Transportation of horses and the implications for health and welfare

by

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ABSTRACT

The equine industry is essential to the social and economic wellbeing of Australia, generating approximately $9 billion annually. Transportation is a crucial component of horse management, but there are serious welfare concerns and economic losses associated with transport-related health and behavioural problems. To try to prevent these problems the Australian Code of Land Transportation was published in 2012 with new standards and guidelines based on the most recent research. However, since the Code appeared no new research has been reported and there is no idea as to how the industry complies with the Code. The current literature only reports on epidemiological studies that affect the welfare of horses travelling to abattoirs. They do not consider what best practices should be applied and why horses develop disease after travelling (Chapter 1). Thus, while the physical and physiological stressors of each phase of transportation have been deeply described, there are still many gaps of knowledge on the impacts of transportation on horse health and welfare (Chapter 1).

The aim of this thesis was to fill some of these gaps of knowledge collecting data from the Australian transport industry and improving the understanding of the implications for performance horse health and welfare arising from transportation by road.

The hypothesis for the first part of the thesis was that there would be an association between the transport management and the development of transport-related behavioural and health problems during and after journeys. In order to address this, an epidemiological study and a questionnaire were undertaken. The epidemiological study aimed to investigate the incidence of transport-related diseases and risk factors for performance horses during a long journey (form Perth to Sydney). Only 3% of horses developed a transport-related disease and journey duration (>20 hours) and season (spring) were identified as risk factors (Chapter 2). The questionnaire explored the most common transport practices and issues in Australia across the different sectors of the equine industry. It was found that 67% of the respondents had experienced a transport-related problem moving their horses over the past two years and that equine transportation management was often not compliant with the Australian Code (Chapter 3.1). Significant associations between the transport management and the development of transport-related health problem were identified. Transport-related injuries were associated with the age of the horse keeper and the use of sedation and protective equipment pre-journey; diarrhoea and heat stroke were more likely to happen in journeys organised by amateurs than professionals (Chapter 3.2). Significant associations were also found between training procedures and transport-related problem behaviours: habituation and self-loading techniques reduced the risk of problem behaviours and subsequent injuries, but they were applied only by 20.2% and 10.8% of respondents (Chapter 3.3). Analysing 214 of transported-related health cases, respiratory diseases was the most frequently reported in Australia and journeys longer than 24 hours were seen to increase the risk for the development of a severe transport-related health problem (gastrointestinal and respiratory problems or death) (Chapter 3.4). Overall, the questionnaire highlighted the need for new welfare indicators, in particular for horses moved over long distances and for identifying horses at risk of respiratory diseases.

In order to address this need, the second part of the thesis was composed of two multidisciplinary studies, exploring the effects of a four day journey and of an eight hour journey on horse health and welfare, with particular emphasis on the immunological and respiratory systems (Chapters 4 and 5). The four day journey was associated with changes in the clinical examination, an acute phase response, impaired lymphocyte proliferation, dehydration and a mobilisation of antioxidants. The results supported the hypothesis that a horse's immunological capacity would be decreased after a long distance transportation predisposing to the development of more severe diseases (Chapter 4). The hypothesis of Chapter 5 was that stress related behavioural and physiological responses to transport would be associated with changes in clinical, haematological, oxidative and respiratory parameters. The tested eight hour journey without water and feed induced an acute phase response, dehydration, mobilisation of antioxidant, fatigue, electrolyte imbalance and an increase in mucus and bacteria (mainly *Pasteurellaceae*) in the lower respiratory tract. Significant association between the horse behaviour *en route*, particularly the time spent with the head in an elevated position and the frequency of stress related behaviours, and the increases in mucus, bacteria and free radicals were found. While transport-related health problems are multifactorial, clinical examination including auscultation before and after travel, behavioural observation *en route*, monitoring of redox-balance and fibrinogen concentrations may aid in the identification of horses at risk of transport-related respiratory disease (Chapter 5).

Overall, this thesis provides a better understanding of transportation stress and collects important data from the Australian transport and equine industry. A number of welfare indicators and management practices were recommended to safeguard horse welfare during transport, and new research areas were identified for further studies.

Keywords: transportation, welfare, health, horse, management.

DEDICATION

To Giampaolo,

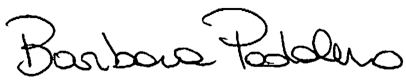
who has followed and supported his mum during this adventure

PREFACE

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

All Chapters of this thesis have been written in publication style. Chapters 1, 2, 3.1, 3.2, 3.3 3.4 and 4 have already been published in peer-reviewed journals; Barbara Padalino is first and corresponding author on all the papers. Sections of Chapter 5 are intended for publication in peer-reviewed journals with some modifications. The assistance to the candidate given by others is indicated in the authorship attribution statement and the acknowledgments.

I acknowledge that an electronic copy of my thesis must be lodged with the University Library and, subject to the General Award Rules of The University of Sydney, immediately made available for research and study in accordance with the Copyright Act 1968.



LIST OF PUBLICATIONS FROM THIS THESIS

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Padalino B, Hall E, Raidal SL, Celi P, Knight P, Jeffcott L, Muscatello G. 2015. Health problems and risk factors associated with long haul transport of horses in Australia. Animals, 5, 1296-1310, doi: 10.3390/ani5040412.

Padalino B, Raidal SL, Hall E, Celi P, Knight P, Jeffcott L, Muscatello G. 2016. Survey of horse transportation in Australia: issues and practices. Australian Veterinary Journal, 10, 349-357, doi:10.1111/avj.12486.

Padalino B, Raidal SL, Hall E, Celi P, Knight P, Jeffcott L, Muscatello G. 2016. A survey on transport management practices associated with injuries and health problems in horses. PLoS ONE, 9, 1-16, doi:10.1371/journal.pone.0162371.

Padalino B, Henshall C, Raidal SL, Celi P, Knight P, Jeffcott L, Muscatello G. 2017. Investigations into transport-related problem behaviours: survey results. Journal of Equine Veterinary Science, 48, 166-173, doi:10.1016/j.jevs.2016.07.001.

Padalino B, Raidal SL, Hall E, Celi P, Knight P, Jeffcott L, Muscatello G. 2016. Risk factors in equine transport-related health problems: a survey of the Australian equine industry. Equine veterinary Journal, doi: 10.1111/evj.12631.

Padalino B, Raidal SL, Carter N, Celi P, Muscatello G, Jeffcott L, de Silva K. 2017 Immunological, clinical, haematological and oxidative responses to long distance transportation in horses. Research in Veterinary Science, 115, 78-87, doi: 10.1016/j.rvsc.2017.01.024

AUTHORSHIP ATTRIBUTION STATEMENT

This thesis includes 7 original papers published in peer-reviewed journals. The core theme of the thesis is the understanding of the implications of transportation of horses on health and welfare.

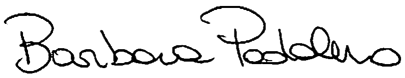
The ideas, development and writing up of all the papers in this thesis were the principal responsibility of the candidate, working under the supervision of Dr Gary Muscatello, Dr Pietro Celi and Prof Leo Jeffcott (Faculty of Veterinary Science, School of Life and Environmental Sciences, The University of Sydney) and of Associate Prof. Sharanne Lee Raidal (School of Animal and Veterinary Sciences, Charles Sturt University).

The inclusion of other co-authors (Dr Peter Knight, Dr Evelyn Hall, Dr Kumikunda de Silva, Ms Cathrynne Henshall, and Ms Nicole Carter) reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

Chapter 1 of this thesis is published as a review in Journal of Veterinary Behavior. I cohesive and wrote the manuscript.

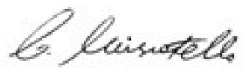
Chapter 2 of this thesis is published as a research article in Animals. I designed the study, analysed the data and wrote the drafts of the manuscript. Chapter 3 of this thesis includes 4 published research articles in Australian Veterinary Journal, PLoS ONE, Journal of Equine Veterinary Science and Equine Veterinary Journal, respectively. I co-designed the study with the co-authors, I interpreted and analysed the data, and wrote the drafts of the manuscripts.

Chapter 4 of this thesis is published as a research article in Research in Veterinary Science. I designed the study, conducted the experiment, analysed the data and wrote the drafts of the manuscript.



As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

Dr Gary Muscatello, 31/10/2016



CONFIRMATION OF CO-AUTHORSHIP OF PUBLISHED WORK

The following details the contribution of each of the co-authors to one or more peer-reviewed publications within this thesis.

Dr Gary Muscatello contributed to study design, laboratory analysis methods and finalizing of all manuscripts prior to publication.

Associate Prof Sharanne Raidal contributed to study design, animal experiments, laboratory analysis methods, statistical analysis, and finalizing of all manuscripts prior to publication.

Prof Leo Jeffcott contributed to study design and finalizing of all manuscripts prior to publication.

Dr Pietro Celi contributed to study design and laboratory analysis methods of all manuscripts.

Dr Peter Knight contributed to study design, and finalizing of the manuscripts (Chapters 2, 3.1, 3.2, 3.3, 3.4) prior to publication.

Dr Evelyn Hall contributed to study design, statistical analysis and finalizing of the manuscripts (Chapters 2, 3.1, 3.2, 3.4) prior to publication.

Dr Kumudika de Silva contributed to study design, laboratory analysis methods and finalizing of the manuscript (Chapter 4) prior to publication.

Mrs Catherine Hall contributed to statistical analysis and finalizing of the manuscript (Chapter 3.3) prior to publication.

Miss Nicole Carter contributed to study design, laboratory analysis methods of the manuscript (Chapter 4).

Candidate Signature

I, as a co-author, endorse that this level of contribution by myself and the candidate indicated above is appropriate.

Name	Signature
Dr Gary Muscatello	

Prof. Sharanne Raidal	
Prof Leo Jeffcott	
Dr Pietro Celi	
Dr Peter Knight	
Dr Evelyn Hall	
Dr Kumudika de Silva	
Ms Cathrynne Henshall	
Ms Nicole Carter	

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LIST OF ABBREVIATIONS

The following abbreviated terms have been used throughout the thesis and are defined at first use in each Chapter. Abbreviations used exclusively in tables are not listed and are defined below the table.

%	percent
µg	microgram(s)
µL	microliter(s)
µm	micrometer(s)
µmol	micromole(s)
AJ	after journey
AOPP	advanced oxidation protein products
APPs	acute phase proteins
BCS	body condition score
BJ	before journey
BW	body weight
CI	confidence interval
CFU	coliform forming units
CP	ceruloplasmin
CRT	capillary refill time
d	day(s)
DNA	deoxyribonucleic acid
g	gram(s)
GSH-Px	glutathione peroxidase
H	habituation
h	hour(s)
HI	heat index
kg	kilogram(s)
L	liter(s)
Log_{10}	logarithm base 10
LPB	loading problem behaviours
m	meter(s)
mg	milligram(s)
min	minute(s)
mL	milliliter(s)
mmol	millimole(s)
mM/L	millimole(s) per liter
NSW	New South Wales
NT	no training
^{o}C	degree Celsius
OC	operant conditioning
OR	odds ratio
OSI	oxidative stress index
OTU	operational taxonomic unit
P	probability
PCR	polymerase chain reaction
pH	hydrogen ion concentration

PLPB	preloading problem behaviours
PTAS	plasma total antioxidant status
qPCR	real-time polymerase chain reaction
r	correlation coefficient
R^2	R-squared (coefficient of correlation)
ROMs	reactive oxygen metabolites
s	second(s)
SB	Standardbred
SE	standard error
SL	self-loading
spp.	species
TB	thoroughbred
TPB	travelling problem behaviours
TRPB	transport-related problem behaviours
TW	tracheal wash
U. Carr	units of Carratelli
U. Cor	units of Cornelli; 1 U.Cor = 1.4 µMol/L of ascorbic acid
UK	United Kingdom
UPB	unloading problem behaviours
vs	versus
wk	week(s)

GENERAL INTRODUCTION

Introduction

Transportation is a part of everyday life for most horses, and much more so than for other farm animals (Friend, 2001). Unfortunately travelling horses by any means of transportation can cause deleterious effects (Leadon, 1994; Mansmann and Woodie, 1995; Weeks et al., 2012). There are therefore important welfare concerns, particularly with road transport (Broom, 2008), as well as potentially serious economic losses for the Australian horse industry (Gordon, 2001). The most common transport-related conditions are traumatic injuries, respiratory problems, colic, laminitis and thermal stress, with pneumonia and enterocolitis considered to be the most significant equine transport related diseases (Austin et al., 1995; Hillyer et al., 2002; Leadon, 2015; McClintock and Begg, 1990; Noble et al., 2013; Raidal et al., 1997a).

An international code of practice for farm animals being transported by road was published in Europe in 2005 (http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=uriserv:f83007). This code is also meant to cover horses, but a study showed that no journeys for horses transported for slaughter complied with this code, and in 65% of the examined journeys horses welfare was compromised resulting in horses arriving at the slaughterhouse in poor condition (Marlin et al., 2011). An Australian code for animal transportation was published in 2012 which set the maximum time without water at 24 hours, and proposed guidelines to assess fitness for travel and how the journey should be managed (http://www.animalwelfarestandards.net.au/land-transport/). However, Australia is a huge country and horses often have to travel great distances and in extreme conditions of heat and humidity compared to those in Europe. Therefore transport related equine health and welfare issues in Australia may be different from those in Europe. Since the publication of the Australian Code, there have been no studies examining horse movement practices in Australia, thus no statistics, no information on how owners/trainers comply with the Code and no data on the frequency and types of transport-related diseases and injuries have been reported.

The objective of this thesis was to address these gaps of knowledge, to increase our understanding of transportation and the implications for horse health and welfare, and to suggest some practical recommendations for safer travel. The research included in this thesis is based on two underlying hypotheses:

1. As a mental and physical stressor of horses, transportation will result in the development of behavioural and health problems during or after travelling by road;

there would be an association between the transport management and the development of those problems.

2. Horses' behaviour *en route*, physiology and the redox balance will be affected by transportation increasing oxidative stress and predisposing to diseases.

In order to address these hypotheses a number of relevant research questions were considered:

1. What are the most common diseases related to equine transportation in Australia?
2. Is there an association between transport management and development of disease?
3. How does transportation affect horse health and why is the respiratory system so susceptible?
4. How does transportation affect the equine immune system?
5. Does the stress of transportation reactivate equine respiratory viruses?
6. How does transportation affect the oxidative balance of the horse?
7. Can oxidative biomarkers be used to identify horses at risk of transport-related disease?
8. Can behavioural responses act as predictors for transport-related disease?
9. What management practices are likely to improve the welfare and wellbeing of the transported horse?

Outline of the thesis

A large amount of data was collected from the Australian transport industry for this PhD project. This comprised a unique multifactorial approach covering many aspects of travelling horses by road in Australia. The work undertaken has been incorporated into 5 Chapters. The first 4 Chapters have been published in appropriate peer-reviewed journals and the fifth one is intended for publication at a later date.

- <u>Chapter 1</u>: This review summarises the research on equine transportation over the last 30 years. The transportation process was divided into 5 phases – preloading handling, loading, transport itself, unloading, and adaptation to a new environment. The effects of each phase on horse health and welfare were described, and how best practice for each phase should be managed. The review highlights the gaps in knowledge, the areas of debate and where more research is needed.
- <u>Chapter 2</u>: This is the first epidemiological study conducted on horses undertaking a unique multi-day road trip across Australia. It analysed reports of transport-related

health problems identified by drivers and horse owners for 180 journeys between Perth and Sydney from 2013 to 2015. The journeys all complied with the Australian Code. Health problems occurred in 2.8% of the transported horses, and fatalities in 0.24%. Respiratory and gastrointestinal problems were the most common problems (27%); journey duration (> 20 hours) and season (i.e. spring) were identified as risk factors. Transportation was confirmed to be a human-related risk to horses and also a horse-related risk to humans. Moving horses over long distances successfully is possible, but it should be always carried out by experienced horse handlers who respect the welfare standards of transportation.

- Chapter 3: A survey was developed to collect data from the Australian horse industry about transport management and the most common complications experienced by horses being moved across Australia. The survey was composed by 40 questions (Appendix 1). It was published online and was open for 12 weeks from July to September 2015. Almost 1000 responses were collected and since the quantity of data was so large, 4 different articles were written. However, the results needed to be interpreted with caution because the questionnaire was limited by many factors. The main limitations of the questionnaire were: self-selected participation in the study and self-reported nature of transport-related behavioural and health problems.
- Chapter 3.1: The first article from this survey addressed the following 4 research questions: i) what are the most common transport management practices applied in the Australian equine industry?, ii) do the applied practices comply with the Australian Code of land transport?, iii) what are the most common transport-related behavioural and health problems suffered by horses transported from 2013 to 2015?, and iv) are practices and problems different between amateur or professional members of the industry? It also reports on how the questionnaire was designed, developed, and distributed, its response rate and the descriptive statistics of all the questions. Amateurs and professional organised transportation differently, and both often did not comply with the Australian Code. Transport-related behavioural and health problems were experienced by horses in the care of 38% and 67% of respondents, respectively. The incidence of behavioural and health problems related to transport was similar between

amateurs and professionals, indicating that transportation remains a substantial risk of adverse health and welfare consequences and economic loss.

- Chapter 3.2: The second article explored the association between transport management strategies and transport-related injuries and diseases. All the responses (n=797) were analysed using univariate and multivariate regression analysis to identify risk factors. The risk of traumatic injuries was higher when the respondent was young (<40 years), took care of many horses (>30 horses), used protective equipment and tranquilisers prior to transport. An increased risk of thermal stress was linked to the restriction of hay and water prior to transportation. Muscular problems appeared to be greater when the health of the horse was not assessed prior to transport. The risk of laminitis was approximately 3 times greater when post-transport recovery strategies were not applied. Associations were made between transport pneumonia and duration of the journey, and with racehorses in training. No associations were seen between the incidence of colic and the predictive variables examined. The findings provide evidence to support many current recommendations for safe transportation of horses, and highlight the need to refine existing policies and practices.
- Chapter 3.3: The third article explored the association between transport-related problem behaviours (TRPB) and transport management, with particular emphasis on the training method for travelling. The association between TRPB and transport-related injuries was also explored. Training methods were classified into: operant conditioning (OC), self-loading (SL), habituation (H) and no training (NT). Univariate and multivariate regression analysis were conducted to identify risk factors. The training method was identified as a risk factor for all types of TRPB and their odds were higher in horses trained by OC and NT compared to SL and H. The training method, loading and travelling problem behaviours were found to be risk factors for transport-related injuries. Based on the results, H and SL training are recommended to reduce the incidence of TRPB and subsequent injuries, safeguarding horse and handler wellbeing during transport.
- Chapter 3.4: The fourth article explored the association between transport-related health problems, the journey (i.e. duration, vehicle, commercial/non-commercial) and the horse characteristics (i.e. sex, age, breed, use, amateur or professional status). The

respondents provided details of the last case of a transport-related health problem that had affected their horse. Multivariable multinomial regression analysis was performed to identify risk factors. Respiratory problems were the most frequently reported problems (33.7%), followed by gastrointestinal problems (23.8%) and traumatic injuries (16.3%). The type of health problem was associated with journey duration (P<0.001) and horse breed (P=0.001). Injuries were more likely to occur on short journeys, while more severe illnesses (e.g. gastrointestinal and respiratory problems or death) were more likely to occur on long journeys. Using Standardbreds as the baseline comparison, Thoroughbreds, Arabians and Warmbloods were found to be more likely to experience a severe illness than an injury. The study confirmed that journey-duration was associated with the type of health problem (Chapter 2); it highlighted the need of further studies on long-haul transportation effects (Chapter 4) and on transport-related respiratory disease (Chapter 5).

- Chapter 4: This is the first multidisciplinary study reporting on the impact of long-transportation on the health and welfare of performance horses. It tests the hypothesis that a horse's immunological capacity to react to a stimulus would decrease after a long journey, and that this decrease might be associated with increased cortisol concentration and other clinical, haematological, inflammatory and oxidative stress parameters. The effects of a 4 day journey on these parameters were compared with a group of similar horses that had not undergone transportation. The 4 day journey was associated with changes on clinical examination, neutrophilia, an acute phase response, impaired lymphocyte proliferation and a mobilisation of antioxidants. However, the observed changes were not associated with increased cortisol level, as was expected, nor with the development of clinical diseases. The tested journey complied with the Australian Code and horses were monitored carefully for a week after the journey. The findings of this study confirm that appropriate transport management is crucial to reduce the risk of transport-related diseases and monitoring hydration and redox balance might be proposed as tools to assess the welfare of horses during and after transportation.
- Chapter 5: This multidisciplinary study was undertaken to evaluate relationships between transport environment, behaviour *en route*, redox balance, respiratory viruses shedding/reactivation and respiratory disease in horses undergoing an 8 hours transport

event devoid of water and feed. The findings supported the hypothesis that an 8 hour journey devoid of water and feed was a stressful event for horses with association seen between stress-related behavioural responses *en route* and haematological, oxidative and respiratory outcomes. This is the first study where conventional and molecular bacteriology support the role of *Pasteurellaceae* as early opportunistic invaders of the respiratory tract associated with transportation. Based on these findings, clinical examination before and after journey, behavioural observations, monitoring of redox-balance and fibrinogen concentration are recommended to safeguard the welfare of the travelling horses.

