
MAX	*0.17*		*0.24*	*0.66*	*4.28*	*0.41*		
H1	0.20	0.12	0.43	1.11	NP	1.68	3.80	7.37
H2	0.08	0.81	1.23	2.60	NP	6.47	6.73	7.65
H3	0.17	1.02	1.01	3.14	NP	6.61	4.73	6.92
H4	0.03	0.08	0.57	0.80	NP	3.12	1.13	10.84
H5	0.06	2.30	3.32	3.81	NP	4.95	17.29	10.47
H6	0.06	0.06	0.50	0.54	NP	2.71		8.55
H7	0.06	0.14	2.02	2.17	NP	5.52	6.87	16.30
MED.	*0.06*	*0.14*	*1.01*	*2.17*		*4.95*	*5.73*	*8.55*
MIN	*0.03*	*0.06*	*0.43*	*0.54*		*1.68*	*1.13*	*6.92*
MAX	*0.20*	*2.30*	*3.32*	*3.81*		*6.61*	*17.29*	*16.30*

Appendix 7.2: (e) BALF mast cell counts (x10^5/ml) in control (C1-6) and heaves (H1-7) at 6h following challenge with saline, HDS-1[31], HDS-1[57], HDS-1[100], HDS-1[316], HDS-2, HDS-2[R] and HDS-3. MED. = median value, MIN. = minimum value, MAX. = maximum value.

	SALINE	HDS-1 [31]	HDS-1 [57]	HDS-1 [100]	HDS-1 [316]	HDS-2	HDS-2[R]	HDS-3
C1	0.23	NP	0.19	0.13	0.18	0.23	NP	NP
C2	0.30	NP	0.12	0.18		0.12	NP	NP
C3	0.26	NP	0.16	0.12	0.08	0.15	NP	NP
C4	0.06	NP	0.05	0.24	0.09	0.06	NP	NP
C5	0.39	NP	0.18	0.15	0.08	0.33	NP	NP
C6	0.09	NP	0.08	0.11	0.03	0.13	NP	NP
MED.	*0.25*		*0.14*	*0.14*	*0.08*	*0.14*		
MIN	*0.06*		*0.05*	*0.11*	*0.03*	*0.06*		
MAX	*0.39*		*0.19*	*0.24*	*0.18*	*0.33*		
H1	0.11	0.07	0.30	0.08	NP	0.13	0.11	0.06
H2	0.15	0.04	0.06	0.07	NP	0.10	0.06	0.06
H3	0.19	0.08	0.09	0.11	NP	0.29	0.10	0.09
H4	0.13	0.05	0.08	0.05	NP	0.04	0.05	0.08
H5	0.09	0.06	0.02	0.02	NP	0.04	0.00	0.00
H6	0.10	0.12	0.12	0.14	NP	0.06		0.09
H7	0.17	0.16	0.06	0.02	NP	0.08	0.12	0.06
MED.	*0.13*	*0.07*	*0.08*	*0.07*		*0.08*	*0.08*	*0.06*
MIN	*0.09*	*0.04*	*0.02*	*0.02*		*0.04*	*0.00*	*0.00*
MAX	*0.19*	*0.16*	*0.30*	*0.14*		*0.29*	*0.12*	*0.09*

Appendix 7.2: (f) BALF basiphiloid cell counts (x10^5/ml) in control (C1-6) and heaves (H1-7) at 6h following challenge with saline, HDS-1[31], HDS-1[57], HDS-1[100], HDS-1[316], HDS-2, HDS-2[R] and HDS-3. MED. = median value, MIN. = minimum value, MAX. = maximum value.

	SALINE	HDS-1 [31]	HDS-1 [57]	HDS-1 [100]	HDS-1 [316]	HDS-2	HDS-2[R]	HDS-3
C1	0.07	NP	0.00	0.20	0.24	0.09	NP	NP
C2	0.01	NP	0.00	0.00		0.08	NP	NP
C3	0.00	NP	0.03	0.03	0.01	0.00	NP	NP
C4	0.03	NP	0.03	1.04	0.00	0.08	NP	NP
C5	0.22	NP	0.02	0.00	0.00	0.01	NP	NP
C6	0.01	NP	0.00	0.00	0.00	0.00	NP	NP
MED.	*0.02*		*0.01*	*0.02*	*0.00*	*0.04*		
MIN	*0.00*		*0.00*	*0.00*	*0.00*	*0.00*		
MAX	*0.22*		*0.03*	*1.04*	*0.24*	*0.09*		
H1	0.01	0.00	0.00	0.00	NP	0.01	0.07	0.00
H2	0.05	0.01	0.01	0.00	NP	0.06	0.00	0.02
H3	0.11	0.02	0.00	0.00	NP	0.03	0.00	0.02
H4	0.01	0.02	0.02	0.00	NP	0.00	0.00	0.00
H5	0.02	0.00	0.02	0.00	NP	0.00	0.02	0.03
H6	0.00	0.02	0.00	0.10	NP	0.02		0.00
H7	0.01	0.01	0.02	0.00	NP	0.03	0.04	0.00
MED.	*0.01*	*0.01*	*0.01*	*0.00*		*0.02*	*0.01*	*0.00*
MIN	*0.00*	*0.00*	*0.00*	*0.00*		*0.00*	*0.00*	*0.00*
MAX	*0.11*	*0.02*	*0.02*	*0.10*		*0.06*	*0.07*	*0.03*

Appendix 7.2: (g) BALF eosinophil counts ($x10^5$/ml) in control (C1-6) and heaves (H1-7) at 6h following challenge with saline, HDS-1[31], HDS-1[57], HDS-1[100], HDS-1[316], HDS-2, HDS-2[R] and HDS-3. MED. = median value, MIN. = minimum value, MAX. = maximum value.

	SALINE	HDS-1 [31]	HDS-1 [57]	HDS-1 [100]	HDS-1 [316]	HDS-2	HDS-2[R]	HDS-3
C1	0.05	NP	0.00	0.00	0.04	0.05	NP	NP
C2	0.06	NP	0.02	0.01		0.00	NP	NP
C3	0.00	NP	0.00	0.04	0.00	0.01	NP	NP
C4	0.00	NP	0.29	0.23	0.14	0.15	NP	NP
C5	0.02	NP	0.00	0.00	0.00	0.06	NP	NP
C6	0.06	NP	0.14	0.02	0.00	0.03	NP	NP
MED.	*0.03*		*0.01*	*0.01*	*0.00*	*0.04*		
MIN	*0.00*		*0.00*	*0.00*	*0.00*	*0.00*		
MAX	*0.06*		*0.29*	*0.23*	*0.14*	*0.15*		
H1	0.00	0.03	0.57	0.03	NP	0.00	0.01	0.02
H2	0.00	0.00	0.00	0.00	NP	0.00	0.00	0.00
H3	0.01	0.00	0.00	0.00	NP	0.00	0.01	0.00
H4	0.01	0.00	0.14	0.01	NP	0.02	0.07	0.21
H5	0.01	0.07	0.10	0.13	NP	0.25	0.15	0.01
H6	0.01	0.00	0.01	0.01	NP	0.00		0.02
H7	0.00	0.10	0.02	0.02	NP	0.05	0.04	0.00
MED.	*0.01*	*0.00*	*0.02*	*0.01*		*0.00*	*0.03*	*0.01*
MIN	*0.00*	*0.00*	*0.00*	*0.00*		*0.00*	*0.00*	*0.00*
MAX	*0.01*	*0.10*	*0.57*	*0.13*		*0.25*	*0.15*	*0.21*

Appendix 7.2: (h) BALF epithelial cell counts ($x10^5$/ml) in control (C1-6) and heaves (H1-7) at 6h following challenge with saline, HDS-1[31], HDS-1[57], HDS-1[100], HDS-1[316], HDS-2, HDS-2[R] and HDS-3. MED. = median value, MIN. = minimum value, MAX. = maximum value.

	SALINE	HDS-1 [31]	HDS-1 [57]	HDS-1 [100]	HDS-1 [316]	HDS-2	HDS-2[R]	HDS-3
C1	0.03	NP	0.00	0.00	0.00	0.00	NP	NP
C2	0.00	NP	0.00	0.00		0.00	NP	NP
C3	0.00	NP	0.00	0.00	0.00	0.00	NP	NP
C4	0.00	NP	0.00	0.00	0.00	0.00	NP	NP
C5	0.00	NP	0.00	0.00	0.00	0.00	NP	NP
C6	0.00	NP	0.00	0.00	0.00	0.00	NP	NP
MED.	*0.00*		*0.00*	*0.00*	*0.00*	*0.00*		
MIN	*0.00*		*0.00*	*0.00*	*0.00*	*0.00*		
MAX	*0.03*		*0.00*	*0.00*	*0.00*	*0.00*		
H1	0.00	0.00	0.00	0.00	NP	0.00	0.00	0.00
H2	0.00	0.00	0.00	0.00	NP	0.00	0.00	0.00
H3	0.00	0.00	0.00	0.00	NP	0.00	0.00	0.00
H4	0.00	0.00	0.00	0.00	NP	0.00	0.00	0.00
H5	0.10	0.00	0.00	0.00	NP	0.00	0.00	0.00
H6	0.00	0.00	0.00	0.00	NP	0.00		0.00
H7	0.00	0.00	0.00	0.00	NP	0.00	0.00	0.00
MED.	*0.00*	*0.00*	*0.00*	*0.00*		*0.00*	*0.00*	*0.00*
MIN	*0.00*	*0.00*	*0.00*	*0.00*		*0.00*	*0.00*	*0.00*
MAX	*0.10*	*0.00*	*0.00*	*0.00*		*0.00*	*0.00*	*0.00*

Appendix 7.3: Clinical scores in control (C1-6) and heaves (H1-7) horses prior to (0) and at 240min following inhalation challenge with saline and 3 separate batches of HDS (HDS-1 [100], HDS-2 [100] and HDS-3 [100]) and a repeat inhalation challenge with HDS-2 [100] (HDS-2 [100]R). Score based on: a (tracheal auscultation), d (dyspnoea), e (respiratory rate) and f (mucopurulent nasal discharge). NP = challenge not performed, NM = clinical score not measured.

CHALLENGE	SALINE		HDS-1 [100]		HDS-2 [100]		HDS-2 [100]R		HDS-3 [100]	
TIME PT. (min)	0	240	0	240	0	240	0	240	0	240
C1	0	0	0	0	0	0	NP	NP	NP	NP
C2	0	0	0	0	0	0	NP	NP	NP	NP
C3	0	0	0	0	0	0	NP	NP	NP	NP
C4	0	0	0	0	0	0	NP	NP	NP	NP
C5	0	0	0	0	0	0	NP	NP	NP	NP
C6	0	0	0	0	0	0	NP	NP	NP	NP
H1	0	0	0	0	0	0	NM	NM	0	0
H2	0	0	0	1 d	0	1 e	NM	NM	0	1 a
H3	0	0	0	0	0	0	NM	NM	0	0
H4	0	0	0	0	0	0	NM	NM	0	0
H5	0	0	0	0	0	1 f	NM	NM	0	0
H6	0	0	0	0	0	0	NP	NP	0	0
H7	0	0	0	0	1 e	1 e	NM	NM	0	0

Appendix 7.4: Arterial blood pH, pCO_2 (mmHg) and pO_2 (mmHg) in control (C1-6) and heaves (H1-7) horses prior to (0) and at 240min following inhalation challenge with saline, HDS-1[100], HDS-2[100] and HDS-3[100]. MED. = median value, MIN. = minimum value, MAX. = maximum value.

	PH								pCO_2								pO_2							
	SALINE		HDS-1[100]		HDS-2 [100]		HDS-3 [100]		SALINE		HDS-1[100]		HDS-2 [100]		HDS-3 [100]		SALINE		HDS-1[100]		HDS-2 [100]		HDS-3 [100]	
TIME PT.	0	240	0	240	0	240	0	240	0	240	0.0	240.0	0.0	240.0	0.0	240.0	0	240	0	240	0	240	0	240
C1	7.40	7.39	7.37	7.37	7.40	7.43	ND	ND	46.0	49.0	53.5	48.9	52.2	50.5	ND	ND	100.0	114.0	100.0	97.7	94.7	99.8	ND	ND
C2	7.36	7.35	7.32	7.36	7.40	7.40	ND	ND	45.0	48.0	54.0	56.0	44.9	49.1	ND	ND	103.0	115.0	90.4	88.0	101.3	94.7	ND	ND
C3	7.38	7.38	7.42	7.41	7.41	7.42	ND	ND	46.5	46.6	46.4	46.3	51.2	48.8	ND	ND	106.5	104.6	113.3	103.7	89.6	99.1	ND	ND
C4	7.40	7.42	7.36	7.41	7.42	7.40	ND	ND	50.1	44.7	49.2	49.1	43.7	47.3	ND	ND	90.6	88.0	80.7	74.6	86.4	102.3	ND	ND
C5	7.36	7.36	7.43	7.41	7.39	7.41	ND	ND	41.0	39.0	51.0	48.5	48.2	45.0	ND	ND	105.0	110.0	125.0	118.0	93.4	90.9	ND	ND
C6	7.39	7.39	7.43	7.40	7.40	7.40	ND	ND	40.0	41.0	50.0	43.5	48.0	46.8	ND	ND	93.0	104.0	98.0	109.3	98.9	108.7	ND	ND
MED.	*7.39*	*7.39*	*7.39*	*7.40*	*7.40*	*7.41*			*45.5*	*45.7*	*50.5*	*48.7*	*48.1*	*48.1*			*101.5*	*107.3*	*99.0*	*100.7*	*94.1*	*99.5*		
MIN.	*7.36*	*7.35*	*7.32*	*7.36*	*7.39*	*7.40*			*40.0*	*39.0*	*46.4*	*43.5*	*43.7*	*45.0*			*90.6*	*88.0*	*80.7*	*74.6*	*86.4*	*90.9*		
MAX.	*7.40*	*7.42*	*7.43*	*7.41*	*7.42*	*7.43*			*50.1*	*49.0*	*54.0*	*56.0*	*52.2*	*50.5*			*106.5*	*115.0*	*125.0*	*118.0*	*101.3*	*108.7*		
H1	7.36	7.38	7.34	7.39	7.38	7.35	7.35	7.35	45.0	40.0	45.2	46.7	47.0	45.3	50.2	52.4	99.0	98.0	104.4	121.8	124.3	110.8	118.0	116.2
H2	7.35	7.36	7.34	7.38	7.34	7.33	7.38	7.38	47.0	46.0	48.9	50.8	56.8	53.5	56.8	54.3	116.0	102.0	100.8	101.5	108.0	113.8	112.2	103.5
H3	7.40	7.41	7.41	7.39	7.42	7.38	7.37	7.42	42.0	37.0	50.0	48.2	49.1	52.5	50.2	51.9	94.0	98.0	92.0	117.6	116.7	113.8	121.3	112.6
H4	7.39	7.35	7.42	7.43	7.40	7.37	7.42	7.44	43.0	45.0	43.4	46.0	42.0	46.0	43.2	42.3	102.0	97.0	104.1	124.0	116.4	92.4	100.8	101.7
H5	7.37	7.37	7.37	7.37	7.37	7.41			53.0	43.0	51.0	55.6	51.8	52.5			94.0	83.0	97.0	85.7	90.7	95.6		
H6	7.42	7.36	7.37	7.40	7.33	7.32	7.33	7.36	48.4	47.6	51.3	56.4	66.9	61.1	63.7	55.2	90.5	94.5	98.4	81.4	84.5	99.7	87.1	107.5
H7	7.41	7.43	7.36	7.40	7.35	7.35	7.43	7.34	46.0	45.0	43.0	44.4	53.0	48.1	52.0	48.4	100.0	109.0	109.2	108.0	125.8	118.6	96.8	123.3
MED.	*7.39*	*7.37*	*7.37*	*7.39*	*7.37*	*7.35*	*7.38*	*7.37*	*46.0*	*45.0*	*48.9*	*48.2*	*51.8*	*52.5*	*51.1*	*52.2*	*99.0*	*98.0*	*100.8*	*108.0*	*116.4*	*110.8*	*106.5*	*110.1*
MIN.	*7.35*	*7.35*	*7.34*	*7.37*	*7.33*	*7.32*	*7.33*	*7.34*	*42.0*	*37.0*	*43.0*	*44.4*	*42.0*	*45.3*	*43.2*	*42.3*	*90.5*	*83.0*	*92.0*	*81.4*	*84.5*	*92.4*	*87.1*	*101.7*
MAX.	*7.42*	*7.43*	*7.42*	*7.43*	*7.42*	*7.41*	*7.43*	*7.44*	*53.0*	*47.6*	*51.3*	*56.4*	*66.9*	*61.1*	*63.7*	*55.2*	*116.0*	*109.0*	*109.2*	*124.0*	*125.8*	*118.6*	*121.3*	*123.3*

Appendix 7.5(a): Lung function measurements in control (C1-6) and heaves (H1-7) horses prior to (0h) and at 5h following inhalation challenge with HDS-1[100]. MED. = median value, MIN. = minimum value, MAX. = maximum value.

	Cdyn (l/kPa)		dPpl (kPa)		RL_{iso} (kPa/l/sec)		RR (breaths/min.)		VT (l)		Wb' (J/min)		$RL_{E25\%}$ (kPa/l/sec)		$RL_{E50\%}$ (kPa/l/sec)		$RL_{E75\%}$ (kPa/l/sec)		$RL_{I25\%}$ (kPa/l/sec)		$RL_{I50\%}$ (kPa/l/sec)		$RL_{I75\%}$ (kPa/l/sec)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
C1	4.87	3.98	1.05	1.51	0.12	0.09	9.40	8.40	3.71	4.95	13.03	14.23	0.09	0.03	0.10	0.01	0.11	0.04	0.20	0.28	0.18	0.20	0.16	0.13
C2	11.13	15.45	0.54	0.54	0.04	0.05	8.35	8.55	4.38	4.73	6.21	8.08	0.06	0.05	0.05	0.04	0.03	0.03	0.12	0.15	0.08	0.09	0.07	0.08
C3	14.67	13.51	0.47	0.74	0.04	0.06	15.20	10.15	3.97	6.05	11.77	16.49	0.03	0.06	0.02	0.04	0.03	0.02	0.07	0.10	0.06	0.09	0.06	0.07
C4	5.33	4.97	1.47	1.83	0.35	0.41	7.15	7.15	3.77	3.88	17.84	22.75	0.29	0.39	0.19	0.27	0.28	0.35	0.43	0.47	0.38	0.40	0.31	0.36
C5	18.89	18.88	0.36	0.52	0.05	0.08	10.70	6.40	3.59	4.78	6.38	6.63	0.04	0.09	0.05	0.09	0.05	0.09	0.03	0.09	0.04	0.07	0.05	0.07
C6	16.23	8.63	0.61	0.84	0.08	0.06	8.40	8.25	4.61	4.15	8.76	9.42	0.03	0.04	0.04	0.10	0.07	0.07	0.12	0.18	0.09	0.23	0.09	0.10
MED.	*12.90*	*11.07*	*0.58*	*0.79*	*0.06*	*0.07*	*8.90*	*8.25*	*3.87*	*4.75*	*10.26*	*11.83*	*0.05*	*0.05*	*0.05*	*0.06*	*0.06*	*0.06*	*0.12*	*0.17*	*0.08*	*0.14*	*0.08*	*0.09*
MIN.	*4.87*	*3.98*	*0.36*	*0.52*	*0.04*	*0.05*	*7.15*	*6.40*	*3.59*	*3.88*	*6.21*	*6.63*	*0.03*	*0.03*	*0.02*	*0.01*	*0.03*	*0.02*	*0.03*	*0.09*	*0.04*	*0.07*	*0.05*	*0.07*
MAX.	*18.89*	*18.88*	*1.47*	*1.83*	*0.35*	*0.41*	*15.20*	*10.15*	*4.61*	*6.05*	*17.84*	*22.75*	*0.29*	*0.39*	*0.19*	*0.27*	*0.28*	*0.35*	*0.43*	*0.47*	*0.38*	*0.40*	*0.31*	*0.36*
H1	22.62	25.23	0.62	0.61	0.05	0.07	7.25	5.25	7.81	8.26	14.61	11.59	0.06	0.09	0.06	0.09	0.05	0.10	0.11	0.07	0.07	0.06	0.05	0.08
H2	43.80	46.18	0.34	0.41	0.04	0.05	8.05	7.05	5.34	5.87	6.38	7.58	0.01	0.04	0.04	0.04	0.05	0.06	0.04	0.05	0.04	0.05	0.05	0.06
H3	19.80	16.26	0.40	0.70	0.04	0.09	4.55	4.00	4.26	6.81	5.36	7.90	0.04	0.00	0.02	0.01	0.06	0.08	0.12	0.15	0.13	0.24	0.09	0.14
H4	40.77	28.59	0.28	0.37	0.04	0.06	10.85	9.40	4.19	4.11	6.26	7.22	0.01	0.03	0.04	0.05	0.04	0.07	0.04	0.07	0.04	0.07	0.04	0.06
H5	28.55	27.38	0.50	0.63	0.04	0.04	8.05	8.90	7.02	8.29	11.50	22.56	0.05	0.04	0.04	0.05	0.06	0.05	0.05	0.04	0.03	0.03	0.04	0.06
H6	9.20	9.83	0.91	0.88	0.11	0.09	8.30	8.50	5.12	5.77	16.19	15.79	0.12	0.08	0.11	0.08	0.12	0.09	0.15	0.13	0.10	0.11	0.11	0.08
H7	27.43	38.35	0.57	0.64	0.06	0.07	15.95	10.65	4.72	5.24	25.36	22.74	0.06	0.07	0.06	0.08	0.07	0.10	0.05	0.07	0.06	0.07	0.07	0.08
MED.	*27.43*	*27.38*	*0.50*	*0.63*	*0.04*	*0.07*	*8.05*	*8.50*	*5.12*	*5.87*	*11.50*	*11.59*	*0.05*	*0.04*	*0.04*	*0.05*	*0.06*	*0.08*	*0.05*	*0.07*	*0.06*	*0.07*	*0.05*	*0.08*
MIN.	*9.20*	*9.83*	*0.28*	*0.37*	*0.04*	*0.04*	*4.55*	*4.00*	*4.19*	*4.11*	*5.36*	*7.22*	*0.01*	*0.00*	*0.02*	*0.01*	*0.04*	*0.05*	*0.04*	*0.04*	*0.03*	*0.03*	*0.04*	*0.06*
MAX.	*43.80*	*46.18*	*0.91*	*0.88*	*0.11*	*0.09*	*15.95*	*10.65*	*7.81*	*8.29*	*25.36*	*22.74*	*0.12*	*0.09*	*0.11*	*0.09*	*0.12*	*0.10*	*0.15*	*0.15*	*0.13*	*0.24*	*0.11*	*0.14*

	T_E (sec)		T_I (sec)		T_I:T_E		V'_E (l/min)		V'_{Emax} (l/sec)		V'_{Imax} (l/sec)		Wb_{el} (J)		Wb_{res} (J)		Wb_{Eres} (J)		Wb_{Ires} (J)		Wb_{tot} (J)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
C1	2.94	3.37	3.36	3.44	1.16	1.02	34.55	42.058	2.09	2.61	1.94	3.30	1.37	3.22	1.38	1.60	0.53	0.10	0.85	1.70	2.22	4.92
C2	3.92	3.58	3.31	3.51	0.84	0.99	36.66	40.32	2.58	2.62	2.09	2.18	0.92	0.93	0.74	0.90	0.12	0.23	0.62	0.68	1.54	1.61
C3	2.15	3.38	1.83	2.59	0.86	0.77	60.20	61.04	2.89	3.55	3.38	3.65	0.55	1.42	0.78	1.65	0.21	0.45	0.57	1.21	1.12	2.62
C4	4.61	4.62	3.85	3.71	0.84	0.81	26.89	27.55	1.33	1.30	1.47	1.60	1.36	1.51	2.52	3.14	1.04	1.34	1.48	1.80	2.85	3.31
C5	3.07	5.24	2.63	4.05	0.88	0.78	39.19	30.64	2.44	1.95	2.42	2.17	0.36	0.64	0.54	1.03	0.23	0.44	0.31	0.59	0.68	1.23
C6	4.34	4.10	3.50	3.24	0.86	0.76	38.73	33.01	2.07	1.93	2.55	2.27	0.81	1.09	1.17	1.14	0.42	0.40	0.75	0.75	1.56	1.84
MED.	*3.49*	*3.84*	*3.33*	*3.47*	*0.86*	*0.80*	*37.70*	*33.01*	*2.27*	*2.28*	*2.26*	*2.22*	*0.86*	*1.25*	*0.98*	*1.37*	*0.32*	*0.42*	*0.69*	*0.98*	*1.55*	*2.23*
MIN.	*2.15*	*3.37*	*1.83*	*2.59*	*0.84*	*0.76*	*26.89*	*27.55*	*1.33*	*1.30*	*1.47*	*1.60*	*0.36*	*0.64*	*0.54*	*0.90*	*0.12*	*0.10*	*0.31*	*0.59*	*0.68*	*1.23*
MAX.	*4.61*	*5.24*	*3.85*	*4.05*	*1.16*	*1.02*	*60.20*	*61.04*	*2.89*	*3.55*	*3.38*	*3.65*	*1.37*	*3.22*	*2.52*	*3.14*	*1.04*	*1.34*	*1.48*	*1.80*	*2.85*	*4.92*
H1	3.67	5.38	4.71	6.32	1.29	1.20	56.24	42.96	4.75	2.92	3.32	2.90	1.40	1.41	2.03	2.25	1.02	1.32	1.00	0.93	2.40	2.34
H2	4.11	5.02	3.37	3.56	0.82	0.72	42.94	41.15	2.43	2.44	2.80	2.82	0.33	0.42	0.79	1.09	0.43	0.52	0.37	0.56	0.70	0.98
H3	3.95	8.58	5.66	6.15	1.92	0.72	19.65	27.41	1.70	2.45	2.53	2.74	0.73	1.33	0.84	1.94	0.22	0.84	0.62	1.10	1.35	2.43
H4	2.82	3.38	2.50	3.02	0.89	0.89	44.97	38.02	2.24	2.10	2.76	2.38	0.23	0.32	0.56	0.77	0.27	0.38	0.29	0.39	0.51	0.71
H5	4.08	3.36	3.20	2.88	0.79	0.87	56.43	73.40	3.28	4.74	3.91	5.08	0.89	1.22	1.39	2.28	0.59	0.99	0.80	1.29	1.68	2.51
H6	2.97	3.11	4.27	3.86	1.46	1.25	42.15	48.98	3.00	3.01	2.40	2.89	1.45	1.70	1.94	1.84	1.06	0.92	0.88	0.92	2.33	2.62
H7	1.94	2.55	1.87	3.04	0.99	1.22	74.85	56.93	3.61	3.08	3.69	3.07	0.44	0.44	1.58	1.88	0.79	1.04	0.79	0.84	1.23	1.29
MED.	*3.67*	*3.38*	*3.37*	*3.56*	*0.99*	*0.89*	*44.97*	*42.96*	*3.00*	*2.92*	*2.80*	*2.89*	*0.73*	*1.22*	*1.39*	*1.88*	*0.59*	*0.92*	*0.79*	*0.92*	*1.35*	*2.34*
MIN.	*1.94*	*2.55*	*1.87*	*2.88*	*0.79*	*0.72*	*19.65*	*27.41*	*1.70*	*2.10*	*2.40*	*2.38*	*0.23*	*0.32*	*0.56*	*0.77*	*0.22*	*0.38*	*0.29*	*0.39*	*0.51*	*0.71*
MAX.	*4.11*	*8.58*	*5.66*	*6.32*	*1.92*	*1.25*	*74.85*	*73.40*	*4.75*	*4.74*	*3.91*	*5.08*	*1.45*	*1.70*	*2.03*	*2.28*	*1.06*	*1.32*	*1.00*	*1.29*	*2.40*	*2.62*

Appendix 7.5(b): Lung function measurements in control (C1-6) and heaves (H1-7) horses prior to (0h) and at 5h following inhalation challenge with HDS-2[100]. MED. = median value, MIN. = minimum value, MAX. = maximum value.

	Cdyn (l/kPa)		dPpl (kPa)		RL_{iso} (kPa/l/sec)		RR (breaths/min.)		VT (l)		Wb' (J/min)		$RL_{E25\%}$ (kPa/l/sec)		$RL_{E50\%}$ (kPa/l/sec)		$RL_{E75\%}$ (kPa/l/sec)		$RL_{I25\%}$ (kPa/l/sec)		$RL_{I50\%}$ (kPa/l/sec)		$RL_{I75\%}$ (kPa/l/sec)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
C1	5.52	7.60	0.75	0.83	0.07	0.09	10.1	7.05	3.01	4.26	7.10	7.22	0.04	0.05	0.02	0.03	0.08	0.05	0.16	0.22	0.09	0.13	0.08	0.15
C2	24.18	12.92	0.47	0.69	0.05	0.12	9.80	7.05	5.07	4.81	11.19	10.63	0.05	0.11	0.03	0.11	0.04	0.13	0.07	0.10	0.06	0.12	0.08	0.13
C3	10.25	13.44	0.75	0.63	0.10	0.08	9.60	9.30	4.06	4.63	12.84	11.46	0.12	0.09	0.10	0.08	0.10	0.07	0.13	0.10	0.13	0.09	0.11	0.09
C4	9.67	9.03	0.73	0.92	0.14	0.20	9.75	7.35	3.40	3.76	11.61	11.52	0.09	0.13	0.05	0.06	0.11	0.18	0.20	0.27	0.21	0.26	0.16	0.19
C5	16.34	15.23	0.49	0.57	0.05	0.09	11.35	7.00	4.16	5.03	8.64	7.83	0.04	0.12	0.03	0.05	0.06	0.08	0.04	0.10	0.04	0.10	0.06	0.06
C6	14.10	12.87	0.50	0.54	0.04	0.06	8.90	9.90	4.73	4.83	7.46	12.16	0.03	0.07	0.00	0.07	0.01	0.07	0.11	0.08	0.16	0.08	0.08	0.08
MED.	*12.18*	*12.90*	*0.61*	*0.66*	*0.06*	*0.09*	*9.75*	*7.20*	*4.11*	*4.72*	*9.92*	*11.04*	*0.04*	*0.10*	*0.03*	*0.06*	*0.07*	*0.08*	*0.12*	*0.10*	*0.11*	*0.11*	*0.08*	*0.11*
MIN.	*5.52*	*7.60*	*0.47*	*0.54*	*0.04*	*0.06*	*8.90*	*7.00*	*3.01*	*3.76*	*7.10*	*7.22*	*0.03*	*0.05*	*0.00*	*0.03*	*0.01*	*0.05*	*0.04*	*0.08*	*0.04*	*0.08*	*0.06*	*0.06*
MAX.	*24.18*	*15.23*	*0.75*	*0.92*	*0.14*	*0.20*	*11.35*	*9.90*	*5.07*	*5.03*	*12.84*	*12.16*	*0.12*	*0.13*	*0.10*	*0.11*	*0.11*	*0.18*	*0.20*	*0.27*	*0.21*	*0.26*	*0.16*	*0.19*
H1	27.70	20.54	0.47	0.71	0.02	0.05	10.40	7.05	6.81	8.80	13.46	18.80	0.04	0.08	0.03	0.10	0.04	0.06	0.03	0.06	0.03	0.05	0.05	0.06
H2	76.18	20.94	0.38	1.65	0.03	0.17	10.40	13.85	6.65	5.94	12.89	90.89	0.03	0.16	0.03	0.15	0.04	0.17	0.03	0.16	0.03	0.18	0.04	0.19
H3	13.16	8.76	0.57	0.67	0.05	0.12	5.80	5.50	4.55	3.64	5.27	4.94	0.00	0.06	0.00	0.00	0.04	0.05	0.13	0.21	0.19	0.28	0.17	0.20
H4	36.29	27.00	0.48	0.50	0.04	0.05	11.90	11.70	5.92	5.60	16.01	16.37	0.03	0.03	0.04	0.05	0.05	0.06	0.03	0.04	0.04	0.05	0.05	0.05
H5	25.13	19.55	0.48	0.59	0.03	0.06	11.55	12.75	6.52	5.17	14.90	19.88	0.03	0.04	0.04	0.05	0.04	0.06	0.03	0.05	0.03	0.07	0.04	0.07
H6	11.06	10.73	0.77	0.74	0.10	0.09	10.35	11.40	4.65	4.73	17.67	19.84	0.08	0.09	0.09	0.10	0.10	0.10	0.12	0.08	0.11	0.08	0.10	0.08
H7	24.26	21.84	0.48	0.62	0.05	0.07	12.75	9.65	4.67	5.37	13.92	14.18	0.05	0.05	0.06	0.06	0.07	0.09	0.04	0.08	0.04	0.08	0.06	0.07
MED.	*25.13*	*20.54*	*0.48*	*0.67*	*0.04*	*0.07*	*10.40*	*11.40*	*5.92*	*5.37*	*13.92*	*18.80*	*0.03*	*0.06*	*0.04*	*0.06*	*0.04*	*0.06*	*0.03*	*0.08*	*0.04*	*0.08*	*0.05*	*0.07*
MIN.	*11.06*	*8.76*	*0.38*	*0.50*	*0.02*	*0.05*	*5.80*	*5.50*	*4.55*	*3.64*	*5.27*	*4.94*	*0.00*	*0.03*	*0.00*	*0.00*	*0.04*	*0.05*	*0.03*	*0.04*	*0.03*	*0.05*	*0.04*	*0.05*
MAX.	*76.18*	*27.00*	*0.77*	*1.65*	*0.10*	*0.17*	*12.75*	*13.85*	*6.81*	*8.80*	*17.67*	*90.89*	*0.08*	*0.16*	*0.09*	*0.15*	*0.10*	*0.17*	*0.13*	*0.21*	*0.19*	*0.28*	*0.17*	*0.20*

	T_E (sec)		T_I (sec)		$T_I:T_E$		V'_E (l/min)		V'_{Emax} (l/sec)		V'_{Imax} (l/sec)		Wb_{el} (J)		Wb_{res} (J)		Wb_{Eres} (J)		Wb_{Ires} (J)		Wb_{itot} (J)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
C1	2.53	4.12	2.50	4.27	0.97	1.07	40.93	29.98	1.84	1.70	2.45	1.91	0.83	1.22	0.62	1.01	0.14	0.24	0.48	0.78	1.31	1.99
C2	3.52	4.98	2.57	3.50	0.77	0.73	49.54	33.86	2.74	1.61	2.93	1.93	0.55	0.94	1.11	1.48	0.31	0.53	0.81	0.95	1.35	1.89
C3	3.09	3.42	3.12	3.07	1.02	0.90	38.97	42.93	2.41	2.31	1.96	2.55	0.84	0.80	1.33	1.24	0.64	0.53	0.68	0.71	1.53	1.51
C4	3.66	4.69	2.72	3.53	0.77	0.76	32.87	27.60	1.47	1.43	2.02	1.73	0.61	0.80	1.21	1.57	0.39	0.63	0.83	0.94	1.44	1.74
C5	3.10	4.97	2.16	3.41	0.70	0.69	46.84	35.13	2.81	1.82	2.83	2.75	0.54	0.82	0.75	1.13	0.28	0.47	0.47	0.65	1.01	1.47
C6	3.83	3.06	2.95	2.96	0.77	0.99	42.14	47.71	3.23	3.98	2.92	2.99	0.79	0.92	0.84	1.21	0.03	0.49	0.81	0.72	1.60	1.64
MED.	*3.31*	*4.41*	*2.64*	*3.45*	*0.77*	*0.83*	*42.14*	*34.49*	*2.57*	*1.76*	*2.64*	*2.24*	*0.70*	*0.87*	*0.98*	*1.23*	*0.29*	*0.51*	*0.74*	*0.75*	*1.40*	*1.69*
MIN.	*2.53*	*3.06*	*2.16*	*2.96*	*0.70*	*0.69*	*32.87*	*27.60*	*1.47*	*1.43*	*1.96*	*1.73*	*0.54*	*0.80*	*0.62*	*1.01*	*0.03*	*0.24*	*0.47*	*0.65*	*1.01*	*1.47*
MAX.	*3.83*	*4.98*	*3.12*	*4.27*	*1.02*	*1.07*	*49.54*	*47.71*	*3.23*	*3.98*	*2.93*	*2.99*	*0.84*	*1.22*	*1.33*	*1.57*	*0.64*	*0.63*	*0.83*	*0.95*	*1.60*	*1.99*
H1	2.95	4.33	2.99	4.07	1.05	0.95	69.87	61.99	5.32	5.09	3.67	3.61	0.83	1.88	1.34	2.64	0.65	1.50	0.69	1.14	1.52	3.02
H2	3.18	2.58	2.62	1.76	0.83	0.68	69.17	82.17	4.24	3.90	4.00	5.11	0.34	0.86	1.25	6.59	0.56	2.34	0.69	4.25	1.02	5.10
H3	5.35	6.78	4.43	4.25	1.03	0.64	26.43	20.18	1.99	1.65	2.84	1.78	0.87	0.75	0.85	0.84	0.23	0.26	0.62	0.59	1.50	1.34
H4	2.68	2.86	2.29	2.29	0.87	0.81	70.55	65.31	3.73	3.37	4.22	4.01	0.52	0.62	1.31	1.41	0.69	0.74	0.62	0.67	1.14	1.28
H5	3.06	2.85	2.07	1.87	0.69	0.68	75.17	65.66	3.53	3.24	4.76	4.02	0.87	0.70	1.27	1.57	0.43	0.45	0.84	1.12	1.71	1.82
H6	2.74	2.56	2.87	2.70	1.05	1.06	48.26	54.17	2.83	3.02	2.46	2.99	0.97	1.05	1.66	1.74	0.71	0.78	0.95	0.96	1.92	2.01
H7	2.31	3.04	2.45	3.22	1.07	1.06	59.36	51.70	2.75	2.69	2.85	2.71	0.46	0.66	1.09	1.48	0.61	0.78	0.48	0.70	0.94	1.37
MED.	*2.95*	*2.86*	*2.62*	*2.70*	*1.03*	*0.81*	*69.17*	*61.99*	*3.53*	*3.24*	*3.67*	*3.61*	*0.83*	*0.75*	*1.27*	*1.57*	*0.61*	*0.78*	*0.69*	*0.96*	*1.50*	*1.82*
MIN.	*2.31*	*2.56*	*2.07*	*1.76*	*0.69*	*0.64*	*26.43*	*20.18*	*1.99*	*1.65*	*2.46*	*1.78*	*0.34*	*0.62*	*0.85*	*0.84*	*0.23*	*0.26*	*0.48*	*0.59*	*0.94*	*1.28*
MAX.	*5.35*	*6.78*	*4.43*	*4.25*	*1.07*	*1.06*	*75.17*	*82.17*	*5.32*	*5.09*	*4.76*	*5.11*	*0.97*	*1.88*	*1.66*	*6.59*	*0.71*	*2.34*	*0.95*	*4.25*	*1.92*	*5.10*

Appendix 7.5(c): Lung function measurements in heaves (H1-5 and 7) horses prior to (0h) and at 5h following inhalation challenge with HDS-2[R]. MED. = median value, MIN. = minimum value, MAX. = maximum value.