CHAPTER 1

Effects of the different transport phases on equine health status, behaviour and welfare: a review

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1.1. Introduction

Transportation is an integral aspect of horse management, but transport stress is still an issue and many problems are associated with it. Horses are transported more frequently than any other type of livestock (Friend, 2001). They are moved for many different reasons: in the past for war and, today, mostly for competitions, breeding, pleasure activities, sale, or slaughter (Fazio et al., 2008b). Three thousand horses are transported daily in Italy (Giovagnoli, 2008). Around $3 billion annually are spent in transportation in the United States, with an estimated population of 9.2 million horses (Council, 2005). It has been reported that a typical Texan horse owner transports an average of 2.5 horses, 24 trips (average 380 km per trip) per year (Gibbs et al., 1997; Gibbs et al., 1998).

Horses can develop preloading, loading, travelling or after-transportation health and behavioural problems. Many injuries to horses and owners occur during the loading procedure (Ferguson and Rosales-Ruiz, 2001). Some horses move or kick inside the trailer, causing driving problems and fatal road accidents. Poor performance problems, health disorders and infectious diseases are the most common complications after transportation. Thus, assessing best practice transporting procedures for horses warrants comprehensive investigation (Cregier, 2010).

In the last thirty years many scientists have conducted research aimed at improving horse welfare during transport and at reducing the incidence of related problems. Results are often conflicting, and how best to manage transport stress is still a matter of debate. One reason for the conflict is that some results are not comparable because the studies used different trucks or trailer models, in different seasons, on different horse breeds. Moreover, it seems that the level of stress caused by transportation is related to the temperament of the horse and its historical travel experience (Fazio et al., 2013b), orientation during travel, the provision for head movement, and factors such as driver skill, ventilation and/or window placement.

This paper reviews approximately 30 years of publications concerning horse transport. The aim of this review is to split transport into its critical points, highlighting the effects that each phase has on horse behaviour and patho-physiology. Suggestions for managing journeys to encourage practical outcomes that safeguard equine wellbeing before, during and after different types of transport are discussed.

1.2. Different means of transport: ship, train, truck, plane

The earliest form of water borne horse transport likely utilized a waterproofed hide stretched over a wooden frame; a form of transport thousands of years old. Specialized water-borne transport for horses has been in use for centuries, and has evolved with different types of ship/vessel construction for river, lake, or sea transport (Cregier, personal communication, 2014). Byzantine historian Theophanes described such specialized horse transport in 762 AD. Chelandia were relatively small ships designed to carry up to a dozen horses with a specific landing ramp (Pryor, 1982). Later, horses were carried regularly by ships (Cregier, 1984), and sea transport was the only means of transport for horses until the late 19th Century. Today sea transport is still in use because it is the cheapest form of transport, particularly for slaughter horses travelling from South America to Europe (Giovagnoli, 2008). In modern cargo ships, horses travel in boxes whose dimensions range from 4.5 x 4.5 m to 6.0 x 4.5 m with access to a sand yard to exercise during the voyage (Waran et al., 2007). The key disadvantages of sea transport are mainly duration and risk of injury (Judge, 1969). Long transfers by ship are well accepted by horses, although after the journey animals can develop jet lag, medical conditions associated with a change in management and conditions or caused by hierarchical conflicts associated with adapting to the new stall and social group (Cavallone et al., 2002).

The earliest mode of land transport was via horse-drawn wagon. Performance horses were drawn by those of lesser value beginning sometime around the reign of Queen Anne in the early 18th century.

Transport by rail was very common from the mid-nineteenth century to the mid-twentieth century with various wagon designs being used. The smaller horse car with two wheels on each end carried eight horses. The larger car, supported by a four wheeled truck or bogie on each end, could carry 16 to 20 horses at speeds up to 145 km/h. Since more than one animal occupied a wagon, the risk of treading on and kicks from cohorts was high. Thus, there was a special single horse van used for more valuable horses. The disadvantage of train transport has been mainly the rough, uncomfortable journey and, often, prolonged waiting times at rail-heads for collection or delivery (Judge, 1969). Today, transport by rail is still in use in developing countries because it is a relatively low cost transportation method.

After World War II, following the building of interstate highway systems, road-transport became the most popular means of transport (Friend, 2001). The 1960s and 1970s are known as the "Trailer Age", because many different kinds of trailers and trucks were designed and built. Consequently, today there are many different types of road vehicles used to transport horses, the major difference being found between lorries or vans and trucks, and trailers. The "motorized horse box" or "horse lorry van" varies in capacity from 1 to 10, 12 or even 16 or more individually stalled or grouped horses. The most common trailer is for two horses and it is usually attached to the towing vehicle at one point via a tow hitch, which makes trailers less stable than lorries or trucks (Cregier, 1984). Trucks and trailers generally have a rear and/or a side ramp for loading and unloading. In some countries (e.g. the US), there may be no ramp and the horses are trained to "step up" into the trailer. In the UK, Canada and New Zealand, platform loading horse trailers were designed to reduce transport stress and increase loading and unloading safety. The horses step onto the platform from the off side, reverse into the trailer, and face away from the direction of travel to allow hind-quarter resting and freedom to balance with their heads during transit (Equi Balance™).

The first reported air transportation of horses was during the early 1920's with the first race horses being flown across the Atlantic in 1946 (Judge, 1969). To date, air transport has become very common even though it is the most expensive form of horse transport. In the plane, horses travel in air stables or jet stalls designed to accommodate a maximum of three horses, side by side, separated by partitions (Waran et al., 2007). Air transport is used primarily for competitions and breeding purposes: in this manner stallions can be mated with mares in both the Northern and Southern Hemispheres in the same calendar year.

The most stressful phases of air transport are loading, unloading, taking off and landing (Stewart et al., 2003). During the flight horses tend to have resting heart rate values and engage in resting behaviours, indicating that they may settle better to air transport than road transport (Munsters et al., 2013). Quarantine regulations are generally applied after air travel to minimize biosecurity risks, however, restraint in the quarantine boxes can cause an increase in heart rate associated with environmental stress (Ohmura et al., 2012).

The vast majority of studies have focused on effects of transport stress in horses moved by road. It is this mode of transport that is the focus of the remainder of this review.

1.3. Different destination of transport: toward a slaughterhouse, a new stall or a competition

Horses travel for different reasons, but the primary difference that can determine the kind of travel is the destination: a slaughterhouse or a different stall.

1.3.1. Transportation of horses for slaughter

Globally, many horses travel daily to slaughterhouses. Horsemeat consumption became popular in Europe after World War II amongst people with lower-incomes. At the time beef was scarce, and old or lame draft horses were slaughtered for affordable lean meat with a high iron content. Horsemeat has become a delicacy associated with high prices in Europe (Stull, 2001), although the 2013 scandal involving beef tainted with phenylbutazone containing horsemeat may have altered this profile. According to the most recent data from the Food and Agricultural Organization (FAO) the largest producer and consumer of horse meat in the world is China, which produced 170,848 tonnes of horse meat in 2010. The second largest producer and consumer is Kazakhstan. In many European countries horse meat is used as an ingredient in traditional meals, and is imported for this. In the USA, even though Congress cancelled federal funding to inspect horse meat destined for human consumption in 2007, 68,444 tonnes of horse meat was produced in the USA in 2010 for pet food (http://faostat.fao.org/, 2015). In Canada, horsemeat exports, primarily to markets in Europe and Asia, exceeded $60 million in 2011, and it is reported that more than 2,000 tonnes of horsemeat are consumed in Canada each year. There are some countries (e.g. the United Kingdom) that have never accepted horsemeat as part of their diet (Reece et al., 2000).

In South America horsemeat is popular, Argentina is the largest equine meat exporter, and Chile slaughters nearly 50 thousand horses annually (Werner and Gallo, 2008).

Meat horses travel loose in the truck toward the slaughterhouse, and density during transport is variable. In high-density compartments horses can fall during shipment, resulting in injury or death, but injuries due to kicking may be less frequent (Whiting, 1999). In a lower density shipment, horses can escape biting situations and aggression, lowering the mentally stress for the horses (Collins et al., 2000), but aggressive behaviour during transport may be related more to individual horse temperament than to conditions (Iacono et al., 2007a). Further studies are needed to define optimal travel density and environment.

Interestingly, the effects of travel on meat quality have been well studied in cattle and pigs (Ritter et al., 2008), but there are few studies in horses. After transportation increased blood lactate and glucose have been reported in horses, factors which impair meat quality (Werner and Gallo, 2008). The stress associated with transport, lairage and stunning also affects horse meat quality.

1.3.2. Transportation toward a new stall

There are an estimated 58 million horses worldwide, and the equine transport industry is a wealthy one. In industrialized countries horses reared primarily for pleasure or sport are most commonly transported professionally. Tamed horses travel in single stalls within the vehicle, whereas unhandled horses are more inclined to be transported in groups. It is common for mares to travel loose with their foal within a box inside the truck (Weeks et al., 2012).

Every time a horse travels to a new place, there is an associated adaptation period during which the horse needs to adjust to the stall, management and training styles, and diet. Because horses may also be exposed to pathogens which they have limited immunity to or carry exotic pathogens sub-clinically, standard-of-care recommendations include a quarantine period and gradual transitions to new diets. It is advisable to avoid abrupt dietary changes and prevent intense exercise sessions during the period following transportation (Waran et al., 2007).

1.3.3. Travel before competition

Despite many horses being transported specifically for performance purposes, surprisingly little is known about the effect transportation has on performance with the results of studies often conflicting.

It has been suggested that for experienced horses, transport over short distances had little impact on performance (Beaunoyer and Chapman, 1987). However, other research found that a 194 Km journey could negatively affect the performance in a close race, particularly in front facing transported horses, which displayed the effects of slight stress (Slade, 1987). The effect of transport on competition jumping horses' performance was also studied. In this case, only horses with less travelling experience showed signs of major stress and reduced performance (Covalesky et al., 1992).

A recovery period of two hours after three hours travel was suggested on the basis of the increase of muscle enzyme concentration caused by the transport (Tateo et al., 2012). Travel longer than 8 hours is discouraged before a competition, as it could compromise racing performance and require some days of recovery (Linden et al., 1991).

After a 100 km transportation distance in a one-horse trailer, facing in the direction of travel, an increase in the concentration of free triiodothyronine (T3) was recorded. Triiodothyronine (T3) is a thyroid hormone involved in growth, metabolism, heart rate and thermoregulation.

Elevated levels of T3 are commonly associated with sessions of intense exercise (Fazio et al., 2008b). Since T3 is intricately associated with the other thyroid hormone, known as thyroxine or T4, by an endocrinology feedback mechanism, it may be supposed that an elevated T3 value recorded at unloading before a race could impair performance. But there has been no study to date that correlates the specific impact of elevated T3 levels resulting from transportation with performance.

The relationship between transport and performance needs more investigation. However, developing good scientific methodologies for definitively assessing the effects of transportation on performance is deemed very challenging due to the confounding effects of factors including horse temperament, position and orientation in the vehicle, fitness level of the horse, historical transportation experience and driver ability/skill level.

1.4. Phases of transport and their effects on horse behaviour and physiology

The transport of animals is a complex procedure involving several potential stressors. The transportation process includes the following critical factors which can all impact on stress levels: handling, separation from familiar physical and social environments, loading, confinement, vibration, changes in temperature and humidity, inadequate ventilation, often deprivation of food and water and unloading (Waran, 1993). Factors that induce stress during transport are mostly psychological (White et al., 1991), but physical factors such as the trailer motion, noise, the driver's ability and road conditions also may play a major role (Jones, 2003). Confinement itself is stressful for horses (Mal et al., 1991), but for many farm animals a stationary vehicle is generally considered to be less stressful than a moving one (Tarrant, 1990). Indeed, during transportation, horses are subjected to changing forces primarily due to acceleration, deceleration, and the turning movements of the vehicle (Waran and Cuddeford, 1995).

Accordingly one survey reported horses have problems both in loading (53.4%) and travelling (51.5%). During travel the majority of problems were recorded when the vehicle first began to move (53%) or when it went around a curve (47%)(Lee et al., 2001). It has been reported that breed has no effect on the problem type, whereas the trailer orientation and the horses' mental association with negative experiences may be important factors in the aetiology of transport problems (Lee et al., 2001).

It should be highlighted that horses can associate the transportation itself with what they experience during and after the journey. So it seems that horses used for sporting and recreational purposes with a number of positive experiences of loading and transportation are less adversely affected by transportation than horses with no experience and horses who have previously experienced negative situations including falling or over-crowding (Leadon et al., 2008).

1.4.1. Handling

Handling refers to how animals are touched, moved and interacted with during husbandry procedures. The handling methods (reinforcement or punishment) can have a significant impact on animal welfare. It has been found that for horses sent for slaughter, handling without the use of sticks or electric goads resulted in improved welfare and lower risk of poor quality meat (Broom, 2005). Restraint is often part of the handling procedure. During restraint animals are often separated or isolated from their conspecifics, which is a known factor causing stress (Grandin and Shivley, 2015). Both handling and transportation involve the interaction of animals with humans and it is important to know how animals react to human behaviour aimed at effectively moving and restraining them (Fazio and Ferlazzo, 2003). The taming/training method (ethological or traditional), can have an ongoing influence on the horse-human relationship, thereby having long term effects on the horses' behaviour during subsequent handling procedures (Casamassima et al., 2008).

Age, sex and physiological condition also influence the behaviour of horses during handling and transport; indeed handling young animals, such as foals and yearlings, which are usually not tamed extensively, can be significantly more difficult and risky than handling older animals. Although it is generally assumed that intact males are more difficult to handle than castrates, this difference may also be age dependent. Rearing environment and previous experience are generally agreed to be of considerable importance. Animals respond to challenges in their immediate environment through several interacting mechanisms including behavioural, hemato-chemical, physiological and neuro-hormonal (Fazio et al., 2013b). The response of animals to handling and transport can also depend on their sensory capabilities, the visual field and flight zone. Some behavioural indicators of discomfort are vocalizations, attempts to escape, kicking, or struggling. Identifying stressful situations by key behaviours could be useful during handling and transport and would promote greater wellbeing in horses (Siniscalchi et al., 2014).

Overall, it is generally more desirable to transport horses that are already well accustomed to handling so that they do not associate the stress of being handled with the process of being transported.

1.4.2. Loading (Injuries and Fear)

Loading is considered to be one of the most stressful components of transport (Waran, 1993). Loading fear comprises different stimuli, such as fear of entering an enclosed space, the height of the step leading onto the ramp and the instability and incline of the ramp (Houpt and Lieb, 1993). It is these factors that result in inexperienced horses often exhibiting extreme evasive behaviour and a strong reluctance to step up onto the ramp. Accustoming the foal to loading has been proven to reduce behavioural problems associated with loading and transporting later in life (Houpt, 1982).

The heart rate during loading is usually higher than the average heart rate during transport, regardless of the level of experience. In fact, climbing a ramp is probably a frightening experience for a naïve horse, and although horses may become accustomed to the situation, experienced horses are still stimulated in some way. This elevation in heart rate can be ascribed partly to the energy expended in climbing the ramp and partly to the emotional fear (Waran, 1993). Evasive behaviour during loading is typical of very young horses and the time taken to load is influenced by age. In fact, it is reported that on average yearlings took more time to load (368 s) than 2-year-olds (29.5 s), 3-year-olds (21.5 s) and those over 3 years-old (5 s) (Waran and Cuddeford, 1995).

As a fear response, many horses fight during loading which in itself is a source of stress and can result in injury to the horse and/or handler. Behaviours such as rearing, pulling back, head-tossing, pawing, and turning sideways are commonly exhibited. These behaviours are likely to be negatively reinforced when the loading process is aborted by the handler (Baron, 1991). The combination of loading a ‘problem’ horse and an owner who applies physical punishment can produce a very dangerous situation. Rope burns, lost fingers, broken bones, or bruises and lacerations have been reported as most common owner injuries. Lacerations to the head from banging into the trailer, scrapes and cuts on the legs, broken legs from falling, or even a broken back if the animal falls backwards while rearing are the most common horse injuries occurring during loading (Ferguson and Rosales-Ruiz, 2001).

Some studies have been conducted to determine procedures which reduce loading fear. To reduce the likelihood of injury, horses that are difficult to load can be trained to load more willingly. Successful training procedures involve increasing the horses' confidence by breaking loading into simpler, separate tasks that can be accomplished in a relaxed mental and physical state. These tasks include moving forward on command, stepping onto and backing off an unstable floor, and moving into a confined space (Scoggins, 1996).

The Tellington-Touch Equine Awareness Method (TTEAM), developed by Linda Tellington-Jones takes this concept of relaxed, progressive training one step further (Curcio-Wolfe, 1996). This method uses non-aversive touch and commands in novel situations as a means for inducing behavioural changes in horses. Horses are generally neophobic, and TTEAM is specifically designed to teach horses to relax and function in the presence of novel and potentially frightening stimuli. Non-aversive retraining methods (based on TTEAM) were effective in reducing loading time for horses with a history of reluctance to load onto a trailer and were also associated with a decreased post-loading heart rate and saliva cortisol levels (Shanahan, 2003).

Loading fear is innate in the horse, but some environmental stimuli can be attributed to exacerbating loading fear. One such example would be loading a horse directly from a brightly lit arena into a dark trailer (Cross et al., 2008).

People have used winches, whips, war bridles, chains, cattle prods, and a variety of other punitive methods to force horses to load. Although professional horse trainers do not openly advocate extremely aversive methods, most of their methods of loading horses include some form of negative reinforcement and the use of punishment in response to undesirable behaviours.

On the other hand, recent literature has proven that in particular for horses which refused to load, positive reinforcement (PR) provided the fastest training solution with the lowest levels of stress response (Hendriksen et al., 2011). It has been reported that the use of applied equine training systems, based on positive reinforcement, results in increased probability of appropriate behaviour being displayed during handling and loading procedures in loading problem horses (Slater and Dymond, 2011).

1.4.3. Transportation

Transportation involves many stressful factors. During the journey, the internal truck temperature, relative humidity and level of environmental contaminants can change dramatically (Leadon et al., 1991). The horses in the vehicle may also have to adapt to unfamiliar factors including traveling companions, confinement spaces, movement beneath their feet, acceleration and deceleration, ascending and descending, taste of drinking water, exposure to vibrations and noise and so on. Transport stress should be considered as a multi-factorial physical and emotional status, where the sympathetic nervous system shifts from alert to fear many times and where the maximal effort is spent in balance preservation. To maximize the wellbeing of any horse during transportation the following factors should be taken into account and/or checked during the journeys.

1.4.3.1. Confinement and Isolation

Both confinement and isolation are stressful and may suppress feeding behaviour during transportation (Mal et al., 1991). Once loaded into the vehicle, the horse is placed in a restricted space, either due to being confined in an individual stall using partitions, or due to pressure on individual space exerted by the other loose horses with which it may be travelling. Tamed yearlings transported in individual stalls or loose had similarly increased cortisol levels measured both during and after transport in comparison to preloading. In both situations transportation was a significant stressor (Garey et al., 2010).

Since transport often requires the animal to travel alone, the effects of transporting horses alone, in company or with a mirror that provided surrogate companionship were investigated. Behaviours (vocalizing, eating, head-tossing, pawing, and head-turning) and physiological parameters were recorded. When traveling with a live companion significantly less time was spent vocalizing, head-turning, head-tossing and pawing and eating behaviour increased. Heart rate and temperature also significantly decreased when travelling with a live companion. Travelling with the mirror did not significantly affect physiological responses when compared to travelling alone, but did significantly reduce time spent turning the head, vocalising and head-tossing while eating behaviour also increased (Kay and Hall, 2009).

In general, the provision of surrogate companionship in the form of an unbreakable (e.g. stainless steel) mirror is preferable to travelling alone, but where possible a live companion is recommended.

1.4.3.2. Effect of Density

During loose horse transportation, high stocking densities create a situation of constant struggle for the horses. Medium stocking densities likely reduce injury and bruising during transportation, but also increase transport costs. It was suggested that decreasing density would reduce the overall stressfulness of long distance transport by allowing horses more opportunity to avoid aggressive horses, to stand in a more comfortable position, to adopt their preferred travelling orientation, and perhaps allow them to rest during periods when the truck is stopped (Whiting, 1999). However, it was recently proven that there was no difference in the movements recorded in unrestrained horses transported in low or moderate density. It was however apparent that the movements of the unrestrained animals inside the truck were strongly influenced by a small number (one or two) of more active horses causing disruption of the group (Calabrese and Friend, 2009).

For individual horses stall size is important and there is legislation about the minimum space required during road transport according to age and type of horse, but minimum space allowances differ between countries (Waran et al., 2007).

1.4.3.3. Effect of fasting and water intake

Depending on the final destination, horses may or may not have the opportunity to feed and drink *en route*. Sport horses transported to a racetrack are often allowed to feed on some hay, usually offered in a net, because it has been shown that it does not impair performance, whereas horses transported to a slaughterhouse are usually fasted to reduce the risk of soiling and resultant meat contamination (Waran and Cuddeford, 1995). Some studies suggest avoiding hay in the vehicle in all cases, because it affects the air quality. Dry hay is likely to pose a risk to horses prone to recurrent airway obstruction (RAO), which could become symptomatic after allergen exposure: for these horses it is better to use wet/dampened hay or pellets (Hotchkiss et al., 2007). However, if the hay is offered during the journey, it is generally better to dampen it and to place it on the floor, to minimize the dust risk. Furthermore, this location could stimulate travelling horses to eat with a lowered head position, which seems to be fundamental in reducing the development of respiratory diseases (Raidal et al., 1996). Regardless of whether or not feed is provided during travel, weight losses are reported after a journey (Waran, 1993). Weight loss is likely to be due to a combination of reduced feed and water intake and increased energy expenditure and fluid loss through sweating (Smith et al., 1996). It is important to emphasize that horses always tend to reduce feed and water intake during the journey, because they are less willing to eat and drink in unfamiliar and stressful surroundings and from unfamiliar sources (Kay and Hall, 2009). Generally, it is better if familiar water and food are offered to the horses during the journey, as well as during planned rest periods. It has been proven that increasing the resting time and cleaning the interior of the truck during rest stops decreases transport stress and respiratory insults (Oikawa et al., 1995).

During long trips water should be offered to the horses, while the vehicle is stationary, at least every 2 to 4 hours, especially when the environmental temperatures are high (Houpt and Lieb, 1993). During a 24 hour trip, a stop is needed at least every 4 hours to provide the horse with the opportunity to urinate; horses urinate approximately six times daily and male horses in particular need to be able to stand in a particular posture which is difficult to do comfortably *en route* (Weeks et al., 2012).

In many codes of transport practice it is deemed acceptable to remove all access to water and food for up to 8 hours during transportation, however, it is recognized that the impacts of such long periods of fasting have not been deeply investigated yet.

1.4.3.4. Environmental Challenges

Temperature, humidity and ventilation inside the vehicle are critical aspects of a journey. Ventilation for horses during transportation has been a topic of research since the 19th century with links reported between level of ventilation and the occurrence of both heat stress and shipping fever (i.e. equine transport pneumonia/pleuropneumonia). Despite research findings, and proof that trailers are under-ventilated at all speeds (from 13 to 90 Km/h) and ventilation configurations, trailer designs have not been modified (Purswell et al., 2006). The suitability of the trailers' thermal environment is generally well assessed for other livestock but is still lacking with regards to horse transportation. The upper limit of the thermo-neutral zone (TNZ) in horses is also not well defined, but it is estimated to be in the range between 25°C and 30°C. Thermal comfort depends not only on temperature, but also on humidity and it is expressed by a calculated thermal-index. For horses the upper limit of this thermal-index was set at 28°C by the Federation Equestre Internationale (FEI) for competitions (Internationale, 2009). The problem is that inside the truck this limit is not valid and it is often exceeded. In traditional trailers, inside-to-outside temperature differences decrease with increased speed and the open vent area, but it still ranges from 5.1°C to 9.5°C (Purswell et al., 2010). This means that horses should not be transported during hot and humid days, when outdoor temperatures exceed about 30°C. Since it is impractical to restrict horse transportation on days that exceed 30°C (particularly in hot arid countries), future research should include new engineering and design solutions to improve thermal environment and ventilation characteristics of trailers and trucks. Increasing vent and window area, increasing the height of the vehicle and adding fans have all been suggested for vehicles transporting other species, but should also be applied in horse transport vehicles (Mitchell and Kettlewell, 2008). The risk of heat stroke is increased dramatically when the vehicle is stationary and when more than one horse is aboard the vehicle. Under such circumstances it is advisable to park the vehicle in shaded areas with all windows and ramps open during rest stops (Waran et al., 2007).

Animals produce CO_2 through respiration and ammonia with their urine, in addition to secreting microorganisms through faeces and other secretions. As a result, the confined space inside the moving vehicle is not usually conducive to a healthy environment. Consequently, good ventilation is vital to ensure not only acceptable air temperature and relative humidity, but also low levels of airborne contaminants such as gases, dust and microbes. Many studies in this research area were conducted by Leadon et al. (2008) who reported that air usually enters the horse transport vehicle through the windows or vents along the side, but that this air tends to drop toward the floor and become contaminated. As a consequence the air quality in the vehicle becomes very poor over time. Further studies are needed on ventilation design to improve air circulation and air quality.

It is important to limit the concentration of noxious gases in the air because it has been proven that during transportation the horses' respiratory clearance mechanism is compromised following exposure to ammonia, nitric oxide and carbon monoxide. These gases damage the pulmonary epithelial barrier, thereby increasing the permeability to bacteria (Traub-Dargatz et al., 1988). In a study reported by Smith et al. (1996), ammonia concentration was recorded in a standard 2-horse trailer. The mean concentration during a 24 hour journey was 0.8 ppm with no detectable odour. Although there is no recommended upper limit for exposure of equines to ammonia and other gases the safe human limit should be taken into account. The current recommended exposure limit is 25 ppm for ammonia and 9 ppm for carbon monoxide (Pickrell, 1991). Endotoxins in air can also create inflammation of the airways. Whilst it has been proven that stabled horses are exposed to 8-fold higher concentrations of endotoxin than pastured horses (Berndt et al., 2010) there are no reported studies of endotoxin levels in transportation vehicles.

Another critical equine health consideration is the risk of disease spread during and after transportation. In the truck, animals from different farms often travel together, so pathogen (bacteria/viruses/parasites) transmission risk is relatively high. Wherever possible, loading of animals from different farms and of different ages should be avoided. Since trailers and trucks are saturated with potentially harmful bacteria and spores after transporting horses, they must be disinfected prior to reuse. An effective cleaning procedure should involve cleaning with hot water, prior to removing excess water with a suitable vacuum cleaner and the concentration of the disinfectant should be elevated at least by a factor of 3. Mechanical actions, such as scrubbing, will improve disinfection efficacy of vehicles as well as preliminary disinfection prior to cleaning (Böhm, 1998).

Monitoring of the microclimate can be conducted with the use of probes located at various positions within the vehicle to measure temperature, humidity and air movement. Monitors located in the vicinity of the horses head(s) should be used to measure concentrations of ammonia and other gases (Smith et al., 1996). The use of a commercial data logger inside the truck is strongly recommended with the collected data being used to modify the vehicle and management practices in manners that neutralize environmental challenges thereby improving animal welfare (Miranda-de la Lama, 2013).

Finally, to eliminate odours, neutralize some gases and disinfect hard surfaces, the use of vinegar, zeolite or baking soda as alternative anti-microbiological agent can be used for routinely cleaning of the vehicle (Fong et al., 2011).

1.4.3.5. Direction of travel

Body orientation during travelling is an important matter of debate. Equine anatomical conformation is such that 60% of the equine body weight is carried over the forelegs, and the hindquarters are poorly designed for continual shifting of weight and bracing against directional change (Cregier, 1982). It is probably for this reason that the most commonly observed body posture in horses travelling in a forward facing direction involves standing with the front and hind limbs apart and the forelegs stretched forward. This exaggerated limb position during transit likely helps the horse to retain its balance by exerting inclined thrusts with one leg or the other, as the occasion demands (Roberts, 1990).

Inappropriate orientation, and consequential loss of balance, can cause injury during horse transport (Whiting, 1999). There are different opinions amongst experts about travel positions that minimize transport stress and optimize the horses' post-transport performance (Gibbs and Friend, 1999). Several studies have been performed in order to determine the effects of orientation on a horses' ability to maintain balance during transport, but results have often been conflicting due to differences in trailer design and lack of simultaneous comparisons. When transported in a 2-horse trailer, backward-facing horses had fewer side and total impacts and losses of balance when compared with forward-facing horses (Clark et al., 1993). Horses transported in a 2-horse lorry without a saddle compartment and facing backward had a significantly lower heart rate, moved less frequently, and showed a greater tendency to rest their rumps on a partition (Waran et al., 1996).

Comparison of three different positions (backward, forward and sideways) during a three hour journey for Standardbred trotters accustomed to travel, revealed that backward facing was the most ideal orientation. Backward-facing horses moved more but lost their balance less and were able to rest a hind quarter in a three leg position during the journey (Padalino et al., 2012). However, since some horses have demonstrated a superior ability to maintain their balance in a particular orientation, individual characteristics and other factors, rather than travel orientation alone, may impact the ability of horses to maintain their balance during transport (Toscano and Friend, 2001). Therefore, further studies are needed on the travel position.

1.4.3.6. Duration of travel: short versus long trip

In order to understand the effects of road transport on equine physiological and behavioural parameters many studies have been conducted and it seems that a key variable is the duration of the journey.

Long transport times likely have a strong effect on both equine physiological and endocrinal parameters. The effects of nine hours of transport on in-foal mares showed increased concentrations of cortisol and progesterone. Despite the increases, no early embryonic deaths were reported (Baucus et al., 1990a).

After 24 hours transport, equine body weight decreased by 6% immediately after unloading with a 3% deficiency remaining at 24 h post transit. The white blood cell (WBC) counts, haematocrit and total proteins concentrations showed a progressive increase with the duration of travel, peaking at the termination of transport. Serum lactate, creatine kinase and aspartate aminotransferase concentrations increased during transport and in the early post-transit period. They returned to normal levels 24-h after unloading, whereas glucose concentrations tended to rise with the initiation of transport and did not decrease to baseline concentration within the 24 hour post transportation period (Stull and Rodiek, 2000). Plasma cortisol and the neutrophil:lymphocyte ratio also increased during transportation, as a stress response. Those haematological and endocrinal changes may increase disease susceptibility and influence energy availability for athletic performance following transportation of horses over long distances.

Due to the long journey, stress-related respiratory diseases and even death have been reported in horses (Anderson et al., 1985; Oikawa et al., 1994; Racklyeft et al., 2000). It was observed that in healthy horses traveling in a trailer for 36 h (1,100 Km), the number of alveolar macrophages and their bactericidal function decreased and cortisol concentration remained elevated one week after transport; favouring the development of lower airway diseases (Laegreid et al., 1988).

According to European law, a journey is considered to be long if it lasts more than 8 hours, requiring that some rest must be planned to minimize the risk of the above mentioned health problems and to safeguard equine welfare.

Horses are transported mainly over shorter distances, in particular before competitions, so the effects of short journeys are also a topic of importance and relevance.

Comparing a short-duration (300 Km) journey and an exercise bout of cantering 1,500 m, similar effects on serum enzymes and metabolic processes were reported in race horses (Codazza et al., 1974).

In a comparison of one hour vs three hour journeys the number of movements recorded per kilometre was greater during the short journey than the long one. More forward and backward movements were reported, possibly as a result of the greater agitation shown by the horses at the start of the journey. In addition, horses subjected to the short journey showed a higher serum cortisol concentration at unloading, suggesting that they could not adapt to the travel within one hour (Tateo et al., 2012). Many authors have argued that horses need around 5 hours to adapt to a transportation experience, and the first phase is always the most critical (Baucus et al., 1990a; Iacono et al., 2007b). Other studies have confirmed that the period of adaptation to a journey is longer than one hour which increases the importance of animal management during short journeys, because the horse is under an acute stress situation (Fazio et al., 2008a).

In conclusion, long and short journeys both affect equine behaviour and endocrinology differently, but both require a restore time and appropriate management. Long journeys should be planned with frequent and long stops, during which the horses should be fed and watered, the male horses allowed to urinate, and the truck cleaned. It is important to choose quiet and shaded rest areas. The horses could be more nervous than usual, so unload them only if the area is fenced and deemed ‘safe’.

1.4.3.7. Effect of driver behaviour

Driving style is another important factor which influences horse welfare during transportation. The driver’s ability could affect the movement of the vehicle and consequent balance ability of the transported animals, in particular during accelerations, braking, cornering and any other difficult manoeuvres. There are two main components in driver behaviour: style and skill, where “skill” is the ability to control the vehicle and “style” is the way in which the vehicle is driven (West et al., 1993). Driver behaviour and road quality (motorway vs. minor road vs. city traffic) affect the behaviour of transported animals as has been shown for sheep (Cockram et al., 2004), cattle, young calves and pigs (Cockram and Spence, 2012). In horses, heart rate seems to be highly correlated with muscular activity spent in balance preservation, and both are strongly affected by the degree of experience of the driver. Consequently, it has been suggested that the vehicle condition (suspension, tire pressure), road quality and driver's professional ability are crucial factors in determining the magnitude of transport stress (Giovagnoli et al., 2002).

1.4.3.8. Effect of noise and vibration

Animals can perceive high frequency sounds and it is possible that they are disturbed or scared by sounds that are inaudible to man. During loading, transportation and unloading, noises audible to humans can arise from different sources such as human voices, whips, animal vocalizations (including other species e.g. barking dogs), noisy machinery, alarm bells/klaxon, and compressed air brakes on vehicles. We cannot quantify easily all sources of ultrasounds, but animals are exposed to many ‘annoying’ noises. It has been proven that intensive noise results in a central nervous system excitation, which causes immune system suppression, fatigue and cell death (Minka and Ayo, 2009).

Vehicle vibration has been correlated with liver and muscle glycogen depletion and consequent fatigue in bird (Warriss et al., 1999); this effect could contribute to reduced animal performance or horse meat quality after transportation.

1.4.4. Unloading

Unloading at journey's end may be another physical stressor. Some horses are particularly difficult to unload, particularly if the ramp is very steep or if the animal is exhibiting lameness. Heart rate tended to remain higher than 'at rest heart rates' up to 30 minutes after unloading, but it is difficult to apportion this to the unloading vs. the cumulative transport stress (Waran and Cuddeford, 1995). However, it has been recently proven that Quarter Horses were stressed more during loading into a truck and stepping off a 20 centimetre step than during unloading (Siniscalchi et al., 2014).

1.4.5. Adaptation period in a new environment

Little is known of horses' behaviours after a journey, and very often, behavioural alterations noted may be a result of environmental change (Waran, 1993). The behaviours of horses transported both short and long distances were studied. To allow the researchers to account for the confounding effect of environmental change, a group of control horses was included in the investigation. These control horses did not experience a journey but were relocated to unfamiliar stalls. Comparing the 3 groups upon arrival in the new stalls, it was clear that horses which had made the journey sniffed less and snorted later than the control group. In the first 2 hours following travel, horses were attracted immediately to concentrated food and then started feeding on hay. After the long journey, the horses performed more bouts of drinking, and drank earlier, than the short journey and control groups (Tateo et al., 2012). The latter behaviours could be explained due to the slight dehydration caused by a long journey, which could have had been a direct cause of the positive influence on drinking behaviour (Iacono et al., 2007a). It was in agreement with another study where horses, after 6 hours transportation, tended to spend more time standing, playing or resting only after drinking and eating (Waran, 1993).

After a journey, horses are usually more interested in feeding than in other behaviours, including exploration, rest, and play, which are usually concentrated in the post-feeding hours, probably to recover energy spent to maintain balance in the truck. Consequently, to guarantee favourable adaptation to a new stall, it is recommended that food and fresh water are offered to the horses at their arrival (Padalino et al., 2012).

A common practice to encourage rapid adaptation after a journey is to stall horses adjacent to a familiar stable companion on arrival.

1.5. Major Pathology Connected with Travel

Another important research area relating to transport is the development of pathology and disease during and after travelling. Higher risks are connected with long transportation. These include acute enterocolitis, laminitis, transit tetany, shipping fever and mild azoturia (Mansmann and Woodie, 1995). However, short and frequent transportation can also result in injuries and health disorders. Frequently travelled horses are likely to show disrupted feeding patterns, weight loss, and fatigue (Cregier, 1982).

Notably, each horse journey implies some risk of disease transmission and contraction. Safeguarding the horse industry against this danger is one of the major responsibilities borne by equine clinicians. The occurrence in 2002 of West Nile fever in a stallion in post-arrival quarantine and the catastrophic outbreak of equine influenza recorded in 2007 in Australia provide some examples of elevated disease risk to the travelling horse and the importance of quarantine periods to protect domestic equine populations. Clinicians also need to be aware of the potential problems relating to horse transport and the way in which these problems may arise (Herholz et al., 2008).

1.5.1. Traumatic Injuries

The second (second to paddock/yard) most common source of injuries to horses is due to the transportation trailer (Darth, 2014). At loading leg injuries associated with the loading ramp are very common. During the journey halter rubbing at the poll or muzzle and tail rubbing are specific types of abrasion. To avoid these problems, it is useful to use specialized protections such as a head bumper or soft wrapping around the halter and protective bandaging on the tail. Long tie-ropes are suggested to allow lowering of the head, but protective screens must be in place to eliminate the risk of biting between neighbouring horses. Wither wounds can be caused though contact with the vehicle ceiling whilst leg wounds most commonly occur as a result of loss of balance after braking and cornering. Rapid and extreme braking has been reported to result in vertebral fracture and dislocation in horses facing forward and restrained with short tie-ropes (Mansmann and Woodie, 1995).

A recent Australian survey reported that 72% of horse owners blamed the horse's behaviour for travel incidents. However, the author highlighted that many incidents are actually related to the drivers' ability particularly on winding country roads. Among the most commonly reported behaviours were scrambling and slipping during cornering – both of which are exacerbated by wet flooring. Injuries resulting directly from horse-horse interactions and conflict were also common as were injuries caused during loading and unloading (Noble et al., 2013).

Injuries during transportation can also be related to road accidents; 2500 known transport accidents involving horses have occurred in the USA over the past 30 years. Often the blame is attributed largely to 'poor' design of vehicles or inappropriate use of vehicles (e.g. overloading). Whilst traumatic injuries are very common; correct transporting procedures and appropriate levels of care could reduce their prevalence.

1.5.2. Respiratory illness post transport

Recent transportation is frequently associated with the development of pleuropneumonia or 'shipping fever', which is a bacterial pneumonia, commonly associated with respiratory commensals, with varying involvement of the pleural space (Raidal et al., 1995). Although its etiological role is uncertain, *Streptococcus equi* subsp. *zooepidemicus* has been frequently isolated from pneumonic lesions in those cases (Mair and Lane, 1989). *Streptococcus equi* subsp. *zooepidemicus* is a commensally bacterium of the upper respiratory tract and an opportunistic pathogen. It may proliferate and become pathogenic when host susceptibility to respiratory infection is increased by stress and other host compromising factors associated with events such as transportation. It has been suggested that transportation predisposes the upper respiratory tract and the lower airways to invasion by the bacterium, with resultant occurrence of episodic pyrexia and acute pneumonia (Oikawa et al., 1994). Pleuropneumonia has a 5% incidence after long transportation, with a significant negative impact on horse welfare, largely because of the relative high case fatality rate seen in transport associated pleuropneumonia (Wilkins, 2003). Early identification and appropriate treatment results in survival rates ranging from 43 to 76%, but only approximately 60% of clinically affected horses are able to resume their athletic career (Copas, 2011).

The initial clinical signs of shipping fever can be insidious with the most common symptoms being fever, depression, and sometimes stiffness. A lack of coughing or nasal discharge is not uncommon and clinical signs are not specific. Factors that may contribute to transport-related respiratory disease in horses are: presence of subclinical respiratory diseases, prolonged restraint in a “head-up” posture that affects the pulmonary clearance mechanisms, stress-related impairment of the immune response, the presence of noxious gases, high concentrations of airborne dust and bacteria in the truck, poor ventilation, length and duration of journey and any emotional and physical stressor, which results in immune system suppression (Oikawa et al., 1995). Recent studies have also proven that an imbalance in oxidative status could be a related cause in the development of respiratory disease in horses (Po et al., 2013), but validation is required to ascertain the link with transportation.

In the traditional hauling of horses, it is common to place hay in a net at the horses’ nostrils to allow feeding and to keep the horse entertained. Unfortunately, normal, good quality hay contains many dust particles and small mold spores that can be inhaled. Dampening the hay and placing it on the trailer floor, or replacing hay with pellets reduces the risk of significant air contamination.

To reduce the insult to the respiratory system, it is important also to choose a bedding material which is not dusty but which is very absorbent.