	Cdyn (l/kPa)		dPpl (kPa)		RL_{iso} (kPa/l/sec)		RR (breaths/min.)		VT (l)		Wb' (J/min)		$RL_{E25\%}$ (kPa/l/sec)		$RL_{E50\%}$ (kPa/l/sec)		$RL_{E75\%}$ (kPa/l/sec)		$RL_{I25\%}$ (kPa/l/sec)		$RL_{I50\%}$ (kPa/l/sec)		$RL_{I75\%}$ (kPa/l/sec)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
H1	27.72	16.87	0.79	0.93	0.07	0.09	9.30	8.45	7.73	7.49	30.59	29.14	0.09	0.13	0.09	0.12	0.09	0.10	0.05	0.06	0.07	0.08	0.08	0.09
H2	84.77	16.02	0.37	1.39	0.03	0.13	13.60	13.50	5.15	4.98	11.52	49.52	0.02	0.10	0.03	0.11	0.04	0.14	0.02	0.11	0.03	0.13	0.05	0.15
H3	9.52	7.63	1.02	0.97	0.12	0.10	5.70	5.20	6.93	4.79	13.46	7.69	0.03	0.12	0.00	0.00	0.03	0.09	0.19	0.28	0.38	0.35	0.15	0.21
H4	37.74	35.45	0.50	0.50	0.06	0.05	11.50	12.90	5.27	5.63	14.40	17.48	0.04	0.03	0.05	0.05	0.05	0.06	0.04	0.04	0.05	0.04	0.05	0.05
H5	22.05	29.78	0.41	0.48	0.03	0.04	9.45	7.00	5.65	7.76	7.58	10.61	0.04	0.04	0.04	0.05	0.05	0.06	0.03	0.04	0.03	0.04	0.03	0.05
H7	13.61	9.79	0.75	1.25	0.08	0.13	12.70	14.45	4.88	5.34	24.42	53.36	0.09	0.13	0.08	0.14	0.08	0.14	0.09	0.12	0.09	0.13	0.10	0.13
MED.	*24.89*	*16.44*	*0.63*	*0.95*	*0.06*	*0.09*	*10.48*	*10.68*	*5.46*	*5.48*	*13.93*	*23.31*	*0.04*	*0.11*	*0.05*	*0.08*	*0.05*	*0.10*	*0.04*	*0.08*	*0.06*	*0.10*	*0.07*	*0.11*
MIN.	*9.52*	*7.63*	*0.37*	*0.48*	*0.03*	*0.04*	*5.70*	*5.20*	*4.88*	*4.79*	*7.58*	*7.69*	*0.02*	*0.03*	*0.00*	*0.00*	*0.03*	*0.06*	*0.02*	*0.04*	*0.03*	*0.04*	*0.03*	*0.05*
MAX.	*84.77*	*35.45*	*1.02*	*1.39*	*0.12*	*0.13*	*13.60*	*14.45*	*7.73*	*7.76*	*30.59*	*53.36*	*0.09*	*0.13*	*0.09*	*0.14*	*0.09*	*0.14*	*0.19*	*0.28*	*0.38*	*0.35*	*0.15*	*0.21*

	T_E (sec)		T_I (sec)		$T_I:T_E$		V'_E (l/min)		V'_{Emax} (l/sec)		V'_{Imax} (l/sec)		Wb_{el} (J)		Wb_{res} (J)		Wb_{Eres} (J)		Wb_{Ires} (J)		Wb_{tot} (J)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
H1	3.26	3.58	3.18	3.58	0.98	1.02	71.66	62.99	5.17	4.50	3.53	2.93	1.10	1.65	3.27	3.43	1.77	1.96	1.51	1.47	2.61	3.12
H2	2.40	2.51	2.10	1.88	0.89	0.75	69.19	66.85	3.32	3.41	3.67	4.22	0.19	0.81	0.88	3.70	0.40	1.55	0.48	2.15	0.67	2.96
H3	5.49	6.80	4.75	4.92	0.88	0.73	39.58	24.84	2.96	1.65	3.59	2.06	2.58	1.43	2.26	1.48	0.29	0.49	1.98	0.99	4.55	2.42
H4	2.91	2.55	2.45	2.13	0.84	0.84	59.72	72.75	4.15	3.62	3.68	4.18	0.39	0.47	1.29	1.34	0.66	0.66	0.64	0.68	1.02	1.15
H5	3.51	5.34	2.77	3.31	0.77	0.63	52.64	54.37	2.87	2.98	3.41	3.69	0.78	1.01	0.81	1.53	0.37	0.54	0.44	0.99	1.22	2.00
H7	2.31	2.23	2.43	1.97	1.06	0.88	62.19	77.31	3.18	3.56	2.93	3.51	0.92	1.51	1.93	3.73	0.86	1.80	1.08	1.94	1.99	3.45
MED.	*3.08*	*3.06*	*2.61*	*2.72*	*0.88*	*0.79*	*60.95*	*64.92*	*3.25*	*3.49*	*3.56*	*3.60*	*0.85*	*1.22*	*1.61*	*2.48*	*0.53*	*1.10*	*0.86*	*1.23*	*1.61*	*2.69*
MIN.	*2.31*	*2.23*	*2.10*	*1.88*	*0.77*	*0.63*	*39.58*	*24.84*	*2.87*	*1.65*	*2.93*	*2.06*	*0.19*	*0.47*	*0.81*	*1.34*	*0.29*	*0.49*	*0.44*	*0.68*	*0.67*	*1.15*
MAX.	*5.49*	*6.80*	*4.75*	*4.92*	*1.06*	*1.02*	*71.66*	*77.31*	*5.17*	*4.50*	*3.68*	*4.22*	*2.58*	*1.65*	*3.27*	*3.73*	*1.77*	*1.96*	*1.98*	*2.15*	*4.55*	*3.45*

Appendix 7.5(d): Lung function measurements in heaves (H1-7) horses prior to (0h) and at 5h following inhalation challenge with HDS-3[100]. MED. = median value, MIN. = minimum value, MAX. = maximum value.

	Cdyn (l/kPa)		dPpl (kPa)		RL_{iso} (kPa/l/sec)		RR (breaths/min.)		VT (l)		Wb' (J/min)		$RL_{E25\%}$ (kPa/l/sec)		$RL_{E50\%}$ (kPa/l/sec)		$RL_{E75\%}$ (kPa/l/sec)		$RL_{I25\%}$ (kPa/l/sec)		$RL_{I50\%}$ (kPa/l/sec)		$RL_{I75\%}$ (kPa/l/sec)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
H1	21.27	19.81	0.77	0.97	0.06	0.11	7.30	9.80	8.84	6.64	22.41	35.06	0.10	0.14	0.13	0.13	0.08	0.11	0.04	0.08	0.03	0.11	0.05	0.13
H2	50.26	12.57	0.56	1.71	0.05	0.18	9.60	10.60	6.48	6.39	16.57	64.60	0.07	0.17	0.05	0.16	0.06	0.17	0.04	0.14	0.04	0.17	0.07	0.18
H3	10.54	7.75	0.97	0.94	0.07	0.19	5.50	5.55	6.18	4.90	7.21	9.37	0.06	0.11	0.01	0.05	0.03	0.11	0.16	0.24	0.32	0.38	0.15	0.23
H4	39.62	43.75	0.44	0.46	0.03	0.05	11.70	15.20	5.66	3.98	13.50	15.41	0.03	0.03	0.03	0.05	0.05	0.07	0.03	0.05	0.03	0.05	0.04	0.06
H5	31.96	24.16	0.48	0.68	0.04	0.04	10.65	12.10	7.56	7.36	15.94	26.34	0.02	0.05	0.04	0.05	0.05	0.06	0.02	0.02	0.03	0.03	0.04	0.05
H6	10.40	3.68	0.67	1.33	0.08	0.20	11.90	20.60	3.79	2.81	15.79	45.80	0.09	0.21	0.09	0.25	0.10	0.27	0.11	0.17	0.09	0.16	0.10	0.15
H7	25.29	18.64	0.47	0.80	0.05	0.09	12.35	7.70	4.89	6.32	13.10	17.91	0.05	0.07	0.06	0.05	0.06	0.11	0.04	0.12	0.04	0.11	0.05	0.10
MED.	*25.29*	*18.64*	*0.56*	*0.94*	*0.05*	*0.11*	*10.65*	*10.60*	*6.18*	*6.32*	*15.79*	*26.34*	*0.06*	*0.11*	*0.05*	*0.05*	*0.06*	*0.11*	*0.04*	*0.12*	*0.04*	*0.11*	*0.05*	*0.13*
MIN.	*10.40*	*3.68*	*0.44*	*0.46*	*0.03*	*0.04*	*5.50*	*5.55*	*3.79*	*2.81*	*7.21*	*9.37*	*0.02*	*0.03*	*0.01*	*0.05*	*0.03*	*0.06*	*0.02*	*0.02*	*0.03*	*0.03*	*0.04*	*0.05*
MAX.	*50.26*	*43.75*	*0.97*	*1.71*	*0.08*	*0.20*	*12.35*	*20.60*	*8.84*	*7.36*	*22.41*	*64.60*	*0.10*	*0.21*	*0.13*	*0.25*	*0.10*	*0.27*	*0.16*	*0.24*	*0.32*	*0.38*	*0.15*	*0.23*

	T_E (sec)		T_I (sec)		$T_I:T_E$		V'_E (l/min)		V'_{Emax} (l/sec)		V'_{Imax} (l/sec)		Wb_{el} (J)		Wb_{res} (J)		Wb_{Eres} (J)		Wb_{Ires} (J)		Wb_{rot} (J)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
H1	4.24	3.14	4.09	3.22	0.97	1.04	64.61	64.33	4.71	4.15	3.44	2.88	1.87	1.17	3.12	3.68	2.00	1.95	1.12	1.73	2.99	2.90
H2	3.51	3.35	2.86	2.31	0.84	0.69	61.54	67.88	3.64	3.29	3.47	4.35	0.44	1.66	1.76	6.11	0.89	2.27	0.87	3.84	1.31	5.50
H3	6.58	6.22	4.92	4.80	0.76	0.77	34.03	27.09	2.91	1.69	2.90	2.11	2.42	1.55	1.41	1.71	0.39	0.62	1.02	1.08	3.44	2.64
H4	2.84	2.19	2.14	1.77	0.76	0.82	66.14	60.49	3.53	2.93	4.20	3.31	0.46	0.19	1.12	1.01	0.57	0.52	0.56	0.49	1.02	0.68
H5	3.30	2.96	2.65	2.18	0.81	0.76	79.97	89.31	4.11	4.70	4.51	5.18	0.93	1.23	1.58	2.22	0.59	0.99	0.99	1.22	1.92	2.45
H6	2.27	1.50	2.74	1.45	1.22	0.98	44.98	57.88	3.69	3.24	2.86	2.97	0.70	1.11	1.31	2.31	0.57	1.26	0.74	1.05	1.44	2.16
H7	2.46	4.02	2.48	3.76	1.02	0.94	60.02	48.71	2.74	2.59	3.31	2.50	0.51	1.08	1.08	2.31	0.61	1.08	0.47	1.23	0.98	2.31
MED.	*3.30*	*3.14*	*2.74*	*2.31*	*0.84*	*0.82*	*61.54*	*60.49*	*3.64*	*3.24*	*3.44*	*2.97*	*0.70*	*1.17*	*1.41*	*2.31*	*0.59*	*1.08*	*0.87*	*1.22*	*1.44*	*2.45*
MIN.	*2.27*	*1.50*	*2.14*	*1.45*	*0.76*	*0.69*	*34.03*	*27.09*	*2.74*	*1.69*	*2.86*	*2.11*	*0.44*	*0.19*	*1.08*	*1.01*	*0.39*	*0.52*	*0.47*	*0.49*	*0.98*	*0.68*
MAX.	*6.58*	*6.22*	*4.92*	*4.80*	*1.22*	*1.04*	*79.97*	*89.31*	*4.71*	*4.70*	*4.51*	*5.18*	*2.42*	*1.66*	*3.12*	*6.11*	*2.00*	*2.27*	*1.12*	*3.84*	*3.44*	*5.50*

Appendix 8.1: Clinical scores in control (C1-6) and heaves (H1-7) horses prior to (0) and at 240min following inhalation challenge with saline, HDS-1 supernatant (SUP), HDS-1 washed particulates (WP), HDS-1 wash fraction (WF), WP and SUP administered seperately (WP/SUP[s]), WP resuspended in SUP (WP/SUP[m]) and HDS-1. Score based on: a (tracheal auscultation), d (dyspnoea) and f (mucopurulent nasal discharge). NP = challenge not performed, NM = clinical score not measured.

CHALLENGE	SALINE		SUP		WP		WF		WP/SUP[s]		WP/SUP[m]		HDS-1	
TIME PT. (min)	0	240	0	240	0	240	0	240	0	240	0	240	0	240
C1	0	0	0	0	NP	NP	NP	NP	NP	NP	NP	NP	0	0
C2	0	0	0	0	NP	NP	NP	NP	NP	NP	NP	NP	0	0
C3	0	0	0	0	NP	NP	NP	NP	NP	NP	NP	NP	0	0
C4	0	0	0	0	NP	NP	NP	NP	NP	NP	NP	NP	0	0
C5	0	0	0	0	NP	NP	NP	NP	NP	NP	NP	NP	0	0
C6	0	0	0	0	NP	NP	NP	NP	NP	NP	NP	NP	0	0
H1	0	0	0	1 a	0	0	0	0	NM	NM	NM	NM	0	0
H2	0	0	0	0	0	0	0	0	NM	NM	NM	NM	0	1 d
H3	0	0	0	0	0	0	0	0	NM	NM	NM	NM	0	0
H4	0	0	0	0	0	1 d	0	0	NM	NM	NM	NM	0	0
H5	0	0	0	0	1 f	1 f	0	0	NM	NM	NM	NM	0	0
H6	0	0	0	0	0	0	0	0	NM	NM	NM	NM	0	0
H7	0	0	0	0	0	1 d	0	0	NM	NM	NM	NM	0	0

Appendix 8.2: Arterial blood pH, pCO_2 (mmHg) and pO_2 (mmHg) in control (C1-6) and heaves (H1-7) horses prior to (0) and at 240min following inhalation challenge with saline, HDS-1[100], SUP and WP. MED. = median value, MIN. = minimum value, MAX. = maximum value.

	PH								pCO_2								pO_2							
	SALINE		HDS-1[100]		SUP		WP		SALINE		HDS-1[100]		SUP		WP		SALINE		HDS-1[100]		SUP		WP	
TIME PT.	0	240	0	240	0	240	0	240	0	240	0	240	0	240	0	240	0	240	0	240	0	240	0	240
C1	7.40	7.39	7.37	7.37	7.46	7.38	NP	NP	46.0	49.0	53.5	48.9	44.9	55.9	NP	NP	100.0	114.0	100.0	97.7	102.3	92.1	NP	NP
C2	7.36	7.35	7.32	7.36	7.34	7.39	NP	NP	45.0	48.0	54.0	56.0	51.1	50.1	NP	NP	103.0	115.0	90.4	88.0	96.2	95.8	NP	NP
C3	7.38	7.38	7.42	7.41	7.37	7.40	NP	NP	46.5	46.6	46.4	46.3	47.0	52.0	NP	NP	106.5	104.6	113.3	103.7	110.0	95.0	NP	NP
C4	7.40	7.42	7.36	7.41	7.39	7.39	NP	NP	50.1	44.7	49.2	49.1	48.2	51.9	NP	NP	90.6	88.0	80.7	74.6	95.2	81.6	NP	NP
C5	7.36	7.36	7.43	7.41	7.41	7.43	NP	NP	41.0	39.0	51.0	48.5	51.6	49.2	NP	NP	105.0	110.0	125.0	118.0	89.5	99.3	NP	NP
C6	7.39	7.39	7.43	7.40	7.41	7.42	NP	NP	40.0	41.0	50.0	43.5	47.7	48.0	NP	NP	93.0	104.0	98.0	109.3	90.4	91.6	NP	NP
MED.	*7.39*	*7.39*	*7.39*	*7.40*	*7.40*	*7.39*			*45.5*	*45.7*	*50.5*	*48.7*	*48.0*	*51.0*			*101.5*	*107.3*	*99.0*	*100.7*	*95.7*	*93.6*		
MIN.	*7.36*	*7.35*	*7.32*	*7.36*	*7.34*	*7.38*			*40.0*	*39.0*	*46.4*	*43.5*	*44.9*	*48.0*			*90.6*	*88.0*	*80.7*	*74.6*	*89.5*	*81.6*		
MAX.	*7.40*	*7.42*	*7.43*	*7.41*	*7.46*	*7.43*			*50.1*	*49.0*	*54.0*	*56.0*	*51.6*	*55.9*			*106.5*	*115.0*	*125.0*	*118.0*	*110.0*	*99.3*		
H1	7.36	7.38	7.34	7.39	7.38	7.41	7.41	7.39	45.0	40.0	45.2	46.7	47.7	49.7	46.9	49.8	99.0	98.0	104.4	121.8	99.9	103.2	105.7	94.8
H2	7.35	7.36	7.34	7.38	7.38	7.39	7.37	7.38	47.0	46.0	48.9	50.8	51.5	52.7	48.5	51.5	116.0	102.0	100.8	101.5	91.4	90.3	98.9	96.4
H3	7.40	7.41	7.41	7.39	7.42	7.44	7.42	7.44	42.0	37.0	50.0	48.2	49.9	44.8	47.6	46.9	94.0	98.0	92.0	117.6	93.0	94.2	107.4	92.8
H4	7.39	7.35	7.42	7.43	7.40	7.43	7.38	7.40	43.0	45.0	43.4	46.0	48.0	44.8	46.6	48.3	102.0	97.0	104.1	124.0	92.4	102.6	97.7	99.1
H5	7.37	7.37	7.37	7.37	7.39	7.35	7.39	7.41	53.0	43.0	51.0	55.6	51.5	55.5	51.9	49.0	94.0	83.0	97.0	85.7	95.4	90.1	88.8	95.9
H6	7.42	7.36	7.37	7.40	7.37	7.41	7.38	7.40	48.4	47.6	51.3	56.4	55.7	51.3	47.3	49.4	90.5	94.5	98.4	81.4	95.3	88.6	98.3	96.2
H7	7.41	7.43	7.36	7.40	7.41	7.41	7.41	7.44	46.0	45.0	43.0	44.4	44.6	48.1	49.9	47.6	100.0	109.0	109.2	108.0	99.2	97.9	98.1	98.9
MED.	*7.39*	*7.37*	*7.37*	*7.39*	*7.39*	*7.41*	7.39	7.40	*46.0*	*45.0*	*48.9*	*48.2*	*49.9*	*49.7*	47.6	49.0	*99.0*	*98.0*	*100.8*	*108.0*	*95.3*	*94.2*	98.3	96.2
MIN.	*7.35*	*7.35*	*7.34*	*7.37*	*7.37*	*7.35*	7.37	7.38	*42.0*	*37.0*	*43.0*	*44.4*	*44.6*	*44.8*	46.6	46.9	*90.5*	*83.0*	*92.0*	*81.4*	*91.4*	*88.6*	88.8	92.8
MAX.	*7.42*	*7.43*	*7.42*	*7.43*	*7.42*	*7.44*	7.42	7.44	*53.0*	*47.6*	*51.3*	*56.4*	*55.7*	*55.5*	51.9	51.5	*116.0*	*109.0*	*109.2*	*124.0*	*99.9*	*103.2*	107.4	99.1

Appendix 8.3: Lung function measurements in control (C1-6) and heaves (H1-7) horses prior to (0h) and at 5h following inhalation challenge with SUP. MED. = median value, MIN. = minimum value, MAX. = maximum value.