Since it has been proven that horses restrained with their heads elevated for 24 hours developed an accumulation of purulent airway secretions and bacteria, which increased the pulmonary risk (Raidal et al., 1996), cross tying horses should be limited to restraining the horse with a rope which is long enough to allow the horse to lower its head. Horses may be secured by the "log and rope" method that allows head movements up and down as well as side to side. Using the "log and rope" method it is feasible to provide hay at floor level thereby allowing the horse to eat in a natural position (with head lowered). Another approach is to anchor the tie-rope alongside the withers. The operator then has the option to route the tie rope through a second ring positioned such that the horse cannot worry the neighbouring horse (Cregier and Gimenez, 2015). In well-designed trucks where the stall dividers are well designed and there is no risk of horses biting travelling companions, horses can travel untied.

Another preventative measure for reducing shipping fever is to avoid transporting horses which are undergoing drug therapy. For example, phenylbutazone can mask the early signs of pneumonia while corticosteroids are known to further decrease the horses’ defence mechanisms and may also increase the risk of laminitis (Mair and Lane, 1989; Racklyeft et al., 2000).

Increasing the rest time and frequency and cleaning the interior of the vehicle during rest stops reduces transportation stress and respiratory insults which may lead to respiratory diseases (Oikawa et al., 2005).

Diagnosis of early stage pleuropneumonia and other respiratory diseases associated with transport is an area that deserves investigation and is not widely reported in the literature. Infrared thermography (IRT) has been shown to have merit in the early detection of bovine respiratory disease complex before it was otherwise manifested (Schaefer et al., 2012). There are also many other reported examples of the use of IRT for early disease detection across many species including humans.

Since recent significant findings have linked hydrogen peroxide, a common oxidant marker, in exhaled breath condensate to *Rhodococcus equi* pneumonia in foals (Crowley et al., 2013), the latter method could become useful to test the travelled horses at unloading, to evaluate the pulmonary oxidant status and likelihood of subclinical pneumonia.

1.5.3. Dehydration, laminitis and colic syndrome

Even when water is available, horses tend to dehydrate during a journey which predisposes them to other pathologies.

Dehydration status can range from mild to severe after transporting and is largely dependent on the travel conditions (e.g. environmental condition and journey duration). Despite an increased physiological requirement for water, drinking behaviour is generally suppressed by the travel stress (Mars et al., 1992).

The earliest stages of dehydration are difficult to determine clinically, because a horse could have up to 5% dehydration without showing any significant clinical signs. For the athletic horse 2-3% dehydration can affect performance, so preventing even slight dehydration may be extremely important for racehorses or any horse travelling to competition.

Dehydration can also cascade into more serious metabolic situations. Moderate dehydration can initiate abnormalities in blood flow to the hooves thereby inducing laminitis. This problem is probably often accentuated by the hoof inflammation caused by removing shoes (a practice for reducing kick injuries) from a horse that normally wears shoes. Other compounding factors that may significantly increase the risk of laminitis could be the duration of the journey relative to the state of fitness of the horse, the level of carbohydrate intake maintained during the journey and any potential endotoxic disorders initiated by the travel event itself. Preventive measures could include: not changing the shoeing status of the horse, adding frog support to higher risk horses, and reducing carbohydrate intake before and during transportation (Masmann and Woodie, 1995).

Diarrhoea can also be caused by transport stress and increases the risk of laminitis, since it is commonly associated with endotoxemia.

Severe dehydration could induce the development of large colon impaction. In fact, there is a higher risk of colic in horses that have more than six transports/year compared with horses that are not transported and those transported fewer than six times per year (Tinker et al., 1997).

Another potential risk caused by dehydration is the reported decrease in renal function which is particularly relevant for horses due to undergo medical treatments (MacAllister and Tailor-MacAllister, 1994).

There are several techniques which are often implemented to minimize the risk and extent of dehydration. One involves the familiarization of horses with an aqueous normalizing substance, such as apple flavoring which reportedly offsets any difference in water taste during transportation and at the destination (Mars et al., 1992). Mineral oil or electrolyte-enriched water via naso-gastric tube has also been successfully implemented, pre-transport, for the prevention of gastrointestinal impaction. Care should be taken with the administration of both mineral oil which can affect the absorption of other nutrients and electrolyte which should not be given in a concentrate form (such as paste) close to a stress event (including racing, extreme exercise or transportation). Electrolytes should always be administered in an iso-osmotic solution (Pagan and Geor, 2005). The recommendations for stopping frequency during transportation are every 4-6 hours and over-nighting horses at least every 12-16 hours. During stops and over-nighting, to prevent dehydration and correlated pathologies, the horses should have the ability to exercise and to be examined by a veterinarian who can administer fluids as required. Oral electrolytes and water can be easily administered via naso-gastric tube to mild or moderately dehydrated horses or intravenously if dehydration is severe. A 450-500 kg horse’s stomach can tolerate 6-8 litres of electrolyte-enriched water every 15 minutes for 1 to 2 hours (Mansmann and Woodie, 1995).

1.6. Transport and Oxidative Stress

Oxidative stress (OS) occurs when the oxidant/antioxidant imbalance results in excess production of reactive oxygen metabolites (ROMs) leading to cellular and tissue damage. Oxidative stress plays an important role in diseases such as cancer, laminitis, neurological disease and heart and pulmonary diseases (Dunlap et al., 2006).

The imbalance occurs during and after stressful events such as travel, exercise and intensive management in both humans and animals (Kirschvink et al., 2002). Racehorses are often transported long distances and endure maximal exercise during training and races and have been shown to suffer frequently from oxidative stress (Hargreaves et al., 2002). It has been suggested that in horses oxidative stress has an effect on the development of airway diseases, such as chronic obstructive pulmonary disease and exercise induced pulmonary haemorrhage (EIPH) (Kirschvink et al., 2008; Soffler, 2007). However, the potential role of oxidative stress in the pathogenesis of transport pneumonia is still unclear.

A recent study was conducted to evaluate oxidative stress indicators in ten horses, transported 528 km by road. Physical (rectal temperature, respiratory and heart rates), haematological, biochemical and oxidative stress (malonedialdehyde (MDA) and superoxide dismutase (SOD)) parameters were measured before and after transportation. The transportation induced significant increases in respiratory and heart rates, and in haematocrit and glycaemia levels whilst the other clinical, haematological and biochemical parameters remained unchanged. The occurrence of oxidative stress induced by a 12 hour journey was evident by a significant increase in plasma MDA concentrations coupled with a significant reduction in plasma SOD activity compared to baseline values (Onmaz et al., 2011). The effects of an 8-hour journey by road on plasma total antioxidant status (PTAS) and general clinical appearance were also investigated in horses and showed that the average PTAS increased soon after unloading and remained elevated even after 24 hours stall rest (Niedźwiedź et al., 2013).

One approach to reducing the impact of oxidative stress during horse transportation could be to supplement their diets with antioxidants prior to transporting, which has been assessed in other production animals (Adenkola et al., 2011). In goats it is reported that long distance travel affected their oxidant/antioxidant status and decreased their excitability and grazing behaviour after unloading. In contrast, goats supplemented with ascorbic acid (a known antioxidant) before the journey, did not display similar behavioural changes after unloading and their oxidant/antioxidant systems remained in balance after the journey was completed (Minka and Ayo, 2013).

Further study is needed to investigate the relationship between transport stress, redox balance and respiratory pathologies development.

1.7. Conclusions

To safeguard the welfare of the transported horse it is important to minimize transport stress.

A horse which is disturbed when first coaxed into a transport vehicle may show various signs of disturbance, but most of these signs will disappear by the tenth transport experience (Broom and Johnson, 1993; Schmidt et al., 2010). Thus, loading and transport training with appropriate provision of positive reinforcement are strongly recommended (McGreevy and McLean, 2007). Sport horses should be transported occasionally for pleasure riding, decreasing the association between the truck and the competitions. As transport is a stressor even for experienced horses, it may be reasonable to give a rest period (a period with no travelling) to "frequent traveller horses".

To limit health problems post transportation, it is important to examine the health status of the horses before travelling, and to optimize the environmental conditions inside the truck to minimise noxious insults and maximise physiological comfort. Providing horses with electrolytes and antioxidants (vitamin E, C and selenium) prior to and after the journey may also aid in recovery and in reducing the risk for the development of transport-related disease.

During long distance journeys it is essential to plan rest stops (of at least thirty minute duration) every 4 hours for watering, feeding, urination (particularly for male horses), and cleaning the vehicle. Horses should not be loaded more than 18 hours without being unloaded and being able to do some physical exercise. Upon arrival at the destination it is important to offer fresh water and high quality food, since it is apparent that drinking and feeding are the horses' highest priority needs after transportation.

Whilst significant research has been conducted and reported, allowing for the development of best practice management guidelines, further research is required to more fully understand the relationships between transport stress, the immune system status and risk of post transport disease development. Transport stress is caused by a mosaic of stressors and the travelling horse's wellbeing can be best improved only through a multi-factorial approach.

CHAPTER 2

Health problems and risk factors associated with long haul transport of horses in Australia

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2.1. Introduction

Transport stress in horses is caused by a myriad of stressors (e.g. isolation, confinement, noise, vibration and balance problem) which affect them both mentally and physically, causing behavioural and health problems prior to, during and after travel (Chapter 1). Both short and long trips are stressful for horses and require proper management (Tateo et al., 2012). Longer trips have a greater effect on horse health and require particular attention (Leadon and Hodgson, 2014; Marlin et al., 2009), and those longer than 10 hours duration may lead to psychological and physical exhaustion and death (Marlin, 2004). Consequently, many animal transport codes include special requirements for longer journeys. For instance, the EU regulation 1/2005 (http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=uriserv:f83007) has specific requirements if the transport exceeds 8 hours. The Australian Animal Welfare Standards and Guidelines for the Land Transport of Livestock has instead species-specific maximum journey and minimum rest periods that take into account access to water and food *en route* (de Witte, 2009).

Stress activates hormonal changes in animals, which help them to adapt to the stressful situation. This response is commonly referred as "the flight or fight response", and is characterized by the activation of the pituitary and adrenal responses and by a release of adrenaline and cortisol. The most common effects of adrenaline are an increase in heart and respiratory rates, and an increase in sweating and defecation (Moberg and Mench, 2000). During transportation these hormonal responses are often a result of the horse attempting to adapt to the challenging situation (being transported), but they can affect the horse's immune response, making the horse more susceptible to transport-related diseases (Stull et al., 2008; Stull et al., 2004).

Transportation has been associated with physical injuries and heat stress, as well as specific illnesses such as respiratory diseases, colic, laminitis, enterocolitis and rhabdomyolysis (Collins et al., 2000; Leadon and Hodgson, 2014; Mansmann and Woodie, 1995) (Chapter 1). The most serious, potentially fatal respiratory disease is equine pleuropneumonia, commonly referred to as "travel sickness" or "shipping fever" (Copas, 2011). The risk of developing this disease increases with journey duration, especially when the duration exceeds 10 hours (Austin et al., 1995). Predisposing factors for the development of shipping fever include prolonged head elevation (Raidal et al., 1995), poor air quality (Katayama Y, 1995), and pre-existing respiratory diseases (Marlin, 2004). Transport associated dehydration, withholding of food and water, and diet change on arrival have been proposed as risk factors in the development of transport-related gastrointestinal disease in horses (Chapter 1). Colic during or after transportation is commonly reported, with impaction of large colon most often recognised (Mansmann and Woodie, 1995; Padalino, 2015). Enterocolitis caused by *Salmonella* spp. has been also associated with transport stress (Feary and Hassel, 2006) and can be fatal. While many risk factors for the development of transport-related diseases and injuries have been identified, further studies are required to identify additional unrecognised factors, and to determine the relative contribution of different contributing factors to transport-related disease and injury. Knowledge of the full range of risk factors related to equine transportation may help to safeguard the health and welfare of horses.

Surveys on farm animal transportation have been performed to identify risk factors and explore the epidemiological basis of transport-related health and welfare issues. For instance, the incidence of mortality during road transport has been calculated for cattle in North America (0.011%) (González et al., 2012), bobby calves in Australia (0.64%) (Cave et al., 2005), pigs in Europe (0.07%) (Averós et al., 2010) and in broilers in Brazil (from 0.42% in summer to 0.23% in autumn) (Vieira et al., 2011). In horses, surveys have been reported only for transport to abattoirs/slaughter plants (Grandin et al., 1999; Marlin et al., 2011; Stefancic and Martin, 2005). In these studies, transport-related health problems ranged from 7% to 28%. However, large numbers of horses are transported for other commercial activities such as competition and breeding, and for recreational uses. As these animals have a greater economic value than those destined for abattoirs, it is likely that their management and their transport-related health problem incidence will be different.

Millions of horses are moved daily all over the world, with the true global total of horse transport movements so large that it is impossible to estimate (Herholz et al., 2008). Consequently, there is a gap in our knowledge of the incidence of transport-related problems, horse mortality, and risk assessment related to equine commercial transportation. To the authors' knowledge, a survey on commercial equine road transport for any purpose has never been conducted in Australia. As the scientific identification and evaluation of hazards can only be done when the scenario including the animal and the transport environment is defined (Marahrens et al., 2011), the records of a horse transport company specialized in long road trips (~4,000 km, taking 3.5 days) in Australia were collected and analysed. The objective of the present study was to determine the incidence of transport-related injury and illness in horses undertaking commercial long-distance road transportation, and to assess and quantify the relationship between animal (sex, breed, and age), transport conditions (duration, and season) and welfare outcomes measured by the incidence of death, injuries, pyrexia, respiratory and gastrointestinal problems associated with a defined commercial long haul transport in Australia.

2.2. Experimental Section

2.2.1. Materials and Methods

Records of all transport movements from April 2013 to April 2015 were obtained from a commercial horse transport company which regularly transports horses between the east and west coast of Australia (~4,000 km and at least 3.5 days duration). Care and handling of the animals during transportation was not supervised by the research team. This data set was collected as part of a comprehensive survey on horse transportation approved by the Human Research Ethics Committee of the University of Sydney (2015/308).

2.2.2. Trip details

Before booking the trip, each horse owner had to send to the company the following information: breed, sex, age, body measurement (i.e. height, body weight), level of tame, reason for transportation. This information was necessary to allocate the right space to each animal inside the vehicle. As policy the company moved only tamed horses, at least trained to halter and basic commands from ground (e.g. follow and stop at the rope) and advised previous transport experience, the transported animals complied with this policy.

All transportation was performed following a fixed schedule from a collection stable in Sydney. The trip consisted of four stages: Sydney-Melbourne (10 hours), Melbourne-Adelaide (8.5 hours), Adelaide-Kalgoorlie (24 hours) and Kalgoorlie-Perth (6 hours). After each stage, horses were given a twelve hour rest period. The total duration was approximately 85 hours with approximately 49 hours in transit and 36 hours for rest stops. The schedule was reversed for Perth-Sydney trips.

At the collection stable and rest points, horses were individually housed in in-walk out rubber lined stables and/or paddocks that were used only for horses in transit.

All animals travelled on the same type of vehicle (Mega Ark Trailers, MAN®, Munich, Germany, Europe) equipped with 15 horse individual stalls, 6 facing backwards and 9 facing forwards. However, since large horses were allocated 1½ stall spaces, the average number of horses transported per trip was 9.1.

The ventilation system comprised venturi vents, louvres and electric fans generating an airflow which the manufacturer verified was compliant with the Australian Code of transportation throughout the trailer. When the vehicle was moving fresh air entered through the louvres and was extracted by the venturi vents. The fans were used in extreme heat conditions (> 35-40^{o}C), and to ensure constant air flow when the truck was stationary (e.g. feeding and watering times, fuel stops).

The horses travelled in individual stalls, restrained by rubber cords which would break under extreme pressure. Foals, ponies or weanlings were not tied up. Mares and foals travelled in a 3 stall section which allowed them to move around as if in a small box. Horses were fed with hay and watered every 4-5 hours, using the stainless steel feed and water bins in each bay; water and food were refreshed regularly *en route*.

Two drivers were used for all trips for which data were collected. Both were licensed to drive heavy combination vehicles and were experienced horse handlers with ten years' experience in commercial horse enterprises.

All journeys complied with the standards and the guidelines for the transport of horses required by the Australian Code of Livestock Land Transportation (http://www.animalwelfarestandards.net.au/land-transport/).

2.2.3. Monitoring of the animals and identification of pathology

The assessment of the fitness for travel of the horses was performed by the drivers and experienced staff members of the company at the collection stable and at each transit stable before loading the animals to continue the journey. The assessment was made in accordance with the Australian Code of Livestock Land Transportation. The conditions to be assessed included any signs of colic, raised or lowered rectal temperature, lethargy, diarrhoea, wound or abscesses, lameness (no more or equal to the fourth grade) and body condition score (no less of two)(http://www.animalwelfarestandards.net.au/land-transport/). After the assessment the report was sent to the operation manager, who gave final approval for transport.

During transport, there were two opportunities for monitoring horse health. The first was during the mandatory rest stop which is required after four hours of driving. At this stop, the driver undertook a visual examination of the horses. The second opportunity for monitoring occurred at the transit stables where rectal temperature was recorded, and drinking, feeding and eliminating behaviours monitored. At the rest stop in Adelaide, horses were inspected by an Australian government accredited veterinarian who confirmed that horses were fit to continue their journey, administered a triclabendazole drench and collected a faecal sample in compliance with the Western Australian quarantine regulations.

As soon as any health problem was identified by the drivers, the company manager was informed and a veterinarian was called for consultation and for treating the affected horse. The company director had a list of veterinarians to call in emergency. The affected horse did not continue the trip if the veterinarian did not evaluate it fit for travel. When health problems were identified by the owners post transport, the transport company manager was informed and he required a veterinarian's report to prove that the problem was related to the previous journey.

2.2.4. Data set

The data set included 1650 horses transported from Perth to Sydney (~4000 km) or vice versa for 180 journeys. Horse details (breed, sex, age) and the date of the trip (month and year) were recorded. The data set included reports of problems and issues identified by the drivers and horse owners and sent to the company manager, including the type of problem(s) and where (e.g. location) and when it occurred (i.e. an estimation of the approximate time at which the issues or incidents were first identified). As after the identification of each problem, horses were checked and treated by veterinarians, and after death necroscopy was conducted, these veterinary records were also included in the data set.

For statistical analysis, the recorded transport-related issues and problems were classified according to the time of occurrence in the following categories: preloading (from the horse's home stable to the company's collection stable in Sydney or Perth); in transit (during the trip or at rest stops); and post-transport (within 3 days after arrival at destination).

Based on the veterinary records, the transport-related health problems were also classified into five categories according to clinical signs/body system affected (Table 1).

Table 1. Description of the transport-related issues.

Category	Definition
Injuries	Laceration, abrasion, contusion, swelling.
Pyrexia	Rectal temperature >38.5°C, in the absence of other localising signs.
Gastrointestinal problems	Colic, enterocolitis, large quantity of internal parasites eliminated after triclabendazole treatment.
Respiratory problems	Nasal discharge, coughing, inflammation/infection of the upper or lower respiratory tract, and pneumonia.
Death	Horses found dead or humanely destroyed.

2.2.5. Statistical Analysis

Descriptive analysis of the dataset was conducted using statulor^{beta} (http://statulator.com/descriptive.html); data were reported as number of injuries or illnesses and as percentages. All further statistical analyses were performed using Gen Stat® Version 14 (VSNi International, Hemel Hempstead, UK). For all statistical analyses, a P value of <0.05 was considered statistically significant.

The details of all the travelled horses were categorized according to sex (mare/filly, gelding, stallion/colt), age (weaning/foal, yearling, 2-5 years, 6-10 years, >10 years), and breed (Arab, Quarter horse, Standardbred, Thoroughbred, Warm Blood). Univariate logistic regression analysis was conducted with development of a transport-related problem as the outcome (1/0: affected/non affected), and sex, breed, and age as the explanatory variables. Wald tests were obtained along with mean predictions of disease rate for each variable.

The date of recorded transport-related issues was categorized into the 4 Southern hemisphere seasons: winter (June-August), spring (September-November), summer (December-February), autumn (March-May). The time of the recorded transport-related issues (calculated from departure to when the recorded transport-related issue or problem was identified) was classified into three categories of journey duration: <20, 21-40, and >40 hours. Based on the veterinary records, considering the severity of the clinical signs, the required treatments and the time of recovery, the type of transport-related issues was listed in order of increasing severity as follow: injuries, pyrexia, gastrointestinal problems, respiratory problems, death. Ordinal regression analysis was then conducted to study the association between the type of transport-related issues (outcome) and the journey duration (<20, 21-40, >40 hours), and the season of the year (winter, autumn, summer, spring) (factors).

2.3. Results

2.3.1. Descriptive statistics

The general demographics of the population of horses studied is shown in table 2. Horses were transported for the following reasons: sale-purchase (30%), competition (50%), and breeding (20%).

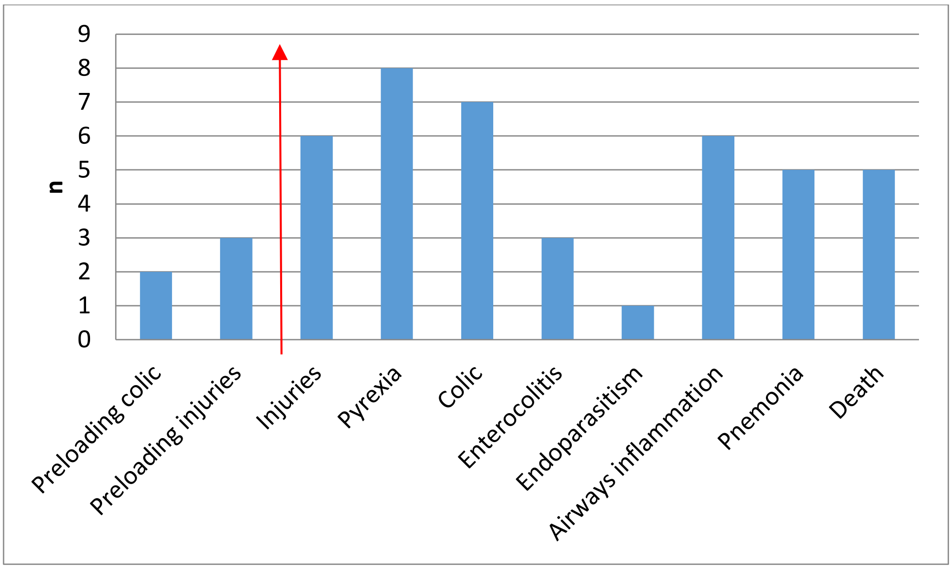
Table 2. Frequency of the total transported horses by sex, breed and age category.

Variable	Category	Frequency (%)
Sex	Gelding	35.7
	Mare/Filly	49.5
	Stallion/Colt	14.8
Breed	Arab	9.6
	Quarter horse	8.7
	Standardbred	27.5
	Thoroughbred	43.0
	Warm blood	11.2
Age	Weaning/Foal	11.2
	Yearling	12.9
	2-5yrs	34.7
	6-10yrs	27.9
	>10yrs	13.3

Approximately ninety-seven per cent (1604/1650) of the horses arrived at destination in good health, without any pain, signs of lameness or other pathology and did not develop any diseases post journey.

Only 2.8% (46/1650) were included in the company data set for a transport-related issue at preloading, in transit, or post-transit (Figure 1). Of the 46 cases, five cases related to preloading events, three were injuries that had occurred before the trip commenced or during transport to the collection stable, and two were cases of colic identified at the departure stable. Of the remaining cases, two horses were injured during loading while resisting loading and 4 injuries happened in transit.

Figure 1. Incidence of transport-related issues as reported by the transport company. The arrow divides issues related to preloading from those related to transit and post transit phase.



All the injuries were minor and the horses were treated topically and continued their journey. Six horses were identified as febrile at rest stops, and another two were identified as febrile upon arrival. No localising signs were identified in any of these cases and all were treated with anti-inflammatory medications. Four horses showed signs of colic at rest stops with another three showing signs of colic post journey. All cases were interpreted as impaction colic; two resolved without treatment (required only monitoring) and 5 were treated medically. Enterocolitis was identified in one horse during transport, and in two horses post transport, with all horses requiring hospitalisation. One horse eliminated a massive quantity of parasites after the anti-parasitic treatment. Five horses developed respiratory signs, including nasal discharge, coughing, and pyrexia during the journey, and one developed signs after arrival. The veterinary diagnosis was inflammation of the upper or lower airways, without pneumonia and all cases were treated medically. The specific diagnosis of pneumonia was made on the basis of signs that developed in four horses during transport, and in one horse after arrival. All horses recovered after appropriate medical treatment. There were four transport-related deaths, giving an overall death rate of 0.24%. Two occurred during transport, 1 horse was found dead within 24 hours after transportation, and 1 was humanely destroyed due to enterocolitis post transit. Another horse was found dead two days after transport, however this death could not be

confirmed to be transport-related. Post-mortem examination failed to reveal the cause of death in the four horses that were found dead. If the horse that was found dead two days after transport is included in the statistics, the death rate increases to 0.30%.
The incidence of the transport-related issues grouped by category is shown in Table 3.

Table 3. Incidence of transportation issues grouped in 5 major categories according to clinical signs and body system affected.

Category	**n**	**Incidence in the affected animals (n=41)**	**Incidence in all transported animals (n=1650)**
Injuries	6	15%	0.36%
Pyrexia	8	19%	0.48%
Gastrointestinal problems	11	27%	0.66%
Respiratory problems	11	27%	0.66%
Death	5	12%	0.30%

2.3.2. Logistic regression

Univariate logistic regression analysis of horses experiencing transport-related health issues showed no significant effect of sex, breed**,** or age (Table 4).

Table 4. Results of the univariate logistic regression analysis with development of a transport-related problem as the outcome (1/0: affected/non affected), with sex, breed, and age as explanatory variable.

Variable	**Category**	**Disease rate prediction (%)±s.e.**	**Estimate± SE (%)**	**OR**	**Lower 5%CI**	**Upper 95%CI**	**P value**
Sex	Gelding	7.0 ±1.5	Ref				
	Mare/Filly	5.3±1.1	-0.29±0.3	0.74	0.39	1.424	0.611
	Stallion/Colt	7.1±2.4	0.02±0.4	1.02	0.43	2.403	
Breed	Arab	3.3±2.2	Ref				
	Quarter horse	5.4±3.0	0.14±0.7	1.16	0.25	5.217	
	Standardbred	5.2±1.6	0.56±0.6	1.75	0.50	6.039	0.187
	Thoroughbred	9.2±1.7	0.72±0.6	2.05	0.59	7.141	
	Warm blood	2.5±1.9	0.96±0.6	2.61	0.70	9.743	
Age	Weaning/Foal	1.8±1.0	Ref				
	Yearling	2.1±1.0	0.51±0.9	1.67	0.26	10.41	
	2-5yrs	3.2±0.8	-7.20±9.3	0.00	8.12E-12	68858	0.523
	6-10yrs	3.8±0.9	1.08±0.7	2.94	0.67	12.8	
	>10yrs	4.8±1.5	-0.10±1.0	0.85	0.11	6.246	

Standard error (SE), Odds ratio (OR), Confidence Interval (CI).

2.3.3. Ordinal regression analysis

There was a significant association (P=0.022) between type of transport-related issues and duration of trip, with a higher probability of a more severe disease after 20 hours of transport. Injuries were more likely to occur in the first 20 hours of transport (Figure 2).

Figure 2. Probability of a more severe transport-related issue to be associated with journey-duration. Bar charts with different letter have a different distribution of transport-related issues: a, b: P<0.05.

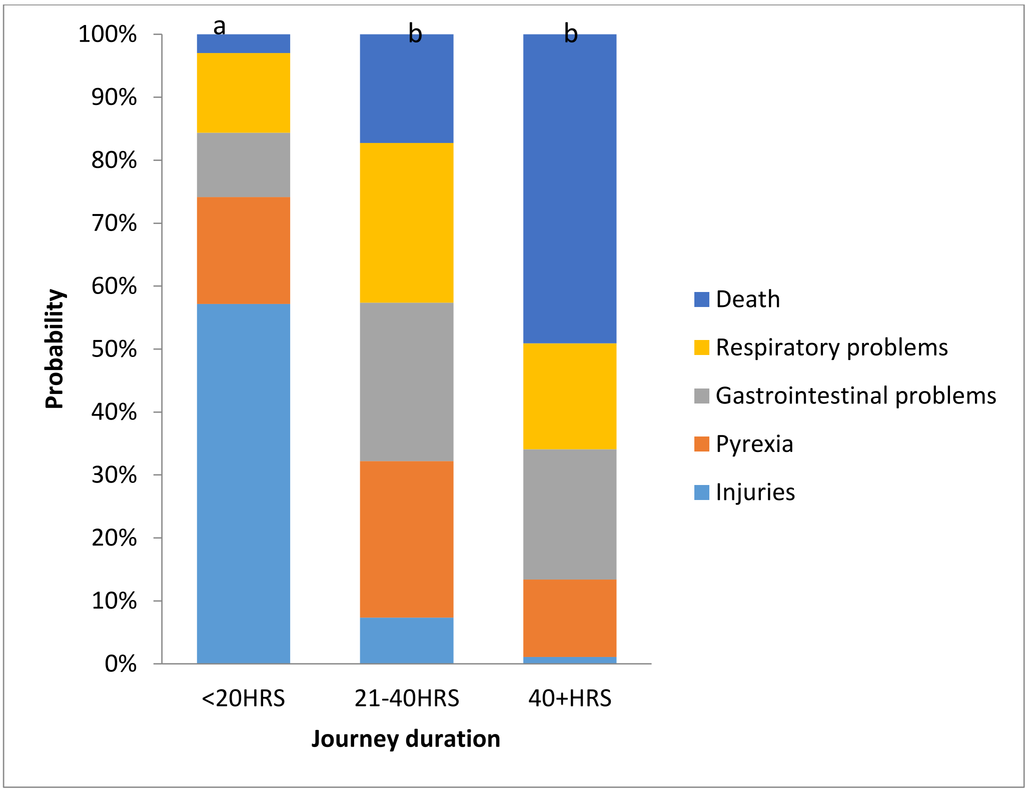


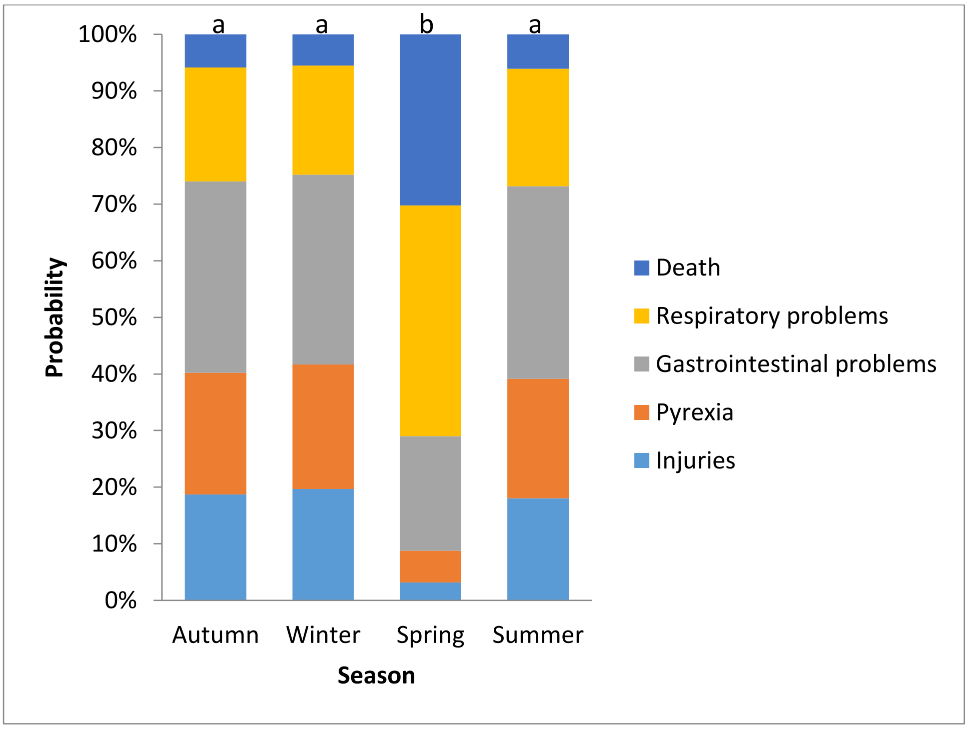
Table 5 shows odd ratio (compared to risk of injury) and confidence interval for each disease occurring in a journey longer than 20 hours.

Table 5. Estimate, odds ratio (OR) and confidence interval (CI) for each transport-related problem on a journey longer than 20 hours.

Transport-related problem	estimate	SE	P value	OR	lower 5% CI	upper 95% CI
Injury	-	-	-	-	-	-
Fever	2.56	1.34	0.057	12.91	0.9271	179.8
Colic	3.19	1.3	0.014	24.37	1.91	311.1
Respiratory	2.93	1.32	0.027	18.69	1.399	249.7
Death	4.54	1.52	0.003	93.49	4.783	1827

Season had a significant effect on the distribution of transport-related issues (P=0.035), with a higher probability to have a more severe transport-related issue (gastrointestinal problems, respiratory problems and death) in spring (Figure 3).

Figure 3. Probability of a more severe transport-related issue to be associated with season. Bar charts with different letter have a different distribution of transport-related issues: a, b: P<0.05.



2.4. Discussion

The present study reports the incidence of transport-related issues and mortality associated with long-haul equine transport by a commercial equine transport company. An overall incidence of transport-related injuries or disease of 2.8% was observed in this study, which is much lower than has been reported by horse owners (Noble et al., 2013) and for horses transported to abattoirs for slaughter (Grandin et al., 1999; Marlin et al., 2011). The data demonstrates that travelling is a risk to equine health, wellbeing and welfare and a correct management of transportation is required for moving horse successfully.

The prevalence of injuries identified in the present study (0.36% of horses) was lower than the rate reported in horses transported to abattoir for slaughter (Grandin et al., 1999; Marlin et al., 2011). This may reflect differences in the way in which the transport was managed (i.e. individual calculated space), and in the tractability and transport experience of the horses. However, it was also lower than reported by owners during non-commercial horse transportation (Noble et al., 2013), which suggests that transport management is a key determinant of the injury rate. The design of the truck (including the floor, suspension and the height of height of the roof) has been identified as a risk factor in the development of injuries and transport-related diseases in farm animals (Marahrens et al., 2011). In cattle, the incidence of injuries during long haul transportation has been associated with the years of experience of the drivers (González et al., 2012), and in horses travelling on non-commercial road transport, many incidents are related to poor driving skills, particularly on winding country roads (Noble et al., 2013). Vehicle design, road quality and driving that allows horses to keep their balance appears to be important in minimising injuries.

Two injuries occurred during the loading process due to resistance to boarding the truck. The horses' fear of being loaded onto the vehicle manifests through various deleterious and dangerous actions and behaviours (e.g. kicking and rearing)(Chapter 1). Handling horses during loading/unloading is therefore considered to be a highly dangerous risk for those handling the transported horse (Riley et al., 2015). Thus it is important that transport procedures are carried out by experienced horse handlers wearing protective equipment, such as, capped boots and gloves, to minimize the risk of injuries to the horse and the handler during the loading and unloading phase of equine transport.

The body systems most commonly affected by transport are the respiratory and the gastrointestinal systems, and a common clinical sign associated with inflammation to these systems is pyrexia (Marlin, 2004). Pyrexia affected 0.48% of all transported horses in this study. Since early identification of pyrexia prompts investigation and implementation of appropriate therapy and recovery, checking temperature during and after long trips should be seen as best practice when dealing with the transported horse and has been recommended previously (Racklyeft et al., 2000; Raidal et al., 1997a).

Transportation has been associated with the development of airway inflammation and equine pleuropneumonia (Copas, 2011). Poor ventilation inside the truck, forced high head position, and dehydration have been identified as predisposition factors in the development of respiratory diseases associated with transport (Leadon and Hodgson, 2014). The horses in the current study travelled in a vehicle equipped with a forced ventilation system, which should assure good air quality and a comfortable temperature inside the trailer, but horses were not allowed to lower their head beyond the height of the wither. In our study 0.66% of all transported horses developed respiratory problems, and only 5 developed pneumonia. This rate is less than expected (Chapter 1), potentially reflecting the importance of a good ventilation system in the transport vehicle. However, there is no evidence to suggest that good ventilation alleviates the need to provide correct watering (Anon, 2014), to minimise the duration of confinement with the head elevated, or to maximize the time available for the horse to physically clear its respiratory tract (Stull and Rodiek, 2002).

Transportation may increase the likelihood of colic for several reasons (Tinker et al., 1997). Firstly during a stressful situation, preferential perfusion of the brain and the muscles may reduce visceral perfusions (flight and fight response)(Moberg and Mench, 2000). Additionally, dehydration during transportation can reduce vascular perfusion of the gut, potentially inducing impaction of the colon (Mansmann and Woodie, 1995). Finally food and water withdrawal, altered diet and/or eating in an unnatural position on route might create change in the pH and gut flora which may influence the chance of enterocolitis. In this study, transport associated gastrointestinal problems were seen in 27% of cases, with enterocolitis seen in 4 out 41 cases, with one requiring euthanasia. Equine enterocolitis, can manifest in sudden death and be associated with over-proliferation of *Salmonella* spp, *Clostridium* spp and *Fusarium* spp in the equine gut (Staempfli et al., 1991). Stress is considered an important predisposing factor for salmonellosis in horses; this pathology has already been associated with transportation, surgery, feed withdrawal, changes in feed, and antimicrobial and anthelminthic therapy (Feary and Hassel, 2006). Avoiding prolonged feed and water restriction, abrupt diet changes, or overuse of antibiotics and anthelminthics before, during and after transportation could therefore potentially reduce the incidence of gastrointestinal transport associated problems.

In this study the mortality rate associated with transport events was 0.24% or 0.30%, which is somewhat higher than rates reported for cattle in North America (0.01%) (González et al., 2012) and pigs in Europe (0.07%)(Averós et al., 2010). A reason for this high rate could be that animal transportation is more risky in Australia due to the long journey duration and the climate; the rate described in this study is indeed in line with mortality rates observed in cattle transported in Queensland by rail (an overall mortality rate of 0.10%, ranging from 0.44% in bulls to 0.06% in calves) (Jarratt et al., 1982), and in bobby calves (> 4 days old) transported by road in Victoria (0.64%)(Cave et al., 2005). Thus, moving horses in Australia may require more detailed and specific strategies to cope with extreme distance and weather.

In the current study, 2 horses were found dead in transit and 2 died soon after transportation. Even after pathological examination the reasons for these deaths were unknown, but maybe contributed by protracted stress. Stress is a physiological and endocrionological response that helps individuals to cope with stressors and to survive. However, when an animal fails to adapt, the stress response can lead to death (Moberg and Mench, 2000). In horses, transport stress is often followed by the stress of living in unfamiliar environments (e.g. new stall, food, social group), further affecting the horse’s health. Consequently, offering similar feed and avoid inserting the recent arrival into a new herd could reduce protracted stress and assist with adaptation to the unfamiliar environment after the journey (Chapter 1) and potentially reduce the risk of death after transportation events. Witnessing a death of any animal can have a negative impact on those who have witnessed it, whether they are professionals working in the animal or veterinary industry or members of the general public owning animals (Foster and Maples, 2014). Consequently, minimising the equine transport associated mortality rate will have a positive impact on the wellbeing of horses and the mental wellbeing of those dealing with the transported horse on a day to day basis.

No significant effect of sex, age, or breed in the development of transport diseases was found, suggesting that individual horse variability and past experience might be more important in influencing the ability of the horse to cope with the transport event (Fazio et al., 2013b). Elevated, but not statistically significant, prediction rate were seen for males, horses aged over 10 years, and Thoroughbreds, consequently dealing with these categories of horses may require specific transport management strategies to reduce the predicted risk of these horses developing transport-related complications.

In agreement with Stefancic and Martin (2005), although number of cases were small, this current study showed transported related death and respiratory diseases were more likely to occur in spring. This may reflect abrupt temperature increases at a time when many horses still have winter coats, with consequent, impaired thermoregulation in transit. Alternatively, animal behaviour and immune system may be affected by the reproductive hormonal profile of the breeding season (Styrt and Sugarman, 1991). Other factors, such as the occurrence of viral respiratory tract infections or increased pollen or other allergens might contribute to increased risk of respiratory disease at this time. Such speculations warrant further research.

The data presented in the current study, confirm the increased risk of mortality and disease in horses associated with longer transport events, with more severe diseases (e.g. enterocolitis, pleuropneumonia) and death/euthanasia more commonly observed after 20 hours of transportation. Horses travelling to abattoirs for slaughter, were similarly more likely to die after protracted transportation (Stefancic and Martin, 2005); and in cattle, higher mortality rates have been associated with trips longer than 36 hours in Australia (Jarratt et al., 1982), and longer than 30 hours in Canada (González et al., 2012). Better understanding of the increasing risk of severe transport-related diseases and death with increasing duration of transport may encourage the adoption of more rigorous preventive strategies, such as a veterinary examination, for horses that are going to be transported for more than 20 hours.

The biggest limitation of this study is that the assessment of the horse health before, during and after journeys was not performed by the authors. Consequently some transport-related problems might have been missed by the drivers and the owners, and the incidence of the transport-related diseases could be underestimated. This is particularly likely if horse owners did not associate illness with recent transport, failed to recognise minor or subclinical disease, or failed to report minor or major illness to the company. Pleuropneumonia and enterocolitis can manifest up to a week after transport (Marlin, 2004), and other effects of transportation stress can take up to one month to manifest after the event (Cregier and Gimenez, 2015). Hence it is possible that owners or agents may have failed to associate disease with transportation, or may have failed to detect mild effects on horse health. The data obtained in this current study is also limited by the lack of environmental parameters measured or recorded during journeys. Extreme hot and cold temperatures have been identified as risk factors in long haul transportation of farm animals (Schwartzkopf-Genswein et al., 2012). Notwithstanding these limitations, this study is the first carried out on horses undertaking this unique multi-day road trip across one of the harshest continents in the world. It has provided important data for the equine industry on the incidence of health problems associated with long haul transportation in the horse. Preliminary evaluation has identified and suggested some predisposing factors associated with transport-related health problems which warrant further evaluation to enhance policy and practices relating to transportation of the horse.