	Cdyn (l/kPa)		dPpl (kPa)		RL_{iso} (kPa/l/sec)		RR (breaths/min.)		VT (l)		Wb' (J/min)		$RL_{E25\%}$ (kPa/l/sec)		$RL_{E50\%}$ (kPa/l/sec)		$RL_{E75\%}$ (kPa/l/sec)		$RL_{I25\%}$ (kPa/l/sec)		$RL_{I50\%}$ (kPa/l/sec)		$RL_{I75\%}$ (kPa/l/sec)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
C1	4.51	4.82	1.26	1.34	0.15	0.16	8.15	8.10	4.35	4.92	11.82	16.53	0.06	0.07	0.02	0.08	0.05	0.08	0.27	0.25	0.22	0.19	0.17	0.16
C2	10.00	8.05	0.88	1.05	0.08	0.09	7.90	9.35	6.17	5.44	16.82	20.70	0.08	0.10	0.06	0.07	0.05	0.07	0.16	0.17	0.14	0.15	0.13	0.14
C3	15.86	32.39	0.61	0.54	0.07	0.07	10.00	10.50	5.10	4.96	12.53	16.249	0.08	0.23	0.07	0.23	0.07	0.47	0.06	0.05	0.07	0.05	0.06	0.06
C4	11.08	3.70	0.57	0.82	0.02	0.08	6.60	7.25	6.01	2.85	2.09	2.82	0.01	0.10	0.00	0.02	0.00	0.01	0.08	0.32	0.13	0.20	0.07	0.14
C5	13.49	19.27	1.20	0.92	0.17	0.13	5.55	6.00	7.54	7.21	22.65	20.36	0.16	0.09	0.15	0.13	0.19	0.16	0.19	0.15	0.15	0.11	0.13	0.11
C6	9.05	7.35	0.81	0.90	0.06	0.08	8.95	8.10	5.69	5.58	13.02	12.00	0.04	0.07	0.01	0.05	0.01	0.03	0.14	0.16	0.16	0.20	0.11	0.13
MED.	*10.54*	*7.35*	*0.84*	*0.92*	*0.07*	*0.09*	*8.03*	*8.10*	*5.85*	*5.44*	*12.77*	*16.53*	*0.07*	*0.09*	*0.04*	*0.07*	*0.05*	*0.07*	*0.15*	*0.17*	*0.14*	*0.19*	*0.12*	*0.14*
MIN.	*4.51*	*3.70*	*0.57*	*0.82*	*0.02*	*0.08*	*5.55*	*6.00*	*4.35*	*2.85*	*2.09*	*2.82*	*0.01*	*0.07*	*0.00*	*0.02*	*0.00*	*0.01*	*0.06*	*0.15*	*0.07*	*0.11*	*0.06*	*0.11*
MAX.	*15.86*	*19.27*	*1.26*	*1.34*	*0.17*	*0.16*	*10.00*	*9.35*	*7.54*	*7.21*	*22.65*	*20.70*	*0.16*	*0.10*	*0.15*	*0.13*	*0.19*	*0.16*	*0.27*	*0.32*	*0.22*	*0.20*	*0.17*	*0.16*
H1	17.02	32.45	0.77	0.72	0.06	0.05	7.60	5.85	8.26	12.44	19.64	22.69	0.08	0.08	0.10	0.07	0.08	0.07	0.07	0.04	0.06	0.04	0.06	0.06
H2	60.46	43.03	0.53	0.54	0.05	0.05	6.75	7.60	7.24	6.88	13.43	12.08	0.02	0.88	0.06	0.65	0.07	0.54	0.05	0.06	0.05	0.05	0.06	0.05
H3	7.36	8.59	0.74	1.04	0.13	0.09	5.75	5.35	4.40	7.43	6.84	16.00	0.07	0.03	0.02	0.01	0.09	0.02	0.19	0.18	0.25	0.29	0.22	0.23
H4	24.11	20.94	0.62	0.65	0.05	0.06	7.40	6.30	7.74	8.26	13.90	14.55	0.03	0.00	0.04	0.04	0.06	0.07	0.05	0.06	0.05	0.06	0.06	0.05
H5	22.12	20.50	0.94	1.13	0.09	0.06	4.55	4.80	12.03	14.42	19.52	34.24	0.13	0.12	0.15	0.13	0.16	0.14	0.06	0.10	0.04	0.06	0.06	0.07
H6	13.42	11.71	0.70	0.66	0.07	0.08	9.00	8.30	5.37	4.88	14.24	9.09	0.08	0.07	0.07	0.05	0.07	0.08	0.10	0.11	0.08	0.15	0.08	0.08
H7	21.85	18.46	0.55	0.87	0.06	0.06	9.75	9.40	5.13	8.08	11.79	28.72	0.06	0.06	0.06	0.05	0.07	0.08	0.06	0.08	0.06	0.07	0.07	0.07
MED.	*21.85*	*20.50*	*0.70*	*0.72*	*0.06*	*0.06*	*7.40*	*6.95*	*7.24*	*8.08*	*13.90*	*16.00*	*0.07*	*0.07*	*0.06*	*0.05*	*0.07*	*0.08*	*0.06*	*0.08*	*0.06*	*0.06*	*0.06*	*0.07*
MIN.	*7.36*	*8.59*	*0.53*	*0.54*	*0.05*	*0.05*	*4.55*	*5.35*	*4.40*	*4.88*	*6.84*	*9.09*	*0.02*	*0.00*	*0.02*	*0.01*	*0.06*	*0.02*	*0.05*	*0.04*	*0.04*	*0.04*	*0.06*	*0.05*
MAX.	*60.46*	*43.03*	*0.94*	*1.13*	*0.13*	*0.09*	*9.75*	*9.40*	*12.03*	*14.42*	*19.64*	*34.24*	*0.13*	*0.88*	*0.15*	*0.65*	*0.16*	*0.54*	*0.19*	*0.18*	*0.25*	*0.29*	*0.22*	*0.23*

	T_E (sec)		T_I (sec)		T_I:T_E		V'E (l/min)		V'Emax (l/sec)		V'Imax (l/sec)		Wb_{el} (J)		Wb_{res} (J)		Wb_{Eres} (J)		Wb_{Ires} (J)		Wb_{tot} (J)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
C1	3.96	3.63	3.72	3.79	0.95	1.05	35.11	39.76	1.78	2.02	2.26	2.29	2.10	2.52	1.50	2.04	0.24	0.58	1.27	1.46	3.36	3.98
C2	4.33	3.72	3.29	2.84	0.76	0.79	48.58	50.46	2.72	2.81	2.66	2.71	1.90	1.88	2.14	2.27	0.47	0.56	1.67	1.71	3.57	3.59
C3	2.94	2.24	3.10	3.05	1.06	6.80	50.95	52.04	2.66	3.72	2.69	3.13	0.82	0.49	1.26	1.50	0.70	0.928	0.56	0.57	1.38	1.06
C4	5.03	4.58	4.13	3.66	0.83	0.81	39.54	20.66	2.20	1.16	2.73	1.27	1.65	1.09	0.31	0.39	0.24	0.00	0.55	0.39	2.20	1.48
C5	6.15	5.39	4.79	4.58	0.78	0.85	41.87	43.30	2.17	2.18	2.62	3.07	2.11	1.36	4.06	3.37	2.10	1.72	1.96	1.65	4.06	3.01
C6	3.47	3.84	3.25	3.59	0.96	0.95	50.86	45.23	3.18	3.84	3.25	2.79	1.78	2.12	1.41	1.48	0.13	0.18	1.28	1.30	3.06	3.42
MED.	*4.15*	*3.84*	*3.50*	*3.66*	*0.89*	*0.85*	*45.23*	*43.30*	*2.43*	*2.18*	*2.67*	*2.71*	*1.84*	*1.88*	*1.46*	*2.04*	*0.35*	*0.56*	*1.27*	*1.46*	*3.21*	*3.42*
MIN.	*2.94*	*3.63*	*3.10*	*2.84*	*0.76*	*0.79*	*35.11*	*20.66*	*1.78*	*1.16*	*2.26*	*1.27*	*0.82*	*1.09*	*0.31*	*0.39*	*0.13*	*0.00*	*0.55*	*0.39*	*1.38*	*1.48*
MAX.	*6.15*	*5.39*	*4.79*	*4.58*	*1.06*	*1.05*	*50.95*	*50.46*	*3.18*	*3.84*	*3.25*	*3.07*	*2.11*	*2.52*	*4.06*	*3.37*	*2.10*	*1.72*	*1.96*	*1.71*	*4.06*	*3.98*
H1	3.39	4.40	4.55	6.04	1.35	1.40	62.47	72.45	4.79	4.43	3.58	4.32	2.03	2.45	2.60	3.96	1.63	2.84	0.97	1.12	2.99	3.57
H2	5.42	4.36	3.48	3.05	0.65	4.72	48.67	52.25	3.42	3.27	4.15	4.14	0.47	0.68	1.98	1.63	1.10	0.73	0.89	0.89	1.35	1.57
H3	6.08	5.83	4.67	4.50	0.79	0.97	25.37	39.65	1.56	2.77	2.12	4.17	1.32	3.76	1.17	2.55	0.41	0.15	0.76	2.40	2.09	6.16
H4	4.69	5.70	3.54	3.78	0.76	0.70	57.59	52.59	3.33	3.35	3.97	3.65	1.29	1.68	1.90	2.28	1.07	1.32	0.83	0.96	2.12	2.64
H5	7.32	7.34	5.75	5.07	0.79	0.70	54.50	63.71	2.78	4.02	4.06	4.64	3.42	4.16	4.21	7.07	2.64	4.64	1.57	2.43	4.99	6.59
H6	2.85	3.15	3.88	4.21	1.38	1.33	48.37	40.55	3.85	2.92	2.77	2.39	1.08	1.01	1.60	1.11	0.79	0.49	0.81	0.62	1.89	1.63
H7	3.15	3.32	3.04	3.07	0.97	0.93	50.11	75.70	2.49	3.49	2.38	4.04	0.62	1.79	1.22	3.04	0.61	1.46	0.61	1.59	1.23	3.38
MED.	*4.69*	*4.40*	*3.88*	*4.21*	*0.79*	*0.97*	*50.11*	*52.42*	*3.33*	*3.35*	*3.58*	*4.14*	*1.29*	*1.79*	*1.90*	*2.55*	*1.07*	*1.32*	*0.83*	*1.12*	*2.09*	*3.38*
MIN.	*2.85*	*3.15*	*3.04*	*3.05*	*0.65*	*0.70*	*25.37*	*39.65*	*1.56*	*2.77*	*2.12*	*2.39*	*0.47*	*0.68*	*1.17*	*1.11*	*0.41*	*0.15*	*0.61*	*0.62*	*1.23*	*1.57*
MAX.	*7.32*	*7.34*	*5.75*	*6.04*	*1.38*	*4.72*	*62.47*	*75.70*	*4.79*	*4.43*	*4.15*	*4.64*	*3.42*	*4.16*	*4.21*	*7.07*	*2.64*	*4.64*	*1.57*	*2.43*	*4.99*	*6.59*

Appendix 8.3: Lung function measurements in heaves (H1-7) horses prior to (0h) and at 5h following inhalation challenge with WP. MED. = median value, MIN. = minimum value, MAX. = maximum value.

	Cdyn (l/kPa)		dPpl (kPa)		RL_{iso} (kPa/l/sec)		RR (breaths/min.)		VT (l)		Wb' (J/min)		$RL_{E25\%}$ (kPa/l/sec)		$RL_{E50\%}$ (kPa/l/sec)		$RL_{E75\%}$ (kPa/l/sec)		$RL_{I25\%}$ (kPa/l/sec)		$RL_{I50\%}$ (kPa/l/sec)		$RL_{I75\%}$ (kPa/l/sec)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
H1	17.53	20.05	1.62	1.27	0.15	0.12	4.75	5.10	13.26	12.35	46.49	37.63	0.19	0.12	0.17	0.13	0.17	0.16	0.11	0.14	0.09	0.11	0.11	0.11
H2	50.27	32.00	0.50	0.54	0.03	0.05	9.85	7.00	8.04	7.48	18.12	12.05	0.05	0.01	0.04	0.04	0.05	0.06	0.03	0.05	0.02	0.05	0.03	0.05
H3	8.13	9.04	1.02	0.98	0.11	0.13	4.95	4.80	6.25	7.18	12.19	13.22	0.09	0.01	0.05	0.01	0.12	0.11	0.17	0.20	0.23	0.32	0.20	0.25
H4	28.46	22.70	0.51	0.50	0.04	0.06	15.50	8.05	5.85	5.76	23.89	9.81	0.03	0.03	0.04	0.06	0.05	0.08	0.03	0.05	0.04	0.06	0.04	0.05
H5	32.71	21.75	0.53	0.84	0.06	0.07	4.45	4.55	9.24	11.64	11.13	19.09	0.07	0.09	0.09	0.11	0.10	0.11	0.08	0.07	0.08	0.07	0.07	0.07
H6	9.95	12.12	0.87	0.74	0.12	0.09	11.35	9.90	4.03	5.31	19.68	16.42	0.13	0.07	0.11	0.06	0.11	0.06	0.13	0.09	0.13	0.06	0.14	0.06
H7	17.56	14.70	1.11	0.93	0.14	0.07	6.30	8.40	7.34	7.26	23.82	22.43	0.15	0.08	0.16	0.07	0.22	0.09	0.14	0.09	0.11	0.06	0.13	0.06
MED.	*17.56*	*20.05*	*0.87*	*0.84*	*0.11*	*0.07*	*6.30*	*7.00*	*7.34*	*7.26*	*19.68*	*16.42*	*0.09*	*0.07*	*0.09*	*0.06*	*0.11*	*0.09*	*0.11*	*0.09*	*0.09*	*0.06*	*0.11*	*0.06*
MIN.	*8.13*	*9.04*	*0.50*	*0.50*	*0.03*	*0.05*	*4.45*	*4.55*	*4.03*	*5.31*	*11.13*	*9.81*	*0.03*	*0.01*	*0.04*	*0.01*	*0.05*	*0.06*	*0.03*	*0.05*	*0.02*	*0.05*	*0.03*	*0.05*
MAX.	*50.27*	*32.00*	*1.62*	*1.27*	*0.15*	*0.13*	*15.50*	*9.90*	*13.26*	*12.35*	*46.49*	*37.63*	*0.19*	*0.12*	*0.17*	*0.13*	*0.22*	*0.16*	*0.17*	*0.20*	*0.23*	*0.32*	*0.20*	*0.25*

	T_E (sec)		T_I (sec)		T_I:T_E		V'_E (l/min)		V'_{Emax} (l/sec)		V'_{Imax} (l/sec)		Wb_{el} (J)		Wb_{res} (J)		Wb_{Eres} (J)		Wb_{Ires} (J)		Wb_{tot} (J)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
H1	5.57	5.37	6.90	6.37	1.27	1.19	62.73	62.56	3.58	3.28	2.98	3.41	5.03	3.87	9.49	7.37	6.69	4.64	2.80	2.73	7.83	6.60
H2	3.30	5.10	2.82	3.56	0.86	0.70	78.78	52.01	4.38	3.36	5.03	4.14	0.65	0.87	1.87	1.74	1.08	0.97	0.79	0.77	1.44	1.63
H3	5.98	7.64	5.43	5.06	1.07	0.67	31.42	34.48	2.12	2.39	2.85	3.19	2.70	2.72	2.26	2.77	0.94	0.87	1.32	1.90	4.02	4.63
H4	2.06	4.20	1.77	3.19	0.87	0.77	90.79	46.34	4.35	2.42	4.83	2.83	0.66	0.73	1.51	1.20	0.76	0.65	0.75	0.54	1.41	1.27
H5	7.14	7.60	5.40	5.00	1.13	0.67	40.97	52.58	2.26	2.99	3.18	4.25	1.40	3.27	2.24	3.79	0.96	1.61	1.28	2.17	2.67	5.44
H6	2.67	2.83	2.66	2.94	1.00	1.11	45.50	52.70	2.84	3.15	2.51	3.38	0.80	1.21	1.75	1.63	0.72	0.79	1.03	0.84	1.83	2.05
H7	4.83	3.44	4.76	3.63	0.99	1.06	46.10	60.90	2.32	3.31	2.16	3.42	1.55	1.79	3.81	2.64	2.31	1.57	1.49	1.07	3.05	2.86
MED.	*4.83*	*5.10*	*4.76*	*3.63*	*1.00*	*0.77*	*46.10*	*52.58*	*2.84*	*3.15*	*2.98*	*3.41*	*1.40*	*1.79*	*2.24*	*2.64*	*0.96*	*0.97*	*1.28*	*1.07*	*2.67*	*2.86*
MIN.	*2.06*	*2.83*	*1.77*	*2.94*	*0.86*	*0.67*	*31.42*	*34.48*	*2.12*	*2.39*	*2.16*	*2.83*	*0.65*	*0.73*	*1.51*	*1.20*	*0.72*	*0.65*	*0.75*	*0.54*	*1.41*	*1.27*
MAX.	*7.14*	*7.64*	*6.90*	*6.37*	*1.27*	*1.19*	*90.79*	*62.56*	*4.38*	*3.36*	*5.03*	*4.25*	*5.03*	*3.87*	*9.49*	*7.37*	*6.69*	*4.64*	*2.80*	*2.73*	*7.83*	*6.60*

Appendix 8.4: PCCdyn70 values (mg/ml) in control (C1-6) and heaves (H1-7) horses at 5h following inhalation challenge with saline, SUP, WP and HDS-1[100]. MED. = median value, MIN. = minimum value, MAX. = maximum value.

	SALINE	SUP	WP	HDS-1[100]
C1	3.82	1.35	NP	3.17
C2	9.91	4.22	NP	7.08
C3	3.46	8.85	NP	5.25
C4	2.82	1.83	NP	2.07
C5	3.87	1.69	NP	4.69
C6	2.29	4.58	NP	2.65
MED.	*3.64*	*3.03*		*4.69*
MIN.	*2.29*	*1.35*		*2.07*
MAX.	*9.91*	*8.85*		*7.08*
H1	7.86	0.72	5.63	8.16
H2	3.06	2.40	4.54	6.91
H3	5.64	4.85	5.74	4.26
H4	10.53	20.13	13.66	11.43
H5	6.40	3.23	3.48	9.96
H6	2.46	4.04	2.89	4.27
H7	5.63	7.12	4.39	6.20
MED.	*5.64*	*4.04*	*4.54*	*6.91*
MIN.	*2.46*	*0.72*	*2.89*	*4.26*
MAX.	*10.53*	*20.13*	*13.66*	*11.43*

Appendix 8.5: (a) BALF total nucleated cell counts (x10^5/ml) in control (C1-6) and heaves (H1-7) at 6h following challenge with saline, HDS-1[100], SUP, WP, WP[R], WF, WP/SUP[s], WP/SUP[m] and H/S B. MED. = median value, MIN. = minimum value, MAX. = maximum value.

	SALINE	HDS-1[100]	SUP	WP	WP[R]	WF	WP/SUP[s]	WP/SUP[m]	H/S B
C1	5.10	3.00	6.60	NP	NP	NP	NP	NP	2.20
C2	3.90	4.30	4.30	NP	NP	NP	NP	NP	2.90
C3	2.00	1.60	1.80	NP	NP	NP	NP	NP	2.10
C4	2.90	8.00	2.90	NP	NP	NP	NP	NP	3.20
C5	9.60	5.00	3.70	NP	NP	NP	NP	NP	4.60
C6	3.80	5.10	2.40	NP	NP	NP	NP	NP	2.20
MED.	*3.85*	*4.65*	*3.30*						*2.55*
MIN	*2.00*	*1.60*	*1.80*						*2.10*
MAX	*9.60*	*8.00*	*6.60*						*4.60*
H1	5.60	4.00	5.50	5.50	3.10	5.00	5.50	5.40	6.20
H2	3.40	3.70	7.40	4.80	3.70	3.90	5.90	4.70	4.20
H3	3.80	6.20	4.70	5.00	3.50	3.10	13.70	10.20	2.80
H4	5.50	4.10	9.40	5.60	5.30	3.20	7.30	5.70	7.00
H5	3.20	7.00	8.20	7.80	3.90	7.70	19.00	7.80	16.20
H6	1.30	2.10	3.40	2.00	3.50	2.30	2.90	2.30	5.70
H7	5.20	4.10	7.30	9.10	NP	4.90	4.40	5.30	6.50
MED.	*3.80*	*4.10*	*7.30*	*5.50*		*3.90*	*5.90*	*5.40*	*6.20*
MIN	*1.30*	*2.10*	*3.40*	*2.00*		*2.30*	*2.90*	*2.30*	*2.80*
MAX	*5.60*	*7.00*	*9.40*	*9.10*		*7.70*	*19.00*	*10.20*	*16.20*

Appendix 8.5: (b) BALF lymphocyte counts (x10^5/ml) in control (C1-6) and heaves (H1-7) at 6h following challenge with saline, HDS-1[100], SUP, WP, WP[R], WF, WP/SUP[s], WP/SUP[m] and H/S B. MED. = median value, MIN. = minimum value, MAX. = maximum value.

	SALINE	HDS-1[100]	SUP	WP	WP[R]	WF	WP/SUP[s]	WP/SUP[m]	H/S B
C1	1.89	0.66	2.96	NP	NP	NP	NP	NP	0.61
C2	1.28	1.24	1.49	NP	NP	NP	NP	NP	0.99
C3	0.99	0.83	1.07	NP	NP	NP	NP	NP	1.07
C4	0.99	1.74	0.88	NP	NP	NP	NP	NP	0.76
C5	6.22	2.29	2.25	NP	NP	NP	NP	NP	3.09
C6	2.41	2.23	0.94	NP	NP	NP	NP	NP	0.79
MED.	*1.59*	*1.49*	*1.28*						*0.89*
MIN	*0.99*	*0.66*	*0.88*						*0.61*
MAX	*6.22*	*2.29*	*2.96*						*3.09*
H1	3.33	1.62	2.94	3.29	0.78	2.16	2.94	2.39	1.44
H2	1.54	0.61	2.76	2.21	1.19	2.17	1.33	1.09	0.71
H3	2.09	1.19	1.63	2.19	0.72	1.16	2.42	2.83	0.60
H4	2.36	1.75	4.32	1.98	1.81	0.68	2.47	2.33	2.30
H5	1.64	1.86	5.61	3.49	1.27	3.08	4.20	1.79	4.26
H6	0.80	0.81	2.33	0.95	1.52	1.28	1.56	1.14	2.46
H7	2.57	1.12	4.37	5.20	NP	2.64	2.36	2.39	2.32
MED.	*2.09*	*1.19*	*2.94*	*2.21*	*1.23*	*2.16*	*2.42*	*2.33*	*2.30*
MIN	*0.80*	*0.61*	*1.63*	*0.95*	*0.72*	*0.68*	*1.33*	*1.09*	*0.60*
MAX	*3.33*	*1.86*	*5.61*	*5.20*	*1.81*	*3.08*	*4.20*	*2.83*	*4.26*

Appendix 8.5: (c) BALF macrophage counts (x10^5/ml) in control (C1-6) and heaves (H1-7) at 6h following challenge with saline, HDS-1[100], SUP, WP, WP[R], WF, WP/SUP[s], WP/SUP[m] and H/S B. MED. = median value, MIN. = minimum value, MAX. = maximum value.

	SALINE	HDS-1[100]	SUP	WP	WP[R]	WF	WP/SUP[s]	WP/SUP[m]	H/S B
C1	2.80	1.72	2.61	NP	NP	NP	NP	NP	1.35
C2	2.25	2.75	2.46	NP	NP	NP	NP	NP	1.89
C3	0.73	0.51	0.52	NP	NP	NP	NP	NP	0.66
C4	1.71	4.19	1.66	NP	NP	NP	NP	NP	1.89
C5	2.57	1.91	1.08	NP	NP	NP	NP	NP	0.98
C6	1.14	2.46	1.21	NP	NP	NP	NP	NP	1.17
MED.	*1.98*	*2.18*	*1.43*						*1.26*
MIN	*0.73*	*0.51*	*0.52*						*0.66*
MAX	*2.80*	*4.19*	*2.61*						*1.89*
H1	1.96	1.16	1.98	1.68	2.15	2.16	1.78	2.21	1.44
H2	1.58	0.41	2.85	2.29	2.13	1.42	1.57	1.19	0.90
H3	1.24	1.75	1.96	1.99	2.47	1.37	5.14	4.81	1.35
H4	2.96	1.48	4.21	3.18	3.22	2.31	4.08	2.10	3.16
H5	1.27	1.18	1.45	3.92	1.82	2.98	5.30	1.37	2.03
H6	0.34	0.50	0.55	0.71	1.67	0.65	0.68	0.38	1.04
H7	2.40	0.77	2.09	1.65	NP	1.76	1.67	1.46	2.57
MED.	*1.58*	*1.16*	*1.98*	*1.99*	*2.14*	*1.76*	*1.78*	*1.46*	*1.44*
MIN	*0.34*	*0.41*	*0.55*	*0.71*	*1.67*	*0.65*	*0.68*	*0.38*	*0.90*
MAX	*2.96*	*1.75*	*4.21*	*3.92*	*3.22*	*2.98*	*5.30*	*4.81*	*3.16*

Appendix 8.5: (d) BALF neutrophil counts (x10^5/ml) in control (C1-6) and heaves (H1-7) at 6h following challenge with saline, HDS-1[100], SUP, WP, WP[R], WF, WP/SUP[s], WP/SUP[m] and H/S B. MED. = median value, MIN. = minimum value, MAX. = maximum value.

	SALINE	HDS-1[100]	SUP	WP	WP[R]	WF	WP/SUP[s]	WP/SUP[m]	H/S B
C1	0.04	0.29	0.25	NP	NP	NP	NP	NP	0.02
C2	0.01	0.12	0.06	NP	NP	NP	NP	NP	0.01
C3	0.02	0.08	0.04	NP	NP	NP	NP	NP	0.24
C4	0.09	0.56	0.18	NP	NP	NP	NP	NP	0.35
C5	0.17	0.66	0.17	NP	NP	NP	NP	NP	0.40
C6	0.09	0.28	0.12	NP	NP	NP	NP	NP	0.11
MED.	*0.06*	*0.28*	*0.15*						*0.17*
MIN	*0.01*	*0.08*	*0.04*						*0.01*
MAX	*0.17*	*0.66*	*0.25*						*0.40*
H1	0.20	1.11	0.28	0.21	0.08	0.40	0.27	0.43	3.14
H2	0.08	2.60	1.64	0.14	0.24	0.12	2.83	2.34	2.47
H3	0.17	3.14	0.98	0.65	0.20	0.40	5.90	2.35	0.74
H4	0.03	0.80	0.53	0.19	0.19	0.05	0.47	1.04	1.47
H5	0.06	3.81	0.98	0.19	0.79	1.54	9.29	4.55	9.83
H6	0.06	0.54	0.28	0.10	0.23	0.21	0.43	0.63	2.05
H7	0.06	2.17	0.31	0.56	NP	0.35	0.12	1.36	1.41
MED.	*0.06*	*2.17*	*0.53*	*0.19*	*0.22*	*0.35*	*0.47*	*1.36*	*2.05*
MIN	*0.03*	*0.54*	*0.28*	*0.10*	*0.08*	*0.05*	*0.12*	*0.43*	*0.74*
MAX	*0.20*	*3.81*	*1.64*	*0.65*	*0.79*	*1.54*	*9.29*	*4.55*	*9.83*

Appendix 8.5: (e) BALF mast cell counts ($x10^5$/ml) in control (C1-6) and heaves (H1-7) at 6h following challenge with saline, HDS-1[100], SUP, WP, WP[R], WF, WP/SUP[s], WP/SUP[m] and H/S B. MED. = median value, MIN. = minimum value, MAX. = maximum value.

	SALINE	HDS-1[100]	SUP	WP	WP[R]	WF	WP/SUP[s]	WP/SUP[m]	H/S B
C1	0.23	0.13	0.28	NP	NP	NP	NP	NP	0.11
C2	0.30	0.18	0.25	NP	NP	NP	NP	NP	0.01
C3	0.26	0.12	0.16	NP	NP	NP	NP	NP	0.11
C4	0.06	0.24	0.08	NP	NP	NP	NP	NP	0.07
C5	0.39	0.15	0.16	NP	NP	NP	NP	NP	0.12
C6	0.09	0.11	0.10	NP	NP	NP	NP	NP	0.11
MED.	*0.25*	*0.14*	*0.16*						*0.11*
MIN	*0.06*	*0.11*	*0.08*						*0.01*
MAX	*0.39*	*0.24*	*0.28*						*0.12*
H1	0.11	0.08	0.23	0.23	0.07	0.18	0.30	0.24	0.12
H2	0.15	0.07	0.13	0.12	0.13	0.19	0.17	0.07	0.11
H3	0.19	0.11	0.09	0.14	0.11	0.16	0.22	0.21	0.10
H4	0.13	0.05	0.11	0.09	0.07	0.13	0.14	0.03	0.08
H5	0.09	0.02	0.02	0.11	0.02	0.03	0.02	0.02	0.08
H6	0.10	0.14	0.23	0.19	0.06	0.15	0.19	0.14	0.10
H7	0.17	0.02	0.20	0.15	NP	0.07	0.19	0.08	0.10
MED.	*0.13*	*0.07*	*0.13*	*0.14*	*0.07*	*0.15*	*0.19*	*0.08*	*0.10*
MIN	*0.09*	*0.02*	*0.02*	*0.09*	*0.02*	*0.03*	*0.02*	*0.02*	*0.08*
MAX	*0.19*	*0.14*	*0.23*	*0.23*	*0.13*	*0.19*	*0.30*	*0.24*	*0.12*

Appendix 8.5: (f) BALF basiphiloid cell counts ($x10^5$/ml) in control (C1-6) and heaves (H1-7) at 6h following challenge with saline, HDS-1[100], SUP, WP, WP[R], WF, WP/SUP[s], WP/SUP[m] and H/S B. MED. = median value, MIN. = minimum value, MAX. = maximum value.

	SALINE	HDS-1[100]	SUP	WP	WP[R]	WF	WP/SUP[s]	WP/SUP[m]	H/S B
C1	0.07	0.20	0.36	NP	NP	NP	NP	NP	0.09
C2	0.01	0.00	0.03	NP	NP	NP	NP	NP	0.00
C3	0.00	0.03	0.00	NP	NP	NP	NP	NP	0.03
C4	0.03	1.04	0.04	NP	NP	NP	NP	NP	0.12
C5	0.22	0.00	0.01	NP	NP	NP	NP	NP	0.00
C6	0.01	0.00	0.00	NP	NP	NP	NP	NP	0.00
MED.	*0.02*	*0.02*	*0.02*						*0.02*
MIN	*0.00*	*0.00*	*0.00*						*0.00*
MAX	*0.22*	*1.04*	*0.36*						*0.12*
H1	0.01	0.00	0.01	0.02	0.01	0.11	0.00	0.00	0.04
H2	0.05	0.00	0.00	0.02	0.00	0.00	0.00	0.01	0.01
H3	0.11	0.00	0.03	0.00	0.00	0.01	0.01	0.00	0.01
H4	0.01	0.00	0.00	0.02	0.00	0.02	0.00	0.00	0.00
H5	0.02	0.00	0.02	0.02	0.00	0.01	0.00	0.02	0.00
H6	0.00	0.10	0.00	0.04	0.01	0.01	0.02	0.00	0.03
H7	0.01	0.00	0.01	0.02	NP	0.05	0.02	0.00	0.08
MED.	*0.01*	*0.00*	*0.01*	*0.02*	*0.00*	*0.01*	*0.00*	*0.00*	*0.01*
MIN	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*
MAX	*0.11*	*0.10*	*0.03*	*0.04*	*0.01*	*0.11*	*0.02*	*0.02*	*0.08*

Appendix 8.5: (g) BALF eosinophil counts ($x10^5$/ml) in control (C1-6) and heaves (H1-7) at 6h following challenge with saline, HDS-1[100], SUP, WP, WP[R], WF, WP/SUP[s], WP/SUP[m] and H/S B. MED. = median value, MIN. = minimum value, MAX. = maximum value.

	SALINE	HDS-1[100]	SUP	WP	WP[R]	WF	WP/SUP[s]	WP/SUP[m]	H/S B
C1	0.05	0.00	0.13	NP	NP	NP	NP	NP	0.01
C2	0.06	0.01	0.02	NP	NP	NP	NP	NP	0.00
C3	0.00	0.04	0.00	NP	NP	NP	NP	NP	0.00
C4	0.00	0.23	0.07	NP	NP	NP	NP	NP	0.02
C5	0.02	0.00	0.03	NP	NP	NP	NP	NP	0.01
C6	0.06	0.02	0.03	NP	NP	NP	NP	NP	0.00
MED.	*0.03*	*0.01*	*0.03*						*0.01*
MIN	*0.00*	*0.00*	*0.00*						*0.00*
MAX	*0.06*	*0.23*	*0.13*						*0.02*
H1	0.00	0.03	0.07	0.07	0.00	0.00	0.21	0.12	0.01
H2	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.01	0.00
H3	0.01	0.00	0.01	0.04	0.00	0.00	0.00	0.00	0.00
H4	0.01	0.01	0.23	0.13	0.01	0.00	0.14	0.20	0.00
H5	0.01	0.13	0.11	0.08	0.00	0.06	0.19	0.06	0.00
H6	0.01	0.01	0.01	0.00	0.00	0.00	0.01	0.01	0.02
H7	0.00	0.02	0.33	1.54	NP	0.02	0.03	0.01	0.02
MED.	*0.01*	*0.01*	*0.07*	*0.07*	*0.00*	*0.00*	*0.03*	*0.01*	*0.00*
MIN	*0.00*	*0.00*	*0.01*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*
MAX	*0.01*	*0.13*	*0.33*	*1.54*	*0.01*	*0.06*	*0.21*	*0.20*	*0.02*

Appendix 8.5: (h) BALF epithelial cell counts ($x10^5$/ml) in control (C1-6) and heaves (H1-7) at 6h following challenge with saline, HDS-1[100], SUP, WP, WP[R], WF, WP/SUP[s], WP/SUP[m] and H/S B. MED. = median value, MIN. = minimum value, MAX. = maximum value.

	SALINE	HDS-1[100]	SUP	WP	WP[R]	WF	WP/SUP[s]	WP/SUP[m]	H/S B
C1	0.03	0.00	0.00	NP	NP	NP	NP	NP	0.00
C2	0.00	0.00	0.00	NP	NP	NP	NP	NP	0.00
C3	0.00	0.00	0.00	NP	NP	NP	NP	NP	0.00
C4	0.00	0.00	0.00	NP	NP	NP	NP	NP	0.00
C5	0.00	0.00	0.00	NP	NP	NP	NP	NP	0.00
C6	0.00	0.00	0.00	NP	NP	NP	NP	NP	0.00
MED.	*0.00*	*0.00*	*0.00*						*0.00*
MIN	*0.00*	*0.00*	*0.00*						*0.00*
MAX	*0.03*	*0.00*	*0.00*						*0.00*
H1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
H2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
H3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
H4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
H5	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
H6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
H7	0.00	0.00	0.00	0.00	NP	0.00	0.00	0.00	0.00
MED.	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*
MIN	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*
MAX	*0.10*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*

Appendix 9.1: Clinical scores in control (C1-6) and heaves (H1-7) horses prior to (0) and at 240min following inhalation challenge with saline, HDS-1 (HDS), LPS depleted HDS-1 (HDS-LPS) and LPS depleted HDS-1 with added back LPS (HDS-LPS+LPS). Score based on: a (tracheal auscultation), d (dyspnoea) and f (mucopurulent nasal discharge). NP = challenge not performed.