2.5. Conclusions

Journey duration and season were identified as risk factors contributing to transport-related health problems in horses undergoing long distance road transportation. Although the trips were well organized and complied with or exceeded the requirements of the Australian Code, serious diseases still occurred. Moving horses should be considered as a human-related risk to horses and also a horse-related risk to humans (Thompson et al., 2015), so it should be always carried out by professional and experienced horse handlers and drivers, wearing adequate protective equipment, to reduce the risk of injuries and diseases in both horses and humans. Further research to confirm preliminary conclusions based on this data and to recognize other risk factors for the development of equine transport-related issues is needed to assist in improvement of the Australian Code of horse transportation.

CHAPTER 3
Survey on horse transportation

CHAPTER 3.1
Survey of horse transportation in Australia: issues and practices

CHAPTER 3.2
A survey on transport management practices associated with injuries and health problems in horses

CHAPTER 3.3
Investigations into equine transport-related problem behaviours: survey results

CHAPTER 3.4

Risk factors in equine transport-related health problems: a survey of the Australian equine industry

Overview of chapter 3

This chapter is based on the results of a survey on horse transportation, which was designed to evaluate horse amateurs/professionals' perspective of horse transportation, both private and commercial, in regards to how transport is conducted and the effect which it has on their horses. We invited people to undertake the survey (Appendix 1) to collect real world data so as to answer the following questions:

1. What are the most common transport practices conducted in Australia?
2. What are the most common pathologies which horses develop during and after transportation in Australia?
3. Is there an association between horse transportation practices and transport-related health problems?
4. Is there an association between horse transportation practices and transport-related problem behaviours?
5. Are horse and journey characteristics associated with transport-related health problems?

Even though the survey was composed of 40 questions (Appendix 1) and anonymus, it got an optimal completion rate and more than 150 respondants left their details sorting feedback from the survey, confirming the considerable interest the Australian horse industry has in improving the health and welfare of the transported horses. To give feedback and to educate horse people on transport best practices, 4 different articles were written. One article was also published in open access to allowed easy access and the results were also presented in a number of seminars to improve the communication of findings.

The ethical aspects of this study were approved by the Human Research Ethics Committee of the University of Sydney *[2015/308]*.

CHAPTER 3.1

Survey of horse transportation in Australia: issues and practices

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3.1.1. Introduction

Horses are transported for competition, sales, breeding, and slaughter (Giovagnoli et al., 2002). However, transport is considered a stressful experience (Grandin and Shivley, 2015) and may lead to serious health disorders (Chapter 1, 2). There is extensive literature on transportation of horses and its effects on physiological, behavioural and health parameters (Fazio et al., 2013b; Friend, 2000; Tateo et al., 2012). Increases in heart rate, respiratory rate, sweating, and blood concentrations of β-endorphin, free triiodothyronine, cortisol and adrenaline have been reported as responses induced by transport stress (Fazio et al., 2013a; Fazio et al., 2015). Anxiety, refusal to load, flight responses, kicking, pawing, scrambling, and stereotypic behaviour are the most common transport-related behavioural problems (Lee et al., 2001). Injuries have been reported as the most common health problem related to transportation (Mansmann and Woodie, 1995), but a variety of other disorders may also result from transportation (Leadon and Hodgson, 2014), such as impaction of large colon (Mansmann and Woodie, 1995), enterocolitis (Feary and Hassel, 2006), and equine pleuropneumonia (Racklyeft et al., 2000). The management of horses before, during and after transportation is a key factor in minimizing transport stress and reducing the incidence of transport-related diseases (Leadon et al., 2008).

A number of strategies have been identified to improve the management of transportation and a number of codes for horse transport have been developed worldwide in the last decade to minimize transport stress and suffering of travelling horses (Anon, 2014; http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=uriserv:f83007; http://www.animalwelfarestandards.net.au/land-transport/). For instance, travelling horses untied (Stull and Rodiek, 2002), allowing the horse to stretch its head and neck downward, and cleaning the truck at rest stops (Oikawa et al., 2005) have been suggested to reduce the incidence of airway inflammation. The Australian Animal Welfare Standards and Guidelines for Land Transport of Livestock was updated in 2012 (http://www.animalwelfarestandards.net.au/land-transport/) and is based on the most recent scientific findings. The standards contained in the Code are requirements that must be met under law for livestock welfare (e.g. horses over 6 months old have a maximum time without water of 24 hours and 12 hours of minimum rest-stop). The guidelines contained in the Code are recommended practices to improve animal welfare outcomes. The Australian Code states that the driver is responsible for the welfare of the transported horse; that animals must “be fit for the intended journey” and that the journey must be planned properly. However, unlike the requirement specified in the European Code (http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=uriserv:f83007), Australian drivers do not need a certificate of competence and a health certificate for the horse before departure is not mandatory. Moreover, in the Australian Code there are no guidelines for the management of horses post-transportation.

Despite the large body of research, a gap remains in our knowledge of implemented transport management practices and their effects on horse health for a number of reasons. Most scientific findings have been based on observational studies of experimental trips on research horses, which may not reflect the status of horses used for economic or recreational purposes (Leadon and Hodgson, 2014). Many studies investigating transport-related welfare outcomes have been conducted during commercial transportation toward abattoirs (Marlin et al., 2011; Roy et al., 2015), but this is unlikely to represent current practices for other commercial or recreational pursuits. Information on compliance with the existing codes is limited (Marlin et al., 2011).

The demographics of horse movements in New Zealand (Rosanowski et al., 2013) and in Great Britain (Robin et al., 2011) have been determined by questionnaire to inform disease control measures, and surveys were developed to document the incidence of particular transport-related issues, such as loading problems (Lee et al., 2001) or injuries (Noble et al., 2013). As no such information is available in Australia, a survey was conducted on transport practices and issues including racing, equestrian and recreational equine industry members. The study had two main objectives: the first was to gain a better understanding of current management practices related to transportation of horses in Australia and to determine whether they complied with the Australian Code; the second was to document the most common behavioural and health problems recognised by horse owners and connections associated with transportation in Australia.

3.1.2. Materials and Methods

3.1.2.1. Respondents

The target population for this survey was people who were experienced in the transport of horses for commercial or recreational purposes. Respondents were required to have organized transport at least monthly over the two years prior to completing the survey. Power calculation (http://statulator.com/samplesize.html) determined that greater than 400 survey responses would provide a representative sample of the estimated 400,000 Australians who care for horses (Smyth and Dagley, 2015) with power of 0.8 and $\alpha = 0.05$.

3.1.2.2. Survey

The survey (Appendix 1) asked respondents for demographic details (gender, age, Australian state or territory in which they were resident) and information on their involvement with equine pursuits (equine industry sector, relation with horses – professional or amateur, number of horses in care, travel frequency and duration), typical transport management (before, during and after journey), and transport-related problems (horse behavioural and health issues) over the two years prior to survey completion (2013 – 2015). The survey was developed by a process of iterative review by the researchers, piloted by the corresponding author with twenty horse owners, published using Survey Monkey (SurveyMonkey Inc., California, USA, www.surveymonkey.com), and promoted in many different ways to a broad and diverse selection of Australian equine-interest groups (Table 1).

Table 1. Distribution and promotion of the survey.

Category	Name	Method
Horse association	Equitana Australia Horsetalk.nz Australian Stock Horse association NSW endurance association Nswhrc.com.au Australian Standarbred Breeders associations Australian Hunter Horse Association	Promotion on their website
Horse Magazine	Horse and People Hoofbeats Australian Horsemanship Sportsman Queensland Endurance West Australian Horse racing Journal	Promotion on their printed and online copies

Horse transport company	Goldners Horse Transport	Emailing of the survey to their clients
Horse Association	Hunter Valley Thoroughbred Breeders Association Equestrian Australia Association International Society of Equitation Science The Australian Endurance Riders Associations	Emailing the survey-link to their members

Briefly, an invitation letter (Appendix 1) and the link to the survey were provided to all associations of Australian horse sports identifiable in the public domain, and were published through their websites. The link was also promoted through several horse magazines and posted on relevant Facebook pages and online horse forums. The survey link was posted to Thoroughbred trainers by at least one commercial horse transport company, and a number of industry associations emailed the survey link to their members. Survey details were shared on the internet and social media, and reached various other websites (e.g. Australian Horse Industry Council) without being directly contacted by the authors. The survey link was available for completion between 6th of July and 7th of September 2015.

<u>3.1.2.3. Statistical Analysis</u>

Descriptive statistics of the survey data were obtained using statulor^{beta} (http://statulator.com/descriptive.html). Data were reported as number of responses and as percentages. Chi squared tests were conducted to determine the association between being an amateur or professional and the sector of the horse industry they were involved in, the number of horses, the frequency of travel, the most common journey duration, transportation management strategies, and incidence of transport-related behavioural and health problems. Chi squared was performed using Gen Stat® Version 14 (VSNi International).

3.1.3. Results

The survey generated 955 responses, of which 158 did not meet the main inclusion criterion of having organized transport on a monthly basis, giving 797 valid responses. Percent responses to some questions did not always add to 100 because some questions allowed for multiple responses.

3.1.3.1. Respondents' details

Respondents' details are summarised in Table 2. Most respondents were female (84%) and approximately 70% were under 50 years old. Consistent with the location of most horse owners (Smyth and Dagley, 2015), more than 66% were from New South Wales, Victoria and Queensland. Almost half (48.8%) were involved in equestrian sport activities (dressage, eventing, jumping, reining, driving, vaulting) and the rest were distributed almost equally across the other equine industry sectors (endurance, breeding, recreational-riding, Thoroughbred and harness racing). Thirty percent of respondents were professionals (people who were involved with horses for financial reward) and 70% were amateurs (people who were involved with horses as a hobby). Respondents who identified a professional relationship with horses were more likely to be involved with racing or breeding sectors ($P<0.001$, Figure 1).

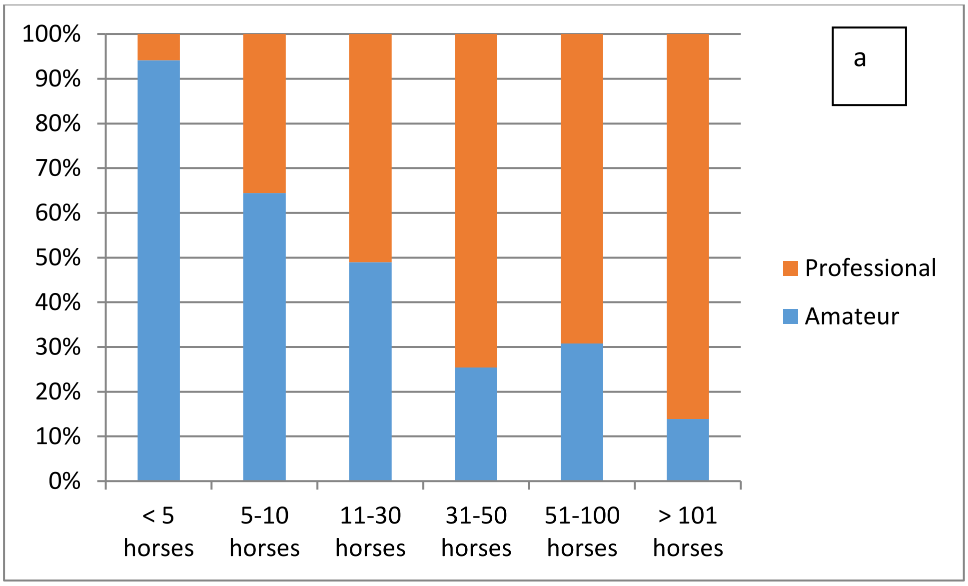
Table 2. Details of respondents' demographics.

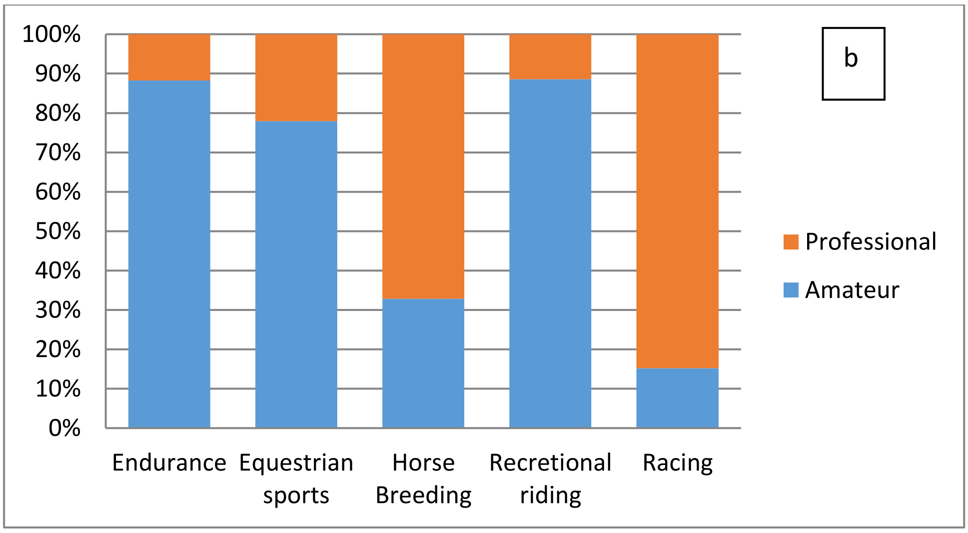
Respondent details	**Frequency (n)**	**Percent (%)**
Sex (n=797)		
Female	671	84.1
Male	126	15.8
Age (n=783)		
20-30	191	24.4
31-40	160	20.4
41-50	198	25.3
51-60	167	21.3
61-70	59	7.5
71 or older	7	0.9
Australian State (n=797)		
Australian Capital Territory	27	3.3
New South Wales	318	39.8
Northern Territory	25	3.1
Queensland	95	11.9
South Australia	54	6.7
Tasmania	18	2.2
Victoria	186	23.3
Western Australia	74	9.2
Equestrian sector (n=797)		
Endurance	51	6.3

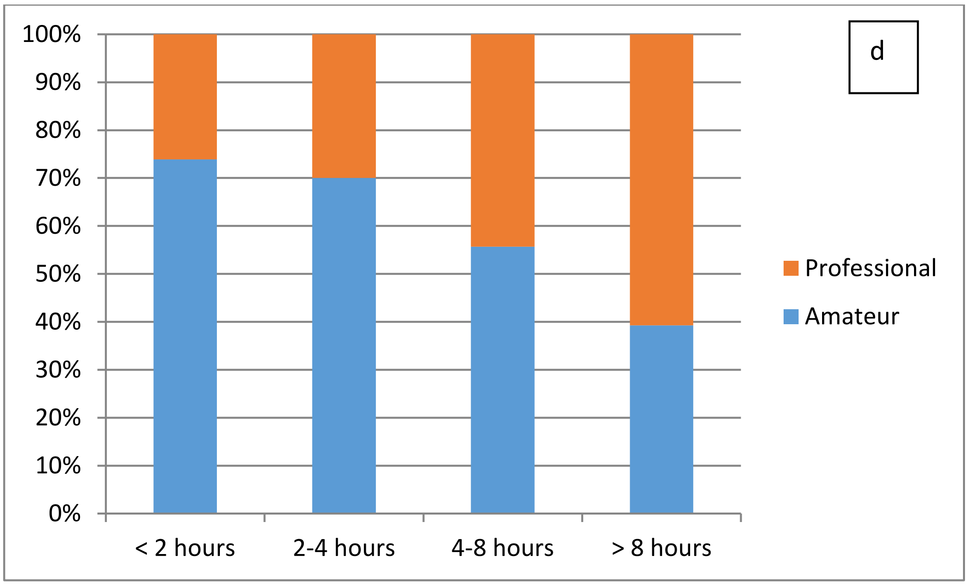
Equestrian sport*	389	48.8
Horse breeding	73	9.1
Recreational- riding	192	24.1
Thoroughbred and harness racing	92	11.5

* Dressage, eventing, jumping, reining, driving, vaulting

Figure 1. $\chi 2$ Amateurs versus professionals: a) number of horse in care; b) sector; c) frequency of transport; d) most common journey-duration. There was a significant association between the amateur/professional status and all the studied variables in this figure (P< 0.001).







<u>3.1.3.2. Number of horses and horse transport event demographics</u>

Information about the number of horses kept on respondents' properties, how often transport events were organized and the most common journey duration is provided in Table 3.

Table 3. Details of horse numbers and horse movements in the survey.

Horse and journey demographics	**Frequency (n)**	**Percent (%)**
Horse on the property (n=797)		
< 5 horses	341	42.7
5-10 horses	192	24.1
11-30 horses	147	18.4
31-50 horses	55	6.9
51-100 horses	26	3.2
> 101 horses	36	4.5
Journey frequency (n=797)		
Daily	55	6.9
Twice a week	124	15.5
Weekly	200	25.1
Fortnightly	161	20.2
Monthly	257	32.2
Journey duration (n=797)		
Less than 2 hours	463	58.1
2-4 hours	227	28.4
4-8 hours	79	9.9
More than 8 hours	28	3.5

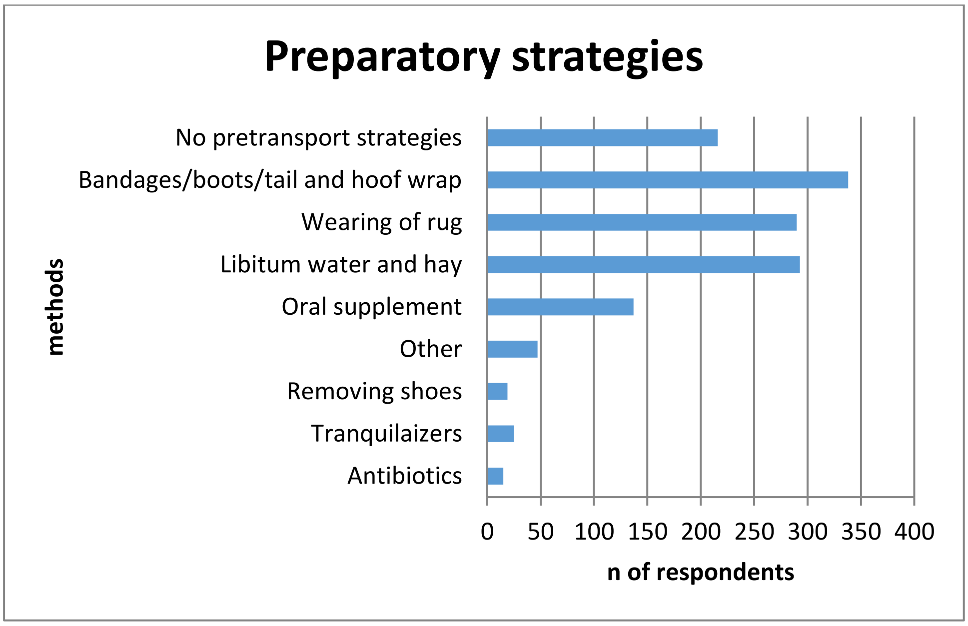
The average number of horses transported in each event (survey question 3) varied from 1 to 20, and the median number of horses transported in each event was 2. Relative to respondents with an amateur involvement, professionals reported a greater number of animals in their care, more frequent transportation (often daily) and journeys of longer duration (all $P<0.001$, Figure 1).

The 797 respondents were responsible for approximately 17,000 horses (question 2) and it was estimated that they organized more than 313,000 individual horse transport events over the two years nominated in the study (question 3). Assuming an estimated population of circa 393,915 horses in Australia (http://faostat.fao.org/, 2015), this study reports recollections of owners on the movements, transport practices and issues arising during transportation of approximately 4.3% of all horses in Australia.