CHALLENGE	SALINE		HDS		HDS-LPS		HDS-LPS+LPS	
TIME PT. (min)	0	240	0	240	0	240	0	240
C1	0	0	0	0	0	0	NP	NP
C2	0	0	0	0	0	0	NP	NP
C3	0	0	0	0	0	0	NP	NP
C4	0	0	0	0	0	0	NP	NP
C5	0	0	0	0	0	0	NP	NP
C6	0	0	0	0	0	0	NP	NP
H1	0	0	0	0	0	0	0	0
H2	0	0	0	1 d	0	1 a	0	0
H3	0	0	0	0	0	0	0	0
H4	0	0	0	0	0	0	0	0
H5	0	0	0	0	0	0	0	2 a, f
H6	0	0	0	0	0	0	NP	NP
H7	0	0	0	0	0	0	0	0

Appendix 9.2a (i and ii): Lung function measurements in control (C1-6) and heaves (H1-5 and 7) horses prior to (0h) and at 5h following inhalation challenge with HDS-1[100]. MED. = median value, MIN. = minimum value, MAX. = maximum value.

(i)

	Cdyn (l/kPa)		dPpl (kPa)		RL_{iso} (kPa/l/sec)		RR (breaths/min.)		VT (l)		Wb' (J/min)		$RL_{E25\%}$ (kPa/l/sec)		$RL_{E50\%}$ (kPa/l/sec)		$RL_{E75\%}$ (kPa/l/sec)		$RL_{I25\%}$ (kPa/l/sec)		$RL_{I50\%}$ (kPa/l/sec)		$RL_{I75\%}$ (kPa/l/sec)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
C1	4.87	3.98	1.05	1.51	0.12	0.09	9.40	8.4	3.71	4.95	13.03	14.23	0.09	0.03	0.10	0.01	0.11	0.04	0.20	0.28	0.18	0.20	0.16	0.13
C2	11.13	15.45	0.54	0.54	0.04	0.05	8.35	8.55	4.38	4.73	6.21	8.08	0.06	0.05	0.05	0.04	0.03	0.03	0.12	0.15	0.08	0.09	0.07	0.08
C3	14.67	13.51	0.47	0.74	0.04	0.06	15.20	10.15	3.97	6.05	11.77	16.49	0.03	0.06	0.02	0.04	0.03	0.02	0.07	0.10	0.06	0.09	0.06	0.07
C4	5.33	4.97	1.47	1.83	0.35	0.41	7.15	7.15	3.77	3.88	17.84	22.75	0.29	0.39	0.19	0.27	0.28	0.35	0.43	0.47	0.38	0.40	0.31	0.36
C5	18.89	18.88	0.36	0.52	0.05	0.08	10.70	6.40	3.59	4.78	6.38	6.63	0.04	0.09	0.05	0.09	0.05	0.09	0.03	0.09	0.04	0.07	0.05	0.07
C6	16.23	8.63	0.61	0.84	0.08	0.06	8.40	8.25	4.61	4.15	8.76	9.42	0.03	0.04	0.04	0.10	0.07	0.07	0.12	0.18	0.09	0.23	0.09	0.10
MED.	*12.90*	*11.07*	*0.58*	*0.79*	*0.06*	*0.07*	*8.90*	*8.25*	*3.87*	*4.75*	*10.26*	*11.83*	*0.05*	*0.05*	*0.05*	*0.06*	*0.06*	*0.06*	*0.12*	*0.17*	*0.08*	*0.14*	*0.08*	*0.09*
MIN.	*4.87*	*3.98*	*0.36*	*0.52*	*0.04*	*0.05*	*7.15*	*6.40*	*3.59*	*3.88*	*6.21*	*6.63*	*0.03*	*0.03*	*0.02*	*0.01*	*0.03*	*0.02*	*0.03*	*0.09*	*0.04*	*0.07*	*0.05*	*0.07*
MAX.	*18.89*	*18.88*	*1.47*	*1.83*	*0.35*	*0.41*	*15.20*	*10.15*	*4.61*	*6.05*	*17.84*	*22.75*	*0.29*	*0.39*	*0.19*	*0.27*	*0.28*	*0.35*	*0.43*	*0.47*	*0.38*	*0.40*	*0.31*	*0.36*
H1	22.62	25.23	0.62	0.61	0.05	0.07	7.25	5.25	7.81	8.26	14.61	11.59	0.06	0.09	0.06	0.09	0.05	0.10	0.11	0.07	0.07	0.06	0.05	0.08
H2	43.80	46.18	0.34	0.41	0.04	0.05	8.05	7.05	5.34	5.87	6.38	7.58	0.01	0.04	0.04	0.04	0.05	0.06	0.04	0.05	0.04	0.05	0.05	0.06
H3	19.80	16.26	0.40	0.70	0.04	0.09	4.55	4.00	4.26	6.81	5.36	7.90	0.04	0.00	0.02	0.01	0.06	0.08	0.12	0.15	0.13	0.24	0.09	0.14
H4	40.77	28.59	0.28	0.37	0.04	0.06	10.85	9.40	4.19	4.11	6.26	7.22	0.01	0.03	0.04	0.05	0.04	0.07	0.04	0.07	0.04	0.07	0.04	0.06
H5	28.55	27.38	0.50	0.63	0.04	0.04	8.05	8.90	7.02	8.29	11.50	22.56	0.05	0.04	0.04	0.05	0.06	0.05	0.05	0.04	0.03	0.03	0.04	0.06
H7	27.43	38.35	0.57	0.64	0.06	0.07	15.95	10.65	4.72	5.24	25.36	22.74	0.06	0.07	0.06	0.08	0.07	0.10	0.05	0.07	0.06	0.07	0.07	0.08
MED.	*27.99*	*27.98*	*0.45*	*0.62*	*0.04*	*0.07*	*8.05*	*7.98*	*5.03*	*6.34*	*8.94*	*9.74*	*0.04*	*0.04*	*0.04*	*0.05*	*0.06*	*0.08*	*0.05*	*0.07*	*0.05*	*0.06*	*0.05*	*0.07*
MIN.	*19.80*	*16.26*	*0.28*	*0.37*	*0.04*	*0.04*	*4.55*	*4.00*	*4.19*	*4.11*	*5.36*	*7.22*	*0.01*	*0.00*	*0.02*	*0.01*	*0.04*	*0.05*	*0.04*	*0.04*	*0.03*	*0.03*	*0.04*	*0.06*
MAX.	*43.80*	*46.18*	*0.62*	*0.70*	*0.06*	*0.09*	*15.95*	*10.65*	*7.81*	*8.29*	*25.36*	*22.74*	*0.06*	*0.09*	*0.06*	*0.09*	*0.07*	*0.10*	*0.12*	*0.15*	*0.13*	*0.24*	*0.09*	*0.14*

(ii)

	T_E (sec)		T_I (sec)		T_I:T_E		V'_E (l/min)		V'_{Emax} (l/sec)		V'_{Imax} (l/sec)		Wb_{el} (J)		Wb_{res} (J)		Wb_{Eres} (J)		Wb_{Ires} (J)		Wb_{tot} (J)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
C1	2.94	3.37	3.36	3.44	1.16	1.02	34.55	42.06	2.09	2.61	1.94	3.30	1.37	3.22	1.38	1.60	0.53	0.10	0.85	1.70	2.22	4.92
C2	3.92	3.58	3.31	3.51	0.84	0.99	36.66	40.32	2.58	2.62	2.09	2.18	0.92	0.93	0.74	0.90	0.12	0.23	0.62	0.68	1.54	1.61
C3	2.15	3.38	1.83	2.59	0.86	0.77	60.20	61.04	2.89	3.55	3.38	3.65	0.55	1.42	0.78	1.65	0.21	0.45	0.57	1.21	1.12	2.62
C4	4.61	4.62	3.85	3.71	0.84	0.81	26.89	27.55	1.33	1.30	1.47	1.60	1.36	1.51	2.52	3.14	1.04	1.34	1.48	1.80	2.85	3.31
C5	3.07	5.24	2.63	4.05	0.88	0.78	39.19	30.64	2.44	1.95	2.42	2.17	0.36	0.64	0.54	1.03	0.23	0.44	0.31	0.59	0.68	1.23
C6	4.34	4.10	3.50	3.24	0.86	0.76	38.73	33.01	2.07	1.93	2.55	2.27	0.81	1.09	1.17	1.14	0.42	0.40	0.75	0.75	1.56	1.84
MED.	*3.49*	*3.84*	*3.33*	*3.47*	*0.86*	*0.80*	*37.70*	*33.01*	*2.27*	*2.28*	*2.26*	*2.22*	*0.86*	*1.25*	*0.98*	*1.37*	*0.32*	*0.42*	*0.69*	*0.98*	*1.55*	*2.23*
MIN.	*2.15*	*3.37*	*1.83*	*2.59*	*0.84*	*0.76*	*26.89*	*27.55*	*1.33*	*1.30*	*1.47*	*1.60*	*0.36*	*0.64*	*0.54*	*0.90*	*0.12*	*0.10*	*0.31*	*0.59*	*0.68*	*1.23*
MAX.	*4.61*	*5.24*	*3.85*	*4.05*	*1.16*	*1.02*	*60.20*	*61.04*	*2.89*	*3.55*	*3.38*	*3.65*	*1.37*	*3.22*	*2.52*	*3.14*	*1.04*	*1.34*	*1.48*	*1.80*	*2.85*	*4.92*
H1	3.67	5.38	4.71	6.32	1.29	1.20	56.24	42.96	4.75	2.92	3.32	2.90	1.40	1.41	2.03	2.25	1.02	1.32	1.00	0.93	2.40	2.34
H2	4.11	5.02	3.37	3.56	0.82	0.72	42.94	41.15	2.43	2.44	2.80	2.82	0.33	0.42	0.79	1.09	0.43	0.52	0.37	0.56	0.70	0.98
H3	3.95	8.58	5.66	6.15	1.92	0.72	19.65	27.41	1.70	2.45	2.53	2.74	0.73	1.33	0.84	1.94	0.22	0.84	0.62	1.10	1.35	2.43
H4	2.82	3.38	2.50	3.02	0.89	0.89	44.97	38.02	2.24	2.10	2.76	2.38	0.23	0.32	0.56	0.77	0.27	0.38	0.29	0.39	0.51	0.71
H5	4.08	3.36	3.20	2.88	0.79	0.87	56.43	73.40	3.28	4.74	3.91	5.08	0.89	1.22	1.39	2.28	0.59	0.99	0.80	1.29	1.68	2.51
H7	1.94	2.55	1.87	3.04	0.99	1.22	74.85	56.93	3.61	3.08	3.69	3.07	0.44	0.44	1.58	1.88	0.79	1.04	0.79	0.84	1.23	1.29
MED.	*3.81*	*4.20*	*3.29*	*3.30*	*0.94*	*0.88*	*50.61*	*42.05*	*2.86*	*2.69*	*3.06*	*2.86*	*0.58*	*0.83*	*1.12*	*1.91*	*0.51*	*0.92*	*0.71*	*0.89*	*1.29*	*1.81*
MIN.	*1.94*	*2.55*	*1.87*	*2.88*	*0.79*	*0.72*	*19.65*	*27.41*	*1.70*	*2.10*	*2.53*	*2.38*	*0.23*	*0.32*	*0.56*	*0.77*	*0.22*	*0.38*	*0.29*	*0.39*	*0.51*	*0.71*
MAX.	*4.11*	*8.58*	*5.66*	*6.32*	*1.92*	*1.22*	*74.85*	*73.40*	*4.75*	*4.74*	*3.91*	*5.08*	*1.40*	*1.41*	*2.03*	*2.28*	*1.02*	*1.32*	*1.00*	*1.29*	*2.40*	*2.51*

Appendix 9.2b (i and ii): Lung function measurements in control (C1-6) and heaves (H1-5 and 7) horses prior to (0h) and at 5h following inhalation challenge with LPS-depleted HDS (HDS-LPS). MED. = median value, MIN. = minimum value, MAX. = maximum value.

(i)

	Cdyn (l/kPa)		dPpl (kPa)		RL_{iso} (kPa/l/sec)		RR (breaths/min.)		VT (l)		Wb' (J/min)		$RL_{E25\%}$ (kPa/l/sec)		$RL_{E50\%}$ (kPa/l/sec)		$RL_{E75\%}$ (kPa/l/sec)		$RL_{I25\%}$ (kPa/l/sec)		$RL_{I50\%}$ (kPa/l/sec)		$RL_{I75\%}$ (kPa/l/sec)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
C1	8.59	6.30	0.67	0.69	0.08	0.09	9.95	21.30	3.64	2.04	7.81	11.13	0.02	0.08	0.02	0.09	0.05	0.11	0.15	0.08	0.12	0.10	0.10	0.11
C2	26.29	14.69	0.59	0.56	0.08	0.06	7.00	6.80	5.06	5.13	9.66	7.78	0.07	0.07	0.06	0.05	0.09	0.01	0.12	0.18	0.10	0.11	0.12	0.10
C3	12.35	6.78	0.55	0.90	0.05	0.08	12.65	12.95	3.83	4.07	10.38	17.62	0.06	0.06	0.05	0.04	0.05	0.05	0.07	0.15	0.06	0.15	0.06	0.13
C4	10.08	8.41	0.59	0.89	0.14	0.18	6.40	5.75	3.76	4.34	5.43	8.79	0.00	0.09	0.01	0.00	0.05	0.04	0.27	0.39	0.26	0.34	0.15	0.23
C5	16.30	16.38	0.57	0.40	0.04	0.06	12.45	11.25	4.92	3.13	11.06	5.94	0.04	0.04	0.06	0.05	0.07	0.07	0.04	0.05	0.03	0.06	0.05	0.08
C6	17.35	19.12	0.40	0.69	0.03	0.07	54.70	34.60	1.66	2.89	14.67	19.07	0.03	0.07	0.03	0.06	0.04	0.06	0.01	0.16	0.02	0.15	0.02	0.08
MED.	*14.32*	*11.55*	*0.58*	*0.69*	*0.06*	*0.07*	*11.20*	*12.10*	*3.79*	*3.60*	*10.02*	*9.96*	*0.04*	*0.07*	*0.04*	*0.05*	*0.05*	*0.06*	*0.09*	*0.16*	*0.08*	*0.13*	*0.08*	*0.10*
MIN.	*8.59*	*6.30*	*0.40*	*0.40*	*0.03*	*0.06*	*6.40*	*5.75*	*1.66*	*2.04*	*5.43*	*5.94*	*0.00*	*0.04*	*0.01*	*0.00*	*0.04*	*0.01*	*0.01*	*0.05*	*0.02*	*0.06*	*0.02*	*0.08*
MAX.	*26.29*	*19.12*	*0.67*	*0.90*	*0.14*	*0.18*	*54.70*	*34.60*	*5.06*	*5.13*	*11.06*	*19.07*	*0.07*	*0.09*	*0.06*	*0.09*	*0.09*	*0.11*	*0.27*	*0.39*	*0.26*	*0.34*	*0.15*	*0.23*
H1	18.65	23.72	0.68	0.64	0.06	0.07	6.50	5.75	8.05	8.86	14.68	15.64	0.09	0.10	0.10	0.15	0.07	0.09	0.08	0.05	0.06	0.05	0.05	0.06
H2	51.39	36.40	0.51	1.24	0.06	0.079	5.50	6.9	7.96	7.56	11.41	19.79	0.03	0.08	0.05	0.08	0.07	0.11	0.06	0.085	0.06	0.149	0.05	0.079
H3	12.46	9.21	0.64	0.82	0.09	0.09	6.65	5.55	4.77	5.32	9.11	10.24	0.08	0.06	0.05	0.02	0.06	0.05	0.71	0.15	0.69	0.20	0.78	0.15
H4	23.87	23.10	0.51	0.53	0.05	0.05	6.60	7.85	7.17	7.19	9.50	9.48	0.01	0.01	0.04	0.04	0.06	0.05	0.06	0.06	0.06	0.06	0.05	0.08
H5	22.47	23.32	0.61	0.47	0.04	0.04	10.40	5.5	7.78	7.52	11.64	5.76	0.09	0.06	0.08	0.06	0.11	0.07	0.05	0.03	0.03	0.03	0.04	0.03
H7	15.82	18.05	0.80	0.75	0.08	0.09	10.10	9.40	6.14	5.96	23.19	24.48	0.07	0.09	0.06	0.09	0.08	0.11	0.09	0.10	0.08	0.11	0.08	0.11
MED.	*20.56*	*23.10*	*0.63*	*0.64*	*0.06*	*0.07*	*6.63*	*6.80*	*7.47*	*7.19*	*11.52*	*10.24*	*0.08*	*0.06*	*0.06*	*0.06*	*0.07*	*0.07*	*0.07*	*0.06*	*0.06*	*0.06*	*0.05*	*0.08*
MIN.	*12.46*	*9.21*	*0.51*	*0.47*	*0.04*	*0.04*	*5.50*	*5.55*	*4.77*	*5.32*	*9.11*	*5.76*	*0.01*	*0.01*	*0.04*	*0.02*	*0.06*	*0.05*	*0.05*	*0.03*	*0.03*	*0.03*	*0.04*	*0.03*
MAX.	*51.39*	*23.72*	*0.80*	*0.82*	*0.09*	*0.09*	*10.40*	*9.40*	*8.05*	*8.86*	*23.19*	*24.48*	*0.09*	*0.10*	*0.10*	*0.15*	*0.11*	*0.11*	*0.71*	*0.15*	*0.69*	*0.20*	*0.78*	*0.15*

(ii)

	T_E (sec)		T_I (sec)		T_I:T_E		V'_E (l/min)		V'_{Emax} (l/sec)		V'_{Imax} (l/sec)		Wb_{el} (J)		Wb_{res} (J)		Wb_{Eres} (J)		Wb_{Ires} (J)		Wb_{tot} (J)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
C1	2.92	1.92	3.24	1.65	1.14	1.03	36.55	37.08	1.90	1.69	2.31	3.07	0.81	0.41	0.79	0.62	0.15	0.18	0.64	0.44	1.45	0.85
C2	4.89	4.96	3.74	3.79	0.79	0.76	35.37	34.96	1.90	2.45	2.25	2.44	0.50	0.94	1.35	1.13	0.46	0.22	0.90	0.91	1.40	1.86
C3	2.35	2.38	2.36	2.23	1.05	0.94	48.31	52.83	3.16	3.58	3.03	2.92	0.63	1.24	0.79	1.35	0.35	0.30	0.44	1.05	1.07	2.29
C4	5.37	6.39	4.04	4.21	0.77	0.67	24.19	24.82	1.33	1.35	1.60	1.86	0.68	1.11	0.84	1.55	0.17	0.24	0.67	1.31	1.34	2.42
C5	2.66	3.11	2.27	2.26	0.86	0.73	60.95	35.11	3.30	1.85	3.36	2.09	0.80	0.31	0.91	0.53	0.41	0.18	0.50	0.35	1.30	0.66
C6	0.52	1.65	0.49	2.06	1.02	1.24	92.24	62.02	5.73	3.36	4.84	3.02	0.15	0.74	0.19	0.90	0.01	0.22	0.18	0.68	0.03	1.42
MED.	*2.79*	*2.74*	*2.80*	*2.24*	*0.94*	*0.85*	*42.43*	*36.10*	*2.53*	*2.15*	*2.67*	*2.68*	*0.65*	*0.84*	*0.82*	*1.02*	*0.26*	*0.22*	*0.57*	*0.80*	*1.32*	*1.64*
MIN.	*0.52*	*1.65*	*0.49*	*1.65*	*0.77*	*0.67*	*24.19*	*24.82*	*1.33*	*1.35*	*1.60*	*1.86*	*0.15*	*0.31*	*0.19*	*0.53*	*0.01*	*0.18*	*0.18*	*0.35*	*0.03*	*0.66*
MAX.	*5.37*	*6.39*	*4.04*	*4.21*	*1.14*	*1.24*	*92.24*	*62.02*	*5.73*	*3.58*	*4.84*	*3.07*	*0.81*	*1.24*	*1.35*	*1.55*	*0.46*	*0.30*	*0.90*	*1.31*	*1.45*	*2.42*
H1	4.46	5.24	4.90	5.24	1.14	1.04	52.28	50.88	4.26	3.95	3.03	2.86	1.79	1.70	2.26	2.66	1.37	1.68	0.89	0.98	2.69	2.68
H2	6.26	5.06	4.64	4.03	0.74	1.020	43.80	45.50	2.92	2.594	4.02	3.980	0.63	2.65	2.07	2.95	1.16	1.15	0.91	1.800	1.53	4.448
H3	5.23	5.62	3.63	4.19	0.79	1.69	31.68	29.42	2.58	1.90	3.04	2.87	0.93	1.68	1.81	1.57	1.10	0.42	0.71	1.15	1.63	2.83
H4	5.32	5.06	4.08	4.19	0.78	0.84	47.35	55.95	2.69	2.99	3.42	3.67	1.12	1.09	1.46	1.47	0.80	0.75	0.66	0.72	1.78	1.81
H5	4.99	6.46	4.92	4.35	1.04	0.67	79.27	38.69	2.20	2.08	2.85	3.13	1.59	1.38	1.91	1.04	1.08	0.48	0.82	0.56	2.41	1.94
H7	2.90	2.78	3.05	3.61	1.06	1.31	62.49	56.35	3.24	3.09	3.35	2.58	1.25	1.05	2.26	2.49	1.00	1.39	1.26	1.11	2.51	2.15
MED.	*5.11*	*5.24*	*4.36*	*4.19*	*0.92*	*1.04*	*49.82*	*48.19*	*2.81*	*2.99*	*3.20*	*2.87*	*1.18*	*1.38*	*1.99*	*1.57*	*1.09*	*0.75*	*0.86*	*0.98*	*2.10*	*2.15*
MIN.	*2.90*	*2.78*	*3.05*	*3.61*	*0.74*	*0.67*	*31.68*	*29.42*	*2.20*	*1.90*	*2.85*	*2.58*	*0.63*	*1.05*	*1.46*	*1.04*	*0.80*	*0.42*	*0.66*	*0.56*	*1.53*	*1.81*
MAX.	*6.26*	*6.46*	*4.92*	*5.24*	*1.14*	*1.69*	*79.27*	*56.35*	*4.26*	*3.95*	*4.02*	*3.67*	*1.79*	*1.70*	*2.26*	*2.66*	*1.37*	*1.68*	*1.26*	*1.15*	*2.69*	*2.83*

Appendix 9.2c (i and ii): Lung function measurements in heaves (H1-5 and 7) horses prior to (0h) and at 5h following inhalation challenge with HDS-LPS with added back LPS (HDS-LPS+LPS). MED. = median value, MIN. = minimum value, MAX. = maximum value.

(i)

	Cdyn (l/kPa)		dPpl (kPa)		RL_{iso} (kPa/l/sec)		RR (breaths/min.)		VT (l)		Wb' (J/min)		$RL_{E25\%}$ (kPa/l/sec)		$RL_{E50\%}$ (kPa/l/sec)		$RL_{E75\%}$ (kPa/l/sec)		$RL_{I25\%}$ (kPa/l/sec)		$RL_{I50\%}$ (kPa/l/sec)		$RL_{I75\%}$ (kPa/l/sec)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
H1	25.00	18.96	0.78	0.92	0.07	0.07	7.40	7.55	8.00	9.44	21.68	31.56	0.11	0.11	0.10	0.11	0.10	0.10	0.05	0.05	0.06	0.06	0.07	0.06
H2	76.89	32.02	0.44	0.84	0.04	0.11	10.45	7.05	6.19	6.30	14.80	18.90	0.02	0.10	0.04	0.10	0.06	0.14	0.04	0.10	0.04	0.10	0.05	0.13
H3	9.77	6.95	0.77	1.03	0.09	0.18	6.50	5.85	5.09	4.94	9.08	11.10	0.07	0.12	0.02	0.00	0.06	0.11	0.15	0.25	0.25	0.41	0.14	0.23
H4	37.44	19.65	0.41	0.70	0.04	0.11	10.75	11.60	5.77	4.13	11.19	17.91	0.04	0.10	0.06	0.10	0.05	0.13	0.03	0.07	0.03	0.09	0.04	0.12
H5	28.22	23.45	0.49	0.58	0.04	0.04	7.55	7.00	7.56	8.03	11.66	12.19	0.03	0.05	0.05	0.06	0.06	0.08	0.03	0.03	0.04	0.03	0.05	0.06
H7	23.29	14.70	0.60	0.81	0.05	0.08	13.55	9.80	6.01	6.02	24.74	21.86	0.05	0.10	0.05	0.09	0.06	0.09	0.04	0.07	0.05	0.08	0.06	0.09
MED.	*26.61*	*19.31*	*0.54*	*0.82*	*0.05*	*0.09*	*9.00*	*7.30*	*6.10*	*6.16*	*13.23*	*18.41*	*0.05*	*0.10*	*0.05*	*0.09*	*0.06*	*0.10*	*0.04*	*0.07*	*0.04*	*0.08*	*0.06*	*0.11*
MIN.	*9.77*	*6.95*	*0.41*	*0.58*	*0.04*	*0.04*	*6.50*	*5.85*	*5.09*	*4.13*	*9.08*	*11.10*	*0.02*	*0.05*	*0.02*	*0.00*	*0.05*	*0.08*	*0.03*	*0.03*	*0.03*	*0.03*	*0.04*	*0.06*
MAX.	*76.89*	*32.02*	*0.78*	*1.03*	*0.09*	*0.18*	*13.55*	*11.60*	*8.00*	*9.44*	*24.74*	*31.56*	*0.11*	*0.12*	*0.10*	*0.11*	*0.10*	*0.14*	*0.15*	*0.25*	*0.25*	*0.41*	*0.14*	*0.23*

(ii)

	T_E (sec)		T_I (sec)		$T_I:T_E$		V'_E (l/min)		V'_{Emax} (l/sec)		V'_{Imax} (l/sec)		Wb_{el} (J)		Wb_{res} (J)		Wb_{Eres} (J)		Wb_{Ires} (J)		Wb_{tot} (J)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
H1	3.78	3.77	4.31	4.29	1.15	1.14	59.05	70.91	4.05	5.92	2.93	3.29	1.27	2.35	2.94	4.26	1.83	2.70	1.11	1.56	2.38	3.91
H2	3.23	5.13	2.50	3.46	0.79	0.68	64.40	44.14	3.23	2.35	3.68	2.57	0.26	0.64	1.42	2.71	0.61	1.27	0.81	1.45	1.06	2.09
H3	4.53	5.53	4.59	4.79	2.17	0.87	33.39	28.80	2.40	2.01	3.05	2.38	1.61	1.76	1.36	1.90	0.36	0.62	1.00	1.28	2.61	3.04
H4	3.13	3.15	2.54	2.16	0.82	0.70	61.81	47.82	3.27	2.05	3.59	2.70	0.48	0.48	1.06	1.53	0.52	0.68	0.53	0.85	1.02	1.32
H5	4.70	4.96	3.03	3.38	0.64	0.69	57.41	55.88	3.70	3.76	4.29	4.01	0.96	1.39	1.43	1.70	0.62	0.58	0.81	1.13	1.77	2.51
H7	2.30	3.17	2.15	2.97	0.95	0.94	81.53	59.00	3.92	3.11	4.10	2.77	0.79	1.24	1.85	2.20	0.83	1.12	1.01	1.08	1.80	2.32
MED.	*3.50*	*4.36*	*2.79*	*3.42*	*0.89*	*0.78*	*60.43*	*51.85*	*3.48*	*2.73*	*3.64*	*2.74*	*0.87*	*1.31*	*1.42*	*2.05*	*0.62*	*0.90*	*0.90*	*1.20*	*1.78*	*2.42*
MIN.	*2.30*	*3.15*	*2.15*	*2.16*	*0.64*	*0.68*	*33.39*	*28.80*	*2.40*	*2.01*	*2.93*	*2.38*	*0.26*	*0.48*	*1.06*	*1.53*	*0.36*	*0.58*	*0.53*	*0.85*	*1.02*	*1.32*
MAX.	*4.70*	*5.53*	*4.59*	*4.79*	*2.17*	*1.14*	*81.53*	*70.91*	*4.05*	*5.92*	*4.29*	*4.01*	*1.61*	*2.35*	*2.94*	*4.26*	*1.83*	*2.70*	*1.11*	*1.56*	*2.61*	*3.91*

Appendix 9.3: PCCdyn70 values (mg/ml) in control (C1-6) and heaves (H1-5 and 7) horses at 5h following inhalation challenge with HDS-1[100], LPS-depleted HDS (HDS-LPS) and HDS-LPS with added back LPS (HDS-LPS+LPS). MED. = median value, MIN. = minimum value, MAX. = maximum value.

	HDS-1[100]	HDS-LPS	HDS-LPS+LPS
C1	3.17	1.67	NP
C2	7.08	3.32	NP
C3	5.25	3.43	NP
C4	2.07	2.70	NP
C5	4.69	0.34	NP
C6	2.65	1.40	NP
MED.	*4.69*	*2.19*	
MIN.	*2.07*	*0.34*	
MAX.	*7.08*	*3.43*	
H1	8.16	9.21	6.18
H2	6.91	1.39	2.71
H3	4.26	3.98	4.46
H4	11.43	10.88	7.16
H5	9.96	12.02	4.71
H7	6.20	4.69	5.34
MED.	*7.53*	*6.95*	*5.02*
MIN.	*4.26*	*1.39*	*2.71*
MAX.	*11.43*	*12.02*	*7.16*

Appendix 9.4: BALF total nucleated cell (TCC), lymphocyte, macrophage, neutrophil, mast cell, basiphiloid cell, eosinophil and epithelial cell counts (x10^5/ml) in heaves (H1-5 and 7) at 6h following inhalation challenge with saline, HDS-1[100], LPS-depleted HDS (HDS-LPS) and HDS-LPS with added back LPS (HDS-L+L). MED. = median value, MIN. = minimum value, MAX. = maximum value.

TCC

	SALINE	HDS-1[100]	HDS-LPS	HDS-L+L
C1	5.10	3.00	1.80	NP
C2	3.90	4.30	3.20	NP
C3	2.00	1.60	3.20	NP
C4	2.90	8.00	3.80	NP
C5	9.60	5.00	4.40	NP
C6	3.80	5.10	2.80	NP
MED.	*3.85*	*4.65*	*3.20*	
MIN	*2.00*	*1.60*	*1.80*	
MAX	*9.60*	*8.00*	*4.40*	
H1	5.60	4.00	3.20	5.50
H2	3.40	3.70	4.10	3.90
H3	3.80	6.20	3.00	4.00
H4	5.50	4.10	4.50	3.40
H5	3.20	7.00	6.20	6.00
H6	1.30	2.10	4.60	NP
H7	5.20	4.10	3.40	4.10
MED.	*3.80*	*4.10*	*4.10*	*4.00*
MIN	*1.30*	*2.10*	*3.00*	*0.00*
MAX	*5.60*	*7.00*	*6.20*	*6.00*

Lymphocytes

	SALINE	HDS-1[100]	HDS-LPS	HDS-L+L
C1	1.89	0.66	0.54	NP
C2	1.28	1.24	0.84	NP
C3	0.99	0.83	1.87	NP
C4	0.99	1.74	1.25	NP
C5	6.22	2.29	1.67	NP
C6	2.41	2.23	1.16	NP
MED.	*1.59*	*1.49*	*1.20*	
MIN	*0.99*	*0.66*	*0.54*	
MAX	*6.22*	*2.29*	*1.87*	
H1	3.33	1.62	1.16	2.35
H2	1.54	0.61	0.90	0.61
H3	2.09	1.19	1.13	0.78
H4	2.36	1.75	1.85	1.36
H5	1.64	1.86	2.43	0.68
H6	0.80	0.81	2.42	NP
H7	2.57	1.12	1.58	1.99
MED.	*2.09*	*1.19*	*1.58*	*1.07*
MIN	*0.80*	*0.61*	*0.90*	*0.61*
MAX	*3.33*	*1.86*	*2.43*	*2.35*

Macrophages

	SALINE	HDS-1[100]	HDS-LPS	HDS-L+L
C1	2.80	1.72	1.00	NP
C2	2.25	2.75	2.13	NP
C3	0.73	0.51	0.78	NP
C4	1.71	4.19	2.19	NP
C5	2.57	1.91	2.11	NP
C6	1.14	2.46	1.39	NP
MED.	*1.98*	*2.18*	*1.75*	
MIN	*0.73*	*0.51*	*0.78*	
MAX	*2.80*	*4.19*	*2.19*	
H1	1.96	1.16	1.37	1.18
H2	1.58	0.41	0.67	0.66
H3	1.24	1.75	1.23	1.10
H4	2.96	1.48	2.16	1.38
H5	1.27	1.18	2.11	1.01
H6	0.34	0.50	0.84	NP
H7	2.40	0.77	1.18	1.03
MED.	*1.58*	*1.16*	*1.23*	*1.07*
MIN	*0.34*	*0.41*	*0.67*	*0.66*
MAX	*2.96*	*1.75*	*2.16*	*1.38*

Neutrophils

	SALINE	HDS-1[100]	HDS-LPS	HDS-L+L
C1	0.04	0.29	0.08	NP
C2	0.01	0.12	0.04	NP
C3	0.02	0.08	0.11	NP
C4	0.09	0.56	0.07	NP
C5	0.17	0.66	0.15	NP
C6	0.09	0.28	0.18	NP
MED.	*0.06*	*0.28*	*0.09*	
MIN	*0.01*	*0.08*	*0.04*	
MAX	*0.17*	*0.66*	*0.18*	
H1	0.20	1.11	0.48	1.78
H2	0.08	2.60	2.48	2.55
H3	0.17	3.14	0.47	1.95
H4	0.03	0.80	0.44	0.56
H5	0.06	3.81	1.62	4.28
H6	0.06	0.54	1.07	NP
H7	0.06	2.17	0.61	0.94
MED.	*0.06*	*2.17*	*0.61*	*1.87*
MIN	*0.03*	*0.54*	*0.44*	*0.56*
MAX	*0.20*	*3.81*	*2.48*	*4.28*

Mast cells

	SALINE	HDS-1[100]	HDS-LPS	HDS-L+L
C1	0.23	0.13	0.17	NP
C2	0.30	0.18	0.12	NP
C3	0.26	0.12	0.30	NP
C4	0.06	0.24	0.06	NP
C5	0.39	0.15	0.25	NP
C6	0.09	0.11	0.05	NP
MED.	*0.25*	*0.14*	*0.15*	
MIN	*0.06*	*0.11*	*0.05*	
MAX	*0.39*	*0.24*	*0.30*	
H1	0.11	0.08	0.10	0.18
H2	0.15	0.07	0.05	0.08
H3	0.19	0.11	0.14	0.14
H4	0.13	0.05	0.03	0.05
H5	0.09	0.02	0.04	0.00
H6	0.10	0.14	0.21	NP
H7	0.17	0.02	0.01	0.05
MED.	*0.13*	*0.07*	*0.05*	*0.07*
MIN	*0.09*	*0.02*	*0.01*	*0.00*
MAX	*0.19*	*0.14*	*0.21*	*0.18*

Basiphiloid cells

	SALINE	HDS-1[100]	HDS-LPS	HDS-L+L
C1	0.07	0.20	0.01	NP
C2	0.01	0.00	0.06	NP
C3	0.00	0.03	0.00	NP
C4	0.03	1.04	0.17	NP
C5	0.22	0.00	0.21	NP
C6	0.01	0.00	0.01	NP
MED.	*0.02*	*0.02*	*0.04*	
MIN	*0.00*	*0.00*	*0.00*	
MAX	*0.22*	*1.04*	*0.21*	
H1	0.01	0.00	0.08	0.00
H2	0.05	0.00	0.00	0.00
H3	0.11	0.00	0.04	0.02
H4	0.01	0.00	0.01	0.01
H5	0.02	0.00	0.00	0.02
H6	0.00	0.10	0.00	NP
H7	0.01	0.00	0.03	0.05
MED.	*0.01*	*0.00*	*0.01*	*0.01*
MIN	*0.00*	*0.00*	*0.00*	*0.00*
MAX	*0.11*	*0.10*	*0.08*	*0.05*

Eosinophils

	SALINE	HDS-1[100]	HDS-LPS	HDS-L+L
C1	0.05	0.00	0.00	NP
C2	0.06	0.01	0.00	NP
C3	0.00	0.04	0.14	NP
C4	0.00	0.23	0.06	NP
C5	0.02	0.00	0.01	NP
C6	0.06	0.02	0.00	NP
MED.	*0.03*	*0.01*	*0.00*	
MIN	*0.00*	*0.00*	*0.00*	
MAX	*0.06*	*0.23*	*0.14*	
H1	0.00	0.03	0.01	0.01
H2	0.00	0.00	0.00	0.00
H3	0.01	0.00	0.00	0.00
H4	0.01	0.01	0.00	0.04
H5	0.01	0.13	0.00	0.01
H6	0.01	0.01	0.06	NP
H7	0.00	0.02	0.00	0.03
MED.	*0.01*	*0.01*	*0.00*	*0.01*
MIN	*0.00*	*0.00*	*0.00*	*0.00*
MAX	*0.01*	*0.13*	*0.06*	*0.04*

Epithelial cells

	SALINE	HDS-1[100]	HDS-LPS	HDS-L+L
C1	0.03	0.00	0.00	NP
C2	0.00	0.00	0.00	NP
C3	0.00	0.00	0.00	NP
C4	0.00	0.00	0.00	NP
C5	0.00	0.00	0.00	NP
C6	0.00	0.00	0.00	NP
MED.	*0.00*	*0.00*	*0.00*	
MIN	*0.00*	*0.00*	*0.00*	
MAX	*0.03*	*0.00*	*0.00*	
H1	0.00	0.00	0.00	0.00
H2	0.00	0.00	0.00	0.00
H3	0.00	0.00	0.00	0.00
H4	0.00	0.00	0.00	0.00
H5	0.10	0.00	0.00	0.00
H6	0.00	0.00	0.00	NP
H7	0.00	0.00	0.00	0.00
MED.	*0.00*	*0.00*	*0.00*	*0.00*
MIN	*0.00*	*0.00*	*0.00*	*0.00*
MAX	*0.10*	*0.00*	*0.00*	*0.00*

Appendix 10.1 (i and ii): Lung function measurements in heaves (H1-7) horses prior to (0h) and at 5h following inhalation challenge with WP+LPS. MED. = median value, MIN. = minimum value, MAX. = maximum value.