3.1.3.3. Management

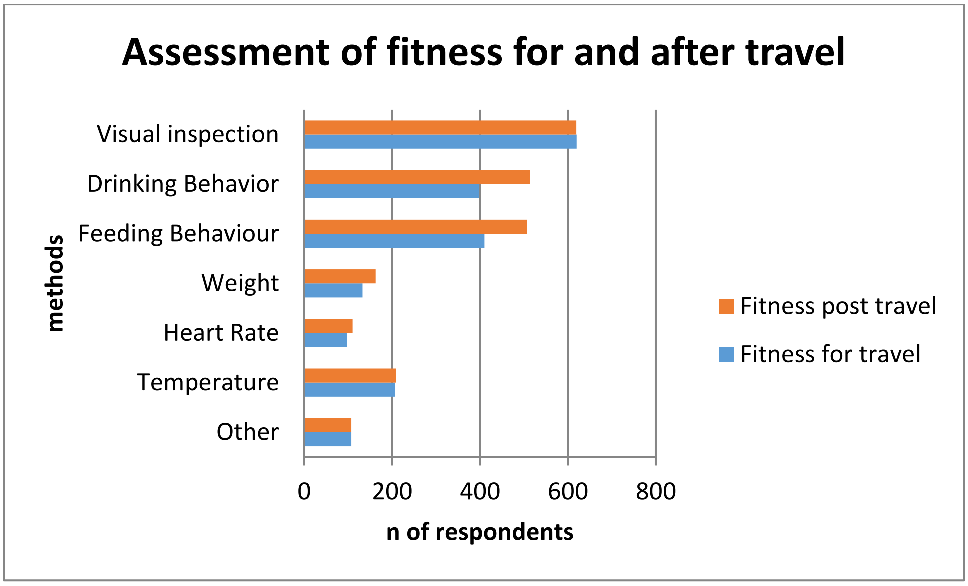
More than half of respondents (56.5%) trained their horses to assist them in loading and travelling, and there was no difference in the proportion of amateurs or professionals who adopted pre-training in loading and travelling (P=0.890). The various strategies reported by respondents to prepare horses for transportation are shown in Figure 2; 34.0% of respondents did not employ any strategy to prepare horses for travelling. There were no differences between amateurs and professionals in the use of protections (P=0.617), or in offering water and hay *ad libitum* (P=0.125). Amateurs rugged horses more frequently (P<0.001), and professionals more frequently reported removing shoes, administering sedation or antibiotics before journeys (P<0.001).

Figure 2. Strategies reported by survey respondents to prepare horses for transportation. The "other" option included: administration of gastro-protector (7/797), probiotics (5/797), or anti-inflammatory medication (2/797); reduction of grain intake (2/797), regular vaccinations against herpes virus prior to long haul transportation (1/797), planning the journey according to the weather condition (3/797). (The question allowed multiple responses).



The health/fitness for travel (http://www.animalwelfarestandards.net.au/land-transport/) was assessed by 76.6% of respondents, almost exclusively (94.4%) by people without veterinary qualifications. The health/fitness after travel was assessed by 88.4% of respondents, with 93.6% of these evaluations conducted by people without any qualifications. Professionals reportedly assessed horse health/fitness more frequently prior to and following transportation, and more often consulted veterinarians than amateurs (all P<0.001). Figure 3 shows how the fitness for and after travel was assessed when this was conducted by non-veterinary staff. Visual examination was the most common method (90%); monitoring of feeding and drinking behaviour pre- (58%) and post-loading (74%) were largely applied; temperature, weight, and heart rate were determined by only 30%, 20%, and 15% of respondents (respectively) both pre- and post-loading. There were no differences between amateurs and professionals in monitoring the weight of the horse before and after journey (P=0.565 and P=0.604), or in the assessment of the fitness by visual inspection before and after transportation (P=0.053, P=0.281). Compared to amateurs, professionals more often reported that they monitored drinking and feeding behaviours before and after transport (both P<0.001), and they were more likely to record heart rate (P=0.015, P=0.002) and temperature (both P<0.001) before and after travel.

Figure 3. How the health/fitness for and after travel was assessed by owners or staff member, without a veterinary qualification. In the ‘other’ category, the following strategies were reported to monitor horses both pre and post-journey by the respondents: inspection of horse(s) legs and shoes and a lameness test (3/797), monitoring of horse mental status (11/797), auscultation of gut and lung sounds (2/797), and monitoring of defaecation and urination (2/797). The following “other” strategies were reported only for post-journey: check for the presence of cuts, lacerations and swelling (11/797), hydration status assessment (6/797). (The question allowed multiple responses).



The most common transport vehicles were a two-horse straight trailer (63.5%), a truck (30.7%), a 3-4 horse angle trailer (18.7%), and a 3-4 horse gooseneck trailer (9.25%); however some respondents had more than one vehicle. There was an association between the vehicles used and the amateurs/professional status (P<0.001): amateurs more frequently used a small trailer, and professionals more frequently used 3-4 horse trailers, gooseneck trailers and/or trucks. Horses were most commonly transported facing forward (59.3%) or sideways (on the angle) (38.2%). Fewer than 2% of the respondents transported their horses facing backwards, and 1% of horses were unrestrained (i.e. free movement and able to choose their position). Amateurs transported their horses facing forwards more frequently than professionals (P<0.001). Even though the majority of respondents (92.1%) had trailers and/or trucks to move their horses, 67.2% of them indicated they also used commercial horse transport companies, especially for journeys longer than 8 hours.

Of the 92.1% of respondents transporting horses privately, only 9.1% did not tie up the horses during transport. Of those who tied their horses, 87.5% reported that the horse(s) were able to lower their head to or below wither height. There were no differences between amateurs and professionals in tying horses during journeys (P=0.104). Food and/or water was offered to the horses inside the vehicle *en route* by 58.5% of the respondents transporting horses privately; 90.8% of them put hay in a net, 5.2% put water and 4% put hard feed in a manger or bucket. Offering food and water *en route* was a practice more frequent applied by amateurs than professionals (P<0.001). Approximately 39% of the private transportation respondents did not monitor the horse or environmental parameters inside the vehicle during transit. Some respondents (37.9%) reported that horses were usually checked visually at every fuel or driving stop, whilst others (21.1%) reported the use of video cameras in the vehicle to monitor horse behaviour constantly. Only 1.7% of the respondents reported the use of equipment to monitor environmental parameters inside the trailer. Amateurs reported monitoring their horses less than professionals during journeys (P<0.001).

Strategies to assist horse recovery from transport stress were employed by 52.8% of respondents (Table 4). Strategies to rehydrate, such as administration of oral electrolytes and administration of water/hay *ad libitum*, were commonly utilised. Professionals reported the use of recovery strategies more frequently than amateurs (P<0.001).

Table 4. Strategies after transportation reported by 421 respondents. As many respondents indicated that they used multiple strategies, the percentage responses sum to more than 100%.

Strategy post transport	**Frequency (n)**	**Percentage (%)**
Oral electrolyte	155	36.8
Walking	147	34.9
Water/hay ad libitum	121	28.7
Grazing	83	19.7
Rest	78	18.5
Monitoring of horse behaviours	24	5.7
Feed on ground	18	4.3
Vitamins/amminoacid	15	3.5
Fluids (nasogastric drench/ i.v.)	10	2.4
Rugging	9	2.1
Cool wash	5	1.2
Anti-inflammatory	4	0.9
Gastric protection	3	0.7
Probiotic	3	0.7
Wet food	3	0.7

3.1.3.4. Behavioural problems

More than one third (38.6%) of the respondents reported that they had encountered behavioural problems in at least one of their horses during transport. There was no association between the amateur/professional status and the incidence of transport-related behavioural problems (P=0.620). Among the respondents who reported experiences of behavioural problems, the most common problem was refuse to load (50.8%), followed by travelling problem behaviours, such as scrambling, kicking, and biting (42.4%). Less common transport-related behavioural problems included preloading problem behaviours (anxiety, vocalization and pawing) (27.8%) and refusing to unload (15.5%).

3.1.3.5. Health problems

At least one transport-related health problem was reported by 67.0% of respondents during the two years reviewed in the study. There was no association between the amateur/professional status and the incidence of transport-related health problems (P=0.236). Transport-related health problems reported in the survey were injuries (including lacerations and fractures, 45.0%), diarrhoea (20.0%), muscular problems (including rhabdomyolysis, 13.0%), respiratory problems (including nasal discharge, coughing, fever, airway infection, 12.3%), heat stroke (10.5%), colic (10.3%), and laminitis (2.9%). Four cases of choke and three of conjunctivitis were also reported. In a question dedicated to transport pneumonia, 9.4% of respondents indicated they had experienced at least one case. Death/euthanasia for one or more horses due to a transport-related problem was reported by 3.5% of survey participants; a total of 35 deaths attributable to transport were recorded giving an approximate mortality rate of 0.01% (35 deaths during an estimated 313,000 individual horse movements). The most common reasons for death/euthanasia were bone fractures (8 in total: 6 limb fractures, 2 cervical fractures), colic (7 cases), pneumonia (7), colitis (5), and dehydration (1). Seven respondents reported they did not know the reason for death and had found the horse dead in transit (3) or soon after arrival (4).

3.1.4. Discussion

The results of this survey documented the demographic characteristics and management practices of equine transportation in Australia and provided information on the perceptions of owners or connections of transport-related behavioural and health problems in horses. The perceptions of industry stakeholders represent a valid perspective and initial data set for understanding current standards of care around equine transportation, and as a basis for comparison against existing industry codes of practice, such as the Australian Animal Welfare Standards and Guidelines for Land Transport of Livestock. To the authors' knowledge, this is the first survey conducted on equine transport management strategies and outcomes worldwide, and the findings are new and relevant for safeguarding horse welfare and wellbeing in Australia.

In the current study, respondents who identified as professionals took care of more horses, and organized transportation more frequently and over longer distances than amateurs. These findings were expected and in agreement with the Australian horse demographics (Gordon, 2001; Smyth and Dagley, 2015) and the higher use of transportation in horses used for competition and racing (Rosanowski et al., 2013; Rosanowski et al., 2015).

Rugging and bandaging were the most common preloading strategies reported by our respondents, with rugs more frequently applied by amateurs. Pre-journey guidelines from the Australian Code recommend that when horses are rugged, airflow should be appropriate to minimize thermal stress and dehydration risk (http://www.animalwelfarestandards.net.au/land-transport/). It has further been suggested that horses should be transported without rugs, head-bumpers, tail guards, bandages, leg wraps or boots, because these devices might become hot or loose and cause heat stroke or panic (Cregier and Gimenez, 2015). Although the provision of water and hay *ad libitum* before transportation is a guideline of the Australian Code, it was reported by only by one third of respondents to this current survey. Other less common pre-transport reported practices included removing shoes, and administration of sedation, antibiotics and anti-inflammatory medications. These practices were more commonly reported by professionals. The current Code recommends that hind shoes must be removed when horses travel with no partitions and in a group (http://www.animalwelfarestandards.net.au/land-transport/), but this circumstance was uncommon in the study population, as most respondents indicated that horses in their care were transported singly or in partitioned vehicles. Unnecessary medications should be avoided before transport, particularly corticosteroids and non-steroidal anti-inflammatories (NSAIDs) should not be administered as they can depress the immune system, mask pyrexia and may potentiate gastric ulcer formation (Kohn, 2000). The Australian Code suggests that sedation should not be used because it can affect the ability of the horse to maintain its balance and increases the risk of falling (Cregier and Gimenez, 2015) and affect also the mucociliary clearance (Raidal et al., 1996). The use of antibiotics should be evaluated carefully considering the risk of antibiotic resistance, and possible development of antibiotic-induced colitis (Feary and Hassel, 2006). The use of a single or multiple doses of procaine penicillin did not prevent the accumulation of mucus or proliferation of bacteria in the trachea of horses restrained with their heads elevated (Raidal et al., 1997b), suggesting that antibiotic administration prior to or during transportation is unlikely to be an effective strategy to prevent transport-associated respiratory infections.

The assessment of the fitness for travel is a requirement of the Australian Code. Horses with clinical signs of colic or other diseases, that are febrile, lame (greater than grade 4 out of 5), or with a body condition score (BCS) less than 2 out of 5 must not be transported (http://www.animalwelfarestandards.net.au/land-transport/). Fitness for travel, or any conscious assessment of health beyond visual inspection, was seldom reported by study respondents. None of our respondents reported they considered the BCS of horses before transportation, and only 10 respondents reported checking horses for lameness. Rectal temperature was taken by fewer than 30% of the respondents; mainly professionals. A standardized assessment of the fitness for travel, including veterinary consultation in difficult cases, is now required in Europe (http://www.fve.org/news/index.php?id=208#208, 2016) and should, in the authors' view, be promoted also in Australia.

To comply with the Australian Code, animals being transported and environmental parameters such as ventilation and temperature inside the trailer should be monitored at least every three hours during journey, the driver should have a direct access to each animal, and alarms or a monitoring system should be fitted to alert the driver to any problems (http://www.animalwelfarestandards.net.au/land-transport/). However, video cameras and/or weather stations in transport vehicles were used by only 21.1% and 1.7% of respondents respectively, and many reported travelling configurations did not allow access to each horse. Monitoring by surveillance cameras for early identifications of animals in distress is mandatory in Europe for journeys of greater than 8 hours (http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=uriserv:f83007) and it should, in the authors' view, become mandatory also in Australia.

The most common position of transport reported in the current study was facing forward, even though travelling facing backward has been reported as the best position to allow horses to maintain balance (Clark et al., 1993), being not more stressful than facing forward (Smith et al., 1994), and to have less impact on muscle enzymes than other positions (Padalino et al., 2012). Similarly, even though it has proved that travelling untied is efficient in minimizing airway inflammation (Stull and Rodiek, 2002), only 9% of the respondents do not tie up the horses during transport.

In Great Britain 90% of horse transport movements were for less than 2 hours (Boden et al., 2013) and in New Zealand the median of journey distance varied from 40 to 80 km (Rosanowski et al., 2013). In Australia, a land of much greater distances and more widely dispersed population centres, journeys of less than 2 hours were reported only by 58.1% of respondents. Thus many horses travelled frequently and over long distances in this country. Because journey-duration has been identified as one of the major risks for the development of transport-related health problems (Kohn, 2000)(Chapter 2), the management of journeys, particularly those longer than 2 hours, should be checked for compliance with the standards and guidelines of the Australian Code to limit the adverse health and welfare incidences.

The Australian Code does not give guidelines for the post-transport management of horses, however many professionals seemed to apply a strategy after transport. In particular, hand walking, grazing or turn out in a small paddock for one hour, and feeding of hay at the floor level were largely applied and in line with the literature (Kohn, 2000; Leadon and Hodgson, 2014). As dehydration is a common consequence of transportation and may lead to other diseases (Chapter1), the reported post-transport practices, such as the administration of oral electrolytes and water and hay *ad libitum*, are also appropriate (Kohn, 2000; Marlin, 2004). Assessing the health of animals by colour and status of gingival mucosa (normally pink and wet) and the capillary refill time (CRT, normally < 2s) were suggested as specific strategies following travel by few respondents. CRT was proved as a reliable welfare indicator (Dalla Costa et al., 2014) and animals with a CRT longer than 3 seconds, with dry and congested mucosa, should be treated by a veterinarian (Leadon and Hodgson, 2014). Even though careful monitoring of temperature and behaviour should be maintained for 3-7 days after journeys longer than 12 hours (Leadon and Hodgson, 2014), this was rarely applied. As astute management following transportation could prevent many transport-related diseases or ensure prompt remediation, post transport strategies should be included as guidelines. However, further research is needed to test different strategies before, during and post transportation in an authentic settings to identify best practice guidelines to include in the Australian Code.

Transport-related behavioural and health problems were reported by amateurs and professionals in similar proportions. Behavioural problems at loading were common, as has been reported in the USA (Lee et al., 2001). Loading is the transport phase most frequently associated with the “flight or fight” response, so avoidance behaviour and injuries to horses and horse handlers are frequent (Chapter 1). While training schemes to desensitize horses to the innate fear of loading and a re-training for loading problematic horses have been reported (Houpt and Wickens, 2014) (Chapter 1), only 56.5% of respondents trained their horses accordingly with these latter mentioned training methods. Evidence-based training methods should be therefore promoted to both amateurs and professionals involved in horse care and transportation.

The incidence of injuries associated with transportation is higher in this study than that reported previously by Australian equestrian horse owners (Noble et al., 2013). This difference may be associated with differences in survey methodology, participants and the number of horses represented. In the current survey, members of racing and breeding sectors were involved and many of them had more than 100 horses. As injuries have been associated with poor driving and animal handling skills, and poor quality of the vehicle (Marahrens et al., 2011), mitigation of these risk factors should be recommended in Australia.

Apart from injuries, other transport-related health disorders reported in this survey were gastrointestinal (including diarrhoea and colic), respiratory (including pneumonia), muscular and metabolic (including laminitis, heat stroke). In Japan the incidence of pleuropneumonia varied from of 1.4% as an overall of all type of journeys to 11.9% on longer journeys (1000-1300km)(Kohn, 2000). Transport pneumonia was reported by 9.4% of respondents in the current study. As prolonged journeys and extreme weather conditions may increase risk to horses in transit, the Australian Code suggests checking weather conditions before departure and travelling by night when possible. This strategy might reduce the incidence of heat stroke, and all transport-related diseases (pneumonia, colic, laminitis) which have dehydration as a predisposing factor (Chapter 1). However, only three respondents reported they plan their journeys in accordance with the weather forecast.

All adverse health outcomes reported by our respondents have been previously identified as risks associated with inappropriate transport management and poor education of horse people (Cregier, 1984). More than two third of our respondents reported management strategy/ies associated with equine transportation that did not comply with the current Transport Code or best practice, suggesting that research findings and changes to transport codes over recent years have not reached horse industry members and may not adequately inform practice. Dissemination of best available scientific evidence relating to preventive measures as a basis for education of amateurs and professionals responsible for horse care and transportation is likely to decrease the incidence of both health and behavioural problems associated with transport.

A number of limitations, many of which are common to survey-based studies (Boden et al., 2013; Rosanowski et al., 2013; Schemann et al., 2011), may be identified with the current study. The surveys were not distributed randomly, but respondents were invited to fill the survey to report management practices and transport-related-problems. This strategy may have induced a selection bias by preferentially recruiting people who are highly concerned and diligent about transportation, or may have selected for respondents with past adverse experiences of transportation. Hence the reported findings may under- or overestimate the incidence of transport-related problems. Transport-related problems were identified by participant recall, hence the diagnosis and incidence of problems may not be accurate, and the technique is vulnerable to recall bias. Women replied more than men to the survey, but this is likely to reflect the higher participation of women in online surveys observed in previous studies (Schemann et al., 2011; Smyth and Dagley, 2015), and/or the greater participation of this gender in horse-related activities (Gordon, 2001; Smyth and Dagley, 2015). Some Australian States were less represented in our responses, however this also reflects the non-homogenous distribution of horses and population in Australia (Gordon, 2001; Smyth and Dagley, 2015). Finally, since there is not an official estimate of the number of Australian horse owners (Smyth and Dagley, 2015) and the horse population (Gordon, 2001; http://faostat.fao.org/, 2015), the calculation of a representative sample size for our survey could be not precise. Notwithstanding these limitations, this study has generated important insight into participants' current understanding and implementation of management strategies relating to transportation of horses.

3.1.5. Conclusions

Overall, respondents reported a number of strategies that are not advocated in the available literature or do not comply with the guidelines of the Australian Code. Adverse welfare and health outcomes were commonly reported and in a similar proportion by both amateurs and professionals associated with transportation. Thus, equine transportation presents still a risk of adverse welfare and health outcomes in Australia. This risk might be mitigated by better communication of current best practice standards, improved policing of compliance with the Australian Code, and improved implementation of research findings. Further research is also recommended to evaluate transport practices, in order to provide evidenced-based guidelines to inform the Australian Code.

CHAPTER 3.2

A survey on transport management practices associated with injuries and health problems in horses

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3.2.1. Introduction

The act of transportation has been associated with traumatic injuries and diseases in horses (Chapter 1). Traumatic injuries, such as lacerations and contusions, to horses and horse handlers are considered to be the most common problem arising from equine transportation (Mansmann and Woodie, 1995). Results of a survey on horse injuries have shown that, apart from the paddock, the trailer is the second most likely place for horses to be injured (Darth, 2014). Transport-related injuries are often minor, but will sometimes be severe enough to warrant euthanasia of the injured horse (Chapter 3.1). Injuries related to transport commonly affect the legs (occurring when the horse contacts the ramp during loading; or against the other leg or part of the trailer/truck during the trip due to loss of balance), the head (due to inadequate height of the trailer or truck) and the tail (occurring when the horse leans its rear against the wall of the truck) (Chapter 1). One in four respondents to a survey conducted in South Eastern Australia reported an incident where horses were injured during non-commercial transport and many of these injuries resulted from scrambling and other “flight or fight response” behaviours (Noble et al., 2013).

Transportation fear seems to be innate in horses (Lee et al., 2001) triggering “flight or fight response” behaviours and other common signs of activation of the sympathetic system including increased defecation and profuse sweating (Moberg and Mench, 2000). An increase in faecal water content and in defecation frequency (diarrhoea) has been previously related to generalize stress responses during travel (Jones, 2003; Marlin, 2004). Sweating may also be a sign of stress, but when it becomes profuse and associated with hyperthermia, weakness and lethargy, it could indicate heat stroke (Reed et al., 2003). Heat stroke can be related to transport events occurring on very hot and humid days. This is not surprising given that in most cases the temperature differences between inside and outside the vehicle usually range from 5.1˚C to 9.5˚C depending on vehicle speed and the open vent area (Purswell et al., 2010). Temperature in the vehicle can be close to 10˚C warmer than the outside temperature in a stationary vehicle (Purswell et al., 2006). However, it should be highlighted that fear level can drastically affect the response to transit where a frightened horse may become overheated and sweat, even in comfortable weather conditions (Weeks et al., 2012).

Other diseases related to transport are rhabdomyolysis, laminitis, colic (caused by impaction of large colon, and enterocolitis), and transport pneumonia (Chapter 1, 2). Transport pneumonia is one of the most studied transport-related pathologies and in the past two decades many predisposing factors have been identified (Austin et al., 1995; Copas, 2011). For instance, it has been shown that confinement and restraint of the head in an elevated position for 6 and 12 hours causes an accumulation of mucus and bacteria in the lower airways (Raidal et al., 1995). Viral infections transmitted during and after the trip (Anzai et al., 2001) can also contribute to the development of transport pneumonia (Cullinane, 2014). Other factors associated with a risk of transport pneumonia include poor internal vehicle environment (e.g. air quality, temperature, humidity) and the lack of appropriate ventilation (Leadon et al., 1989; Leadon et al., 2008). The lack of adaptation to transport has also been identified as a contributor to the risk of transport-associated pneumonia. A Japanese study evaluating many stress indicators showed that horses that adapted well to transport were less likely to be affected by pneumonia during extensive transport events (either 36 or 41 hours)(Oikawa et al., 2004). Most of these identified risk factors have been generated from relatively controlled scientific trials, during which horses were managed by scientists and not by members of the equine industry (either owners or transport companies). Thus, there are still many unknown factors in the development of equine transport pneumonia and other transport-related diseases that need to be identified, in particular relating ‘real world’ transportation management strategies and disease risk.

The association between horse management and the development of behavioural and health problems has been often investigated through surveys (Darth, 2014; Hockenhull and Creighton, 2014; McGreevy et al., 1995). Surveys distributed to horse owners/trainers have generally produced excellent response rates (52.5% and 67.2%, respectively) (Schemann et al., 2011; Smyth and Dagley, 2015), demonstrating a great interest from people associated with horses in research related to the health and wellbeing of their horses. In particular, online surveys have been shown to be an efficient way to gather data from the equine industry (759 in Australia and 4601 in Great Britain return rate) (Boden et al., 2013; Schemann et al., 2011). While transportation is an integral aspect of horse management, to the best of our knowledge a survey has never been conducted across a broad cross section of the Australian equine industry to investigate the associations between transport management practices and health problems.

The aims of this online cross-sectional study were to collect information about transport management and transport-related diseases experienced by members of the equine industry (professional and amateur in all sectors) in Australia and to determine possible associations between them. This study reports these data and comments upon associations that may help those working in the equine industry to instigate changes that will improve the health outcomes of the transported horse.

3.2.2. Materials and Methods

3.2.2.1. Survey

Key design features required to ensure valid questionnaire results, recently reviewed by Dean (Dean, 2015) and Christley (Christley, 2016), were addressed in this study design. Details of the design and distribution of the survey and the description of the demographic characteristics of the study population have been reported previously (Chapter 3.1). Briefly, the survey was digitized using SurveyMonkey (SurveyMonkey Inc., California, USA, www.surveymonkey.com) and it was open from June to September 2015. People involved in any equine industry sector (racing, equestrian and recreational) were invited to take part in the survey if they had organized transport at least monthly over the past two years. An invitation letter and the link to the survey were provided to a wide range of Australian horse sports and organisations, that were asked to publicise the survey. The survey link was also published on equestrian websites and promoted through several horse magazines, relevant Facebook pages and online horse forums. The anonymous survey was organised in 3 sections (Appendix 1). The first section contained questions related to respondents' details (i.e. age, address, equine industry sector, relation with horses, number of horses in care, journey frequency and most common journey duration). The second section focused on their typical management practices related to equine transport (i.e. transport management before, during and after the journey). The last section was on transport-related health problems which the respondents' horses had experienced in the past two years.

This study focused on the association between respondents' details, transport management and transport-related health problems. A power calculation (http://statulator.com/samplesize.html) determined that more than 400 survey responses would provide a representative sample of the estimated 400,000 Australians who care for horses (Smyth and Dagley, 2015) with power of 0.8 and α = 0.05 (Chapter 3.1). The survey had a return rate of 987 responses, of which 797 met inclusion criteria (respondent organized transportation at least monthly and provided usable responses to all survey questions). The 797 completed responses were used as data set for the analysis.

3.2.2.2. Outcome variables

The key transport-related health problems of interest were: traumatic injuries (laceration, bruising, swelling); diarrhoea; heat stroke (profuse sweating with hyperthermia and lethargy); muscular problems (tying up), laminitis, transport pneumonia and colic.

3.2.2.3. Predictive variables

Predictive variables investigated in this study were: respondent details, and transport management before, during and after the journey (Table 1).

Table 1. Predictive variables. Name, description and values of the predictive variables evaluated.

Name	Description	Values
	Respondents details	
Age	Age of the respondents	20-30, 31-40, 41-50, 51-60, >61
Address	In which Australian state they lived	Australian Capital territory, New South Wales, Northern Territory, Queensland, South Australia, Tasmania, Victoria, Western Australia
Sector	In which sector of the horse industry were involved	Thoroughbred (TB) racing, Standarbred (SB) racing, Equestrian sport, Endurance, Horse breeding, Recreational non-competitive
Background	Relationship with the horses	Professional (involved with horses for financial reward), Amateur (involved with horses as a hobby)

Number of horses	Number of horses present on their propriety in the busiest week of the year	<5, 5-10, 11-30, 31-50, >50 horses
Journey frequency	Frequency of organized transport events	Daily, twice a week, weekly, fortnightly, monthly
Journey duration	The most common journey duration	<2, 2-4, 5-8, >8 hours
	Transport management before journey (BJ)	
Antibiotics	Administration of antibiotics	Yes, No
Tranquilizers	Administration of tranquilizers	Yes, No
Oral supplements	Administration of electrolytes, vitamins	Yes, No
Ad libitum hay/water	*Ad libitum* access to hay and water	Yes, No
Protections	Application of any type of protection equipment (travel boots, bandages)	Yes, No
Rug	Wearing of rug	Yes, No
Health assessment	If and by whom fitness for travel was assessed	A veterinarian, non-veterinary staff, no assessment
Temperature	Assessment of body temperature	Yes, No
Heart rate	Assessment of heart rate	Yes, No
Feeding behaviour	Monitoring of feeding behaviour	Yes, No
Drinking behaviour	Monitoring of drinking behaviour	Yes, No
Weight	Registering body weight	Yes, No
General health	Assessment of general health (e.g. horse coat, demeanour) by visual inspection	Yes, No
	Transport management during journey	
Vehicle	Vehicle used to transport horses	Two-horse straight trailer, two-horse angle trailer, 3-4-horse angle trailer, gooseneck-trailer, truck
Tying	Horses tied inside the vehicle	Yes, No
Monitoring	If and how horses are monitored	By video camera, at fuel stop, no monitor
Feeding	If horses had access to food and/or water whist travelling	Yes, No
	Transport management after journey (AJ)	
Health assessment	If and by whom the fitness after travel was assessed	A veterinarian, non-veterinary staff, no assessment
Temperature	Assessment of body temperature	Yes, No
Heart rate	Assessment of heart rate	Yes, No
Feeding behaviour	Monitoring of feeding behaviour	Yes, No
Drinking behaviour	Monitoring of drinking behaviour	Yes, No
Weight	Registering body weight	Yes, No

General health	Assessment of general health (e.g. horse coat, demeanour) by visual inspection	Yes, No
Recovery strategies	If a particular strategy (administration of electrolytes, hand walking) was applied to helping in recovering	Yes, No

3.2.2.4. Statistical Analysis

The overall incidence for each transport-related problem was calculated by descriptive statistical analysis using GenStat®Version 14 (VSN International, Hemel Hempstead, UK). The survey results were analysed by logistic regression using GenStat®Version 14 (VSN International, Hemel Hempstead, UK). A model was derived for each transport-related problem; the outcome was binary (1/0, affected/non-affected); P values were calculated using Wald Test. Each predictor variable returning a P-value <0.25 from the univariate modelling was considered for inclusion in a multivariate model for that outcome (Appendix 2, Table 1). A step-wise backward elimination procedure was then conducted whereby predictive variables were removed until all variables in the final model had a P-value<0.05 indicating significance. The findings are presented as odds ratio (OR) and confidence interval (95% CI) for each predictive variable value. Wald test's P values were reported for each association.

3.2.3. Results

Descriptions of the respondent population, transport management strategies and reported incidence of transport-related problems have been published previously (Chapter 3.1). Briefly, the overall incidence of specific transport-related problems is shown in Table 2. Traumatic injuries were the most prevalent transport-related problem in this survey, with an incidence of 45% reported. The incidence of transport-related diarrhoea was 20%, whilst the incidence of heat stroke, muscular problems, colic and pneumonia associated with transport ranged from 9.2 - 13.0%. The incidence of transport-related laminitis was <3%.

Table 2. Incidence of transport-related health problems.

Transport-related problem	Overall incidence (OI)
Traumatic injuries	45.0%
Diarrhoea	20.0%
Muscular problems	13.0%
Heat stroke	10.5%
Colic	10.3%
Transport pneumonia	9.2%
Laminitis	2.9%

Overall incidence for the examined transport-related health problems from the most frequent to the less frequent was traumatic injuries, diarrhoea, muscular problems, heat stroke, colic, transport pneumonia, laminitis.

The results of univariate analyses are provided in the supplementary materials (Appendix 2, Tables 2-7).

The significant results generated from the final multivariate logistic model for the outcome variables are presented in Tables 3-7. The incidence of traumatic injuries related to transport was significantly associated with age of the respondent, maximum number of horses respondents cared for in a given week, administration of tranquilizers, application of any type of protection, the type of vehicle used to transport horses and the general health of the horse after transportation (Table 3).

Table 3. Results of the multivariate model with traumatic injuries as the outcome variable. Significant respondents details and transport management risk factors for transport-related traumatic injuries identified using a multivariate logistic regression model (n=787).

Variable and Category	Estimate	SE	OR	95%CI	[a] P
Age					<0.001
>61	Ref		1		
51-60	0.17	0.33	1.15	0.60-2.22	
41-50	0.45	0.32	1.57	0.83-2.97	
31-40	0.90	0.33	2.40	1.25-4.61	
20-30	1.52	0.33	4.41	2.31-8.43	
Number of horses					<0.001
<5	Ref		1		
5-10	0.44	0.20	1.64	1.11-2.43	
11-30	0.31	0.22	1.39	0.89-2.15	

31-50	1.48	0.34	4.23	2.16-8.28	
>51	0.74	0.45	3.69	1.96-6.96	
Tranquilizers					0.017
No	Ref		1		
Yes	1.22	0.50	3.30	1.23-8.83	
Protections					0.002
No	Ref				
Yes	0.47	0.16	1.65	1.21-2.25	
Vehicle					0.028
Truck	Ref		1		
Two horses straight trailer	0.59	0.22	1.82	1.17-2.83	
Two horses angle trailer	-0.01	0.32	0.98	0.52-1.86	
3-4 horses angle trailer	0.347	0.28	1.41	0.81-2.44	
3-4 gooseneck trailer	0.05	0.19	1.76	0.50-2.18	
General health AJ					0.004
No	Ref		1		
yes	0.48	0.19	1.76	1.19-2.58	

Ref= Reference category; SE= standard error; OR= odds ratio; CI=95% confidence interval; [a] P value calculated using Wald's test; AJ: after journey.

The incidence of transport-related diarrhoea was significantly associated with one predictor variable, that being the background of the respondents. The odds of transport-related diarrhoea were 1.5 (CI: 1.01-2.24, P=0.046) times greater if the carers were amateurs compared to professionals. The incidence of heat stroke related to transportation was significantly associated with the background of the respondents and access to feed and water prior to transport (*ad libitum* hay and water) (Table 4).

Table 4. Results of the multivariate model with heat stroke as the outcome variable. Significant respondents details and transport management risk factors for transport-related heat stroke identified using a multivariate logistic regression model (n=787).

Variable and Category	estimate	SE	OR	95%CI	[a] P
Backgrounds					
Professionals	Ref				0.045
Amateurs	0.864	0.27	1.75	1.01-3.03	
***Ad libitum* hay/water**					
Yes	Ref				0.002
No	0.864	0.277	2.37	1.37-4.086	

Ref= Reference category; SE= standard error; OR= odds ratio; CI=95% confidence interval; [a] P value calculated using Wald's test.

Transport-related muscular problems were reported significantly more often if health assessment prior to transport was performed by non-veterinary (lay) persons or not at all, and if drinking behaviour after transportation was monitored (Table 5).

Table 5. Results of the multivariate model with muscular problem as the outcome variable. Significant respondents details and transport management risk factors for transport-related muscular problems identified using a multivariate logistic regression model (n=787).

Variable and Category	estimate	SE	OR	95%CI	[a] P
Health assessment BJ					0.016
A veterinarian	Ref				
Non veterinary staff	0.967	0.734	2.62	0.62-11.09	
No assessment	1.731	0.780	5.64	1.22-26.05	
Drinking behaviour AJ					0.031
No	Ref				
Yes	0.557	0.258	1.74	1.05-2.89	

Ref= Reference category; SE= standard error; OR= odds ratio; CI=95% confidence interval; [a] P value calculated using Wald's test; BJ: before journey; AJ: after journey.

The incidence of laminitis related to transport was significantly greater if body weight was monitored prior to transportation, and was lessened if post transport recovery strategies were used (Table 6).

Table 6. Results of the multivariate model with laminitis as the outcome variable. Significant respondents details and transport management risk factors for transport-related laminitis identified using a multivariate logistic regression model (n=787).

Variable and Category	estimate	SE	OR	95%CI	[a] P
Weight BJ					0.035
No	Ref				
Yes	0.995	0.47	2.70	1.07-6.80	
Recovery strategies					0.018
Yes	Ref				
No	1.089	0.46	2.97	1.20-7.33	

Ref= Reference category; SE= standard error; OR= odds ratio; CI=95% confidence interval; [a] P value calculated using Wald's test; BJ: before journey.

Four risk factors were significantly associated with the occurrence of transport pneumonia. This condition was reported less commonly by respondents transporting horses for recreational purposes, by respondents caring for fewer than five horses per week, and when journeys were less than 2 hours duration. The condition was reported more commonly when rectal temperature was monitored following transportation (Table 7).

Table 7. Results of the multivariate model with transport pneumonia as the outcome variable. Significant respondents details and transport management risk factors for transport pneumonia identified using a multivariate logistic regression model (n=787).

Variable and Category	estimate	SE	OR	95%CI	[a] P
Sector					0.002
Recreational	Ref				
Endurance	0.80	0.64	2.24	0.63-7.92	
Equestrian Sport	0.41	0.49	1.50	0.57-3.95	
Horse Breeding	0.48	0.60	1.61	0.49-7.92	
SB racing	1.59	0.63	4.91	1.40-17.15	
TB racing	1.84	0.55	6.34	2.12-18.98	
Number of horse					0.004
<5	Ref		Ref		
5-10	0.82	0.42	2.27	0.98-5.26	
11-30	0.63	0.45	1.89	0.77-4.63	
31-50	1.22	0.53	3.39	1.18-9.67	
>51	1.90	0.50	6.73	2.50-16.11	
Journey duration					<.001
< 2 hours	Ref				
2-4 hours	-0.021	0.34	0.97	0.50-1.90	
4-8 hours	0.92	0.41	2.53	1.13-5.63	
>8 hours	1.84	0.52	6.29	2.26-17.49	
Temperature AJ					<.001
No			Ref		
Yes	1.24	0.28	3.46	1.98-6.06	

Ref= Reference category; SE= standard error; OR= odds ratio; CI=95% confidence interval; [a] P value calculated using Wald's test; SB: Standardbred; TB: Thoroughbred; AJ: after journey.

None of the examined predictive variables were significantly associated with the risk of transport-related colic; this was true for both univariate and multivariate analyses.

3.2.4. Discussion

This cross sectional study explored for the first time associations between equine transport management, including information on those caring for the horses, and respondents' perceptions of transport-related health problems in horses. The occurrence of colic associated with transportation was not associated with any risk factor examined in this current study. Different risk factors were associated with the other transport-related health problems examined. However, the findings of surveys relying upon the retrospective recollection of participants should not be construed as defining causal relationships among factors (Hockenhull and Creighton, 2014). The findings of this study add to the knowledge base that can be used in the development of transport guidelines that are designed to improve the welfare of the travelling horse and decrease the risk of disease or injury. Our findings highlight key areas that require empirical further research. It is worth highlighting that the nature of the relationships identified between transport-related health problems and transport management routines in this study cannot be clearly defined (Cohen et al., 2005; Hockenhull and Creighton, 2014). For instance, management practices may have been applied in an attempt to identify transport-related problems rather than being the cause. Thus, some of the associations between monitoring strategies and transport-related problems (e.g. visual inspection and injuries; monitoring temperature and transport pneumonia) are more likely a reflection of good practices for prompt identification of these problems, and have been interpreted in this way. Thus, based on the observed associations, these practices might be recommended for identifying animals at risk of diseases and not for preventing transport-related health problems. Individual horse details, sex, breed, age, travel experience have not been considered in this cross sectional study and they should form a critical component of future experimental or observational studies.

This study has a number of limitations common to online surveys (Christley, 2016). The response rate and power analyses performed can be based only on estimations of the total number of participants in the Australian horse sector (Smyth and Dagley, 2015), as the true number of horse owners cannot be known. Dean (2015) identified sampling bias, non-response bias, recall bias and social acceptability bias as factors that may confound the interpretation of survey data, and all may apply to this study. Although strenuous efforts were made to recruit participants across all components of the Australian horse industry, and numbers of respondents from each sector have been reported, it is not possible to determine whether there is a difference in response rate between sectors (sampling bias). Certainly online survey distribution selected for participants with internet access. Across sectors, respondents who have experienced transport-related problems may have been more motivated to participate (response bias). Arguably, however, the experiences of such a group is best suited to informing discussion of factors associated with injury or disease after transportation. Conversely, potential respondents who are utilising practices that effectively limit the incidence of such problems may not have responded. In the absence of objective data on disease incidence, recall bias may have influenced findings and some observations, particularly the apparent association between respondent age and the number of adverse events might be explained by this factor. As there was no means to document whether a veterinary diagnosis informed participant perceptions or recall, the diagnosis of specific problems associated with transportation must be treated with some circumspection. However, to mitigate this risk, disease categories were deliberately defined in broad terms designed to be meaningful to lay people in the industry. Further, the health problems rating scale is well described and was reduced into binary responses for analysis to limit the effect of individual interpretation. Finally, participants, although anonymous, may have been reluctant to disclose some aspects of their practice in the survey (accountability bias). Given the aim of the study was to survey a large and diverse component of the equine industry to document perceptions about transport management practices and outcomes, this work reports for the first time findings that may be useful not only to safeguarding horse welfare but also to reducing the economic waste related to transport in the equine industry. Our findings add confirmatory evidence to the European (Anon, 2014; http://www.fve.org/news/index.php?id=208#208, 2016) and Australian (http://www.animalwelfarestandards.net.au/land-transport/) guidelines for equine transportation.

In comparison to horses managed by older respondents, horses managed by respondents younger than 40 years old appeared to be at greater risk of transport-related injuries. These findings support the assertions commonly found in the literature that horses should be managed during loading and travelling by experienced and educated people (Cregier, 1984; Houpt and Wickens, 2014; Weeks et al., 2012). Our finding may be linked not only with limited experience in horse handling but also in driving skills. Driving ability has been identified as a factor associated with transport-related injuries, due to erratic driving related to lack of experience impairing the horse’s ability to balance in the trailer (Giovagnoli et al., 2002). Similarly in cattle, drivers with less than 5 years’ experience reported a higher incidence of injuries associated with transportation (González et al., 2012). Specialized training in equine transport is not mandatory under the Australian Code of transport (http://www.animalwelfarestandards.net.au/land-transport/) and, although such training is mandated in Europe (http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=uriserv:f83007), to the authors' knowledge no country has an explicit requirement for a special driving license for live animal transport. Specialised training in how driving styles impact on balance, injuries and stress of the transported animal may be beneficial in reducing transport-related injury risk. Traumatic injuries during transportation may involve also horse handlers. An elevated risk of injury has been associated with poor experience, misjudging how to handle a situation, reduced attention caused by distraction, taking a general view, and failing to consider other strategies that may reduce risks (DeAraugo et al., 2016). To improve safety for humans and horses, knowledge and experience in horse handling and driving would appear important. The odds of a horse injury associated with transport were more than 4 times greater for respondents who took care of more than 30 horses. This may be related to the fact that moving larger numbers of horses increases the likelihood of traumatic injuries, supporting the assertion that injuries