(i)

	Cdyn (l/kPa)		dPpl (kPa)		RL_{iso} (kPa/l/sec)		RR (breaths/min.)		VT (l)		Wb' (J/min)		$RL_{E25\%}$ (kPa/l/sec)		$RL_{E50\%}$ (kPa/l/sec)		$RL_{E75\%}$ (kPa/l/sec)		$RL_{I25\%}$ (kPa/l/sec)		$RL_{I50\%}$ (kPa/l/sec)		$RL_{I75\%}$ (kPa/l/sec)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
H1	21.48	17.29	0.73	0.96	0.06	0.09	8.35	7.85	7.81	7.69	19.81	25.00	0.08	0.12	0.09	0.12	0.06	0.09	0.04	0.08	0.04	0.07	0.05	0.05
H2	46.81	20.39	0.47	1.19	0.05	0.14	7.50	6.90	6.12	7.56	10.52	31.39	0.04	0.15	0.06	0.12	0.09	0.18	0.06	0.12	0.05	0.13	0.05	0.15
H3	7.96	9.21	0.86	0.80	0.11	0.14	5.40	5.05	4.94	5.42	10.83	7.87	0.05	0.11	0.02	0.03	0.10	0.11	0.22	0.19	0.35	0.31	0.26	0.16
H4	27.98	22.11	0.76	0.61	0.06	0.05	7.85	6.40	7.34	6.98	15.64	11.61	0.09	0.14	0.07	0.08	0.08	0.09	0.07	0.07	0.05	0.03	0.05	0.06
H5	18.68	22.50	0.60	0.56	0.05	0.05	7.10	9.40	7.34	6.33	9.97	13.79	0.04	0.05	0.06	0.06	0.07	0.06	0.04	0.03	0.04	0.05	0.04	0.06
H6	9.17	6.81	0.70	0.97	0.11	0.15	10.45	8.30	4.17	3.95	11.19	13.21	0.09	0.13	0.10	0.14	0.10	0.18	0.25	0.26	0.24	0.16	0.08	0.15
H7	19.20	13.80	0.73	0.67	0.06	0.07	9.95	8.30	6.96	6.12	22.04	13.37	0.05	0.08	0.05	0.06	0.07	0.07	0.07	0.08	0.07	0.08	0.07	0.07
MED.	*19.20*	*17.29*	*0.73*	*0.80*	*0.06*	*0.09*	*7.85*	*7.85*	*6.96*	*6.33*	*11.19*	*13.37*	*0.05*	*0.12*	*0.06*	*0.08*	*0.08*	*0.09*	*0.07*	*0.08*	*0.05*	*0.08*	*0.05*	*0.07*
MIN.	*7.96*	*6.81*	*0.47*	*0.56*	*0.05*	*0.05*	*5.40*	*5.05*	*4.17*	*3.95*	*9.97*	*7.87*	*0.04*	*0.05*	*0.02*	*0.03*	*0.06*	*0.06*	*0.04*	*0.03*	*0.04*	*0.03*	*0.04*	*0.05*
MAX.	*46.81*	*22.50*	*0.86*	*1.19*	*0.11*	*0.15*	*10.45*	*9.40*	*7.81*	*7.69*	*22.04*	*31.39*	*0.09*	*0.15*	*0.10*	*0.14*	*0.10*	*0.18*	*0.25*	*0.26*	*0.35*	*0.31*	*0.26*	*0.16*

(ii)

	T_E (sec)		T_I (sec)		$T_I:T_E$		V'_E (l/min)		V'_{Emax} (l/sec)		V'_{Imax} (l/sec)		Wb_{el} (J)		Wb_{res} (J)		Wb_{Eres} (J)		Wb_{Ires} (J)		Wb_{Itot} (J)	
TP	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h	0h	5h
H1	3.27	3.32	4.13	4.40	1.32	1.37	65.02	60.13	4.93	4.84	3.77	3.05	1.40	1.74	2.39	3.18	1.60	2.12	0.79	1.06	2.20	2.80
H2	4.32	4.72	3.87	4.00	0.90	0.85	45.70	52.25	2.67	2.54	2.88	2.58	0.45	1.43	1.43	4.57	0.83	2.39	0.61	2.18	1.06	3.61
H3	5.48	6.24	5.12	5.54	1.22	0.89	26.77	27.59	1.82	1.58	2.47	2.34	1.70	1.61	1.82	1.54	0.64	0.63	1.19	0.91	2.89	2.52
H4	4.78	5.05	3.76	4.41	0.81	0.87	57.53	44.57	3.57	2.63	3.87	3.00	0.97	1.14	2.19	1.81	1.33	1.15	0.87	0.66	1.84	1.80
H5	5.17	3.67	3.32	2.63	0.65	0.73	52.38	59.25	2.90	3.37	3.90	3.88	1.45	0.90	1.40	1.44	0.52	0.58	0.88	0.86	2.32	1.76
H6	3.02	3.09	3.74	4.16	1.26	1.36	43.43	32.75	3.01	2.27	1.94	1.72	0.95	1.15	1.30	1.57	0.62	0.70	0.68	0.87	1.63	2.02
H7	2.81	3.47	3.19	3.67	1.14	1.06	69.45	50.80	3.78	2.79	3.25	2.53	1.25	1.39	2.21	1.59	1.11	0.81	1.10	0.78	2.35	2.17
MED.	*4.32*	*3.67*	*3.76*	*4.16*	*1.14*	*0.89*	*52.38*	*50.80*	*3.01*	*2.63*	*3.25*	*2.58*	*1.25*	*1.39*	*1.82*	*1.59*	*0.83*	*0.81*	*0.87*	*0.87*	*2.20*	*2.17*
MIN.	*2.81*	*3.09*	*3.19*	*2.63*	*0.65*	*0.73*	*26.77*	*27.59*	*1.82*	*1.58*	*1.94*	*1.72*	*0.45*	*0.90*	*1.30*	*1.44*	*0.52*	*0.58*	*0.61*	*0.66*	*1.06*	*1.76*
MAX.	*5.48*	*6.24*	*5.12*	*5.54*	*1.32*	*1.37*	*69.45*	*60.13*	*4.93*	*4.84*	*3.90*	*3.88*	*1.70*	*1.74*	*2.39*	*4.57*	*1.60*	*2.39*	*1.19*	*2.18*	*2.89*	*3.61*

Appendix 10.2: PCCdyn70 values (mg/ml) in heaves (H1-7) horses at 5h following inhalation challenge with saline, WP+LPS and HDS-1[100]. MED. = median value, MIN. = minimum value, MAX. = maximum value.

	SALINE	HDS-1[100]	WP+LPS
H1	7.86	8.16	5.63
H2	3.06	6.91	4.54
H3	5.64	4.26	5.74
H4	10.53	11.43	13.66
H5	6.40	9.96	3.48
H6	2.46	4.27	2.89
H7	5.63	6.20	4.39
MED.	*5.64*	*6.91*	*4.54*
MIN.	*2.46*	*4.26*	*2.89*
MAX.	*10.53*	*11.43*	*13.66*

Appendix 10.3(a): BALF total nucleated cell counts (x10^5/ml) in heaves (H1-7) horses at 6h following challenge with saline, 20μg LPS, HDS-1[100], WP, WP[R], WP+LPS, WP+LPS[R], WP/SUP[m] and WP/SUP[s]. MED. = median value, MIN. = minimum value, MAX. = maximum value.

	SALINE	20μg LPS	HDS-1	WP	WP[R]	WP+LPS	WP+LPS[R]	WP/SUP[M]	WP/SUP[S]
H1	5.60	6.30	4.00	5.50	3.10	2.80	3.30	5.40	5.50
H2	3.40	3.20	3.70	4.80	3.70	2.90	4.50	4.70	5.90
H3	3.80	4.10	6.20	5.00	3.50	4.20	11.00	10.20	13.70
H4	5.50	5.40	4.10	5.60	5.30	4.20	4.20	5.70	7.30
H5	3.20	4.60	7.00	7.80	3.90	3.80	4.40	7.80	19.00
H6	1.30	2.90	2.10	2.00	3.50	2.30	2.30	2.30	2.90
H7	5.20	7.40	4.10	9.10	NP	3.90	NP	5.30	4.40
MED.	*3.80*	*4.60*	*4.10*	*5.50*		*3.80*		*5.40*	*5.90*
MIN	*1.30*	*2.90*	*2.10*	*2.00*		*2.30*		*2.30*	*2.90*
MAX	*5.60*	*7.40*	*7.00*	*9.10*		*4.20*		*10.20*	*19.00*

Appendix 10.3(b): BALF lymphocyte counts (x10^5/ml) in heaves (H1-7) horses at 6h following challenge with saline, 20μg LPS, HDS-1[100], WP, WP[R], WP+LPS, WP+LPS[R], WP/SUP[m] and WP/SUP[s]. MED. = median value, MIN. = minimum value, MAX. = maximum value.

	SALINE	20μg LPS	HDS-1	WP	WP[R]	WP+LPS	WP+LPS[R]	WP/SUP[M]	WP/SUP[S]
H1	3.33	3.45	1.62	3.29	0.78	1.07	1.42	2.39	2.94
H2	1.54	2.04	0.61	2.21	1.19	0.81	1.06	1.09	1.33
H3	2.09	1.88	1.19	2.19	0.72	1.21	2.51	2.83	2.42
H4	2.36	2.45	1.75	1.98	1.81	2.05	1.22	2.33	2.47
H5	1.64	2.93	1.86	3.49	1.27	1.46	0.76	1.79	4.20
H6	0.80	1.93	0.81	0.95	1.52	1.38	0.94	1.14	1.56
H7	2.57	4.83	1.12	5.20	NP	2.08	NP	2.39	2.36
MED.	*2.09*	*2.45*	*1.19*	*2.21*	*1.23*	*1.38*	*1.14*	*2.33*	*2.42*
MIN	*0.80*	*1.88*	*0.61*	*0.95*	*0.72*	*0.81*	*0.76*	*1.09*	*1.33*
MAX	*3.33*	*4.83*	*1.86*	*5.20*	*1.81*	*2.08*	*2.51*	*2.83*	*4.20*

Appendix 10.3(c): BALF macrophage counts (x10^5/ml) in heaves (H1-7) horses at 6h following challenge with saline, 20μg LPS, HDS-1[100], WP, WP[R], WP+LPS, WP+LPS[R], WP/SUP[m] and WP/SUP[s]. MED. = median value, MIN. = minimum value, MAX. = maximum value.

	SALINE	20μg LPS	HDS-1	WP	WP[R]	WP+LPS	WP+LPS[R]	WP/SUP[M]	WP/SUP[S]
H1	1.96	2.26	1.16	1.68	2.15	0.89	1.28	2.21	1.78
H2	1.58	0.88	0.41	2.29	2.13	0.60	1.64	1.19	1.57
H3	1.24	1.68	1.75	1.99	2.47	1.23	3.18	4.81	5.14
H4	2.96	2.57	1.48	3.18	3.22	1.51	2.36	2.10	4.08
H5	1.27	1.15	1.18	3.92	1.82	0.75	0.88	1.37	5.30
H6	0.34	0.70	0.50	0.71	1.67	0.36	0.63	0.38	0.68
H7	2.40	1.94	0.77	1.65	NP	1.15	NP	1.46	1.67
MED.	*1.58*	*1.68*	*1.16*	*1.99*	*2.14*	*0.89*	*1.46*	*1.46*	*1.78*
MIN	*0.34*	*0.70*	*0.41*	*0.71*	*1.67*	*0.36*	*0.63*	*0.38*	*0.68*
MAX	*2.96*	*2.57*	*1.75*	*3.92*	*3.22*	*1.51*	*3.18*	*4.81*	*5.30*

Appendix 10.3(d): BALF neutrophil counts (x10^5/ml) in heaves (H1-7) horses at 6h following challenge with saline, 20μg LPS, HDS-1[100], WP, WP[R], WP+LPS, WP+LPS[R], WP/SUP[m] and WP/SUP[s]. MED. = median value, MIN. = minimum value, MAX. = maximum value.

	SALINE	20μg LPS	HDS-1	WP	WP[R]	WP+LPS	WP+LPS[R]	WP/SUP[M]	WP/SUP[S]
H1	0.20	0.39	1.11	0.21	0.08	0.71	0.45	0.43	0.27
H2	0.08	0.20	2.60	0.14	0.24	1.35	1.70	2.34	2.83
H3	0.17	0.23	3.14	0.65	0.20	1.62	5.08	2.35	5.90
H4	0.03	0.30	0.80	0.19	0.19	0.55	0.53	1.04	0.47
H5	0.06	0.28	3.81	0.19	0.79	1.54	2.74	4.55	9.29
H6	0.06	0.18	0.54	0.10	0.23	0.40	0.63	0.63	0.43
H7	0.06	0.53	2.17	0.56	NP	0.56	NP	1.36	0.12
MED.	*0.06*	*0.28*	*2.17*	*0.19*	*0.22*	*0.71*	*1.16*	*1.36*	*0.47*
MIN	*0.03*	*0.18*	*0.54*	*0.10*	*0.08*	*0.40*	*0.45*	*0.43*	*0.12*
MAX	*0.20*	*0.53*	*3.81*	*0.65*	*0.79*	*1.62*	*5.08*	*4.55*	*9.29*

Appendix 10.3(e): BALF mast cell counts (x10^5/ml) in heaves (H1-7) horses at 6h following challenge with saline, 20μg LPS, HDS-1[100], WP, WP[R], WP+LPS, WP+LPS[R], WP/SUP[m] and WP/SUP[s]. MED. = median value, MIN. = minimum value, MAX. = maximum value.

	SALINE	20μg LPS	HDS-1	WP	WP[R]	WP+LPS	WP+LPS[R]	WP/SUP[M]	WP/SUP[S]
H1	0.11	0.18	0.08	0.23	0.07	0.13	0.15	0.24	0.30
H2	0.15	0.07	0.07	0.12	0.13	0.09	0.08	0.07	0.17
H3	0.19	0.30	0.11	0.14	0.11	0.13	0.23	0.21	0.22
H4	0.13	0.07	0.05	0.09	0.07	0.09	0.08	0.03	0.14
H5	0.09	0.08	0.02	0.11	0.02	0.00	0.02	0.02	0.02
H6	0.10	0.07	0.14	0.19	0.06	0.14	0.11	0.14	0.19
H7	0.17	0.06	0.02	0.15	NP	0.08	NP	0.08	0.19
MED.	*0.13*	*0.07*	*0.07*	*0.14*	*0.07*	*0.09*	*0.09*	*0.08*	*0.19*
MIN	*0.09*	*0.06*	*0.02*	*0.09*	*0.02*	*0.00*	*0.02*	*0.02*	*0.02*
MAX	*0.19*	*0.30*	*0.14*	*0.23*	*0.13*	*0.14*	*0.23*	*0.24*	*0.30*

Appendix 10.3(f): BALF basiphiloid cell counts (x10^5/ml) in heaves (H1-7) horses at 6h following challenge with saline, 20μg LPS, HDS-1[100], WP, WP[R], WP+LPS, WP+LPS[R], WP/SUP[m] and WP/SUP[s]. MED. = median value, MIN. = minimum value, MAX. = maximum value.

	SALINE	20μg LPS	HDS-1	WP	WP[R]	WP+LPS	WP+LPS[R]	WP/SUP[M]	WP/SUP[S]
H1	0.01	0.00	0.00	0.02	0.01	0.00	0.00	0.00	0.00
H2	0.05	0.01	0.00	0.02	0.00	0.03	0.02	0.01	0.00
H3	0.11	0.01	0.00	0.00	0.00	0.01	0.00	0.00	0.01
H4	0.01	0.01	0.00	0.02	0.00	0.00	0.00	0.00	0.00
H5	0.02	0.12	0.00	0.02	0.00	0.02	0.00	0.02	0.00
H6	0.00	0.02	0.10	0.04	0.01	0.01	0.00	0.00	0.02
H7	0.01	0.01	0.00	0.02	NP	0.02	NP	0.00	0.02
MED.	*0.01*	*0.01*	*0.00*	*0.02*	*0.00*	*0.01*	*0.00*	*0.00*	*0.00*
MIN	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*
MAX	*0.11*	*0.12*	*0.10*	*0.04*	*0.01*	*0.03*	*0.02*	*0.02*	*0.02*

Appendix 10.3(g): BALF eosinophil counts (x10^5/ml) in heaves (H1-7) horses at 6h following challenge with saline, 20μg LPS, HDS-1[100], WP, WP[R], WP+LPS, WP+LPS[R], WP/SUP[m] and WP/SUP[s]. MED. = median value, MIN. = minimum value, MAX. = maximum value.

	SALINE	20μg LPS	HDS-1	WP	WP[R]	WP+LPS	WP+LPS[R]	WP/SUP[M]	WP/SUP[S]
H1	0.00	0.01	0.03	0.07	0.00	0.00	0.00	0.12	0.21
H2	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.00
H3	0.01	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00
H4	0.01	0.00	0.01	0.13	0.01	0.00	0.00	0.20	0.14
H5	0.01	0.00	0.13	0.08	0.00	0.03	0.00	0.06	0.19
H6	0.01	0.00	0.01	0.00	0.00	0.01	0.00	0.01	0.01
H7	0.00	0.01	0.02	1.54	NP	0.01	NP	0.01	0.03
MED.	*0.01*	*0.00*	*0.01*	*0.07*	*0.00*	*0.01*	*0.00*	*0.01*	*0.03*
MIN	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*
MAX	*0.01*	*0.01*	*0.13*	*1.54*	*0.01*	*0.03*	*0.00*	*0.20*	*0.21*

Appendix 10.3(h): BALF epithelial cell counts (x10^5/ml) in heaves (H1-7) horses at 6h following challenge with saline, 20μg LPS, HDS-1[100], WP, WP[R], WP+LPS, WP+LPS[R], WP/SUP[m] and WP/SUP[s]. MED. = median value, MIN. = minimum value, MAX. = maximum value.

	SALINE	20μg LPS	HDS-1	WP	WP[R]	WP+LPS	WP+LPS[R]	WP/SUP[M]	WP/SUP[S]
H1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
H2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
H3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
H4	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00
H5	0.10	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00
H6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
H7	0.00	0.02	0.00	0.00	NP	0.00	NP	0.00	0.00
MED.	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*
MIN	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*
MAX	*0.10*	*0.05*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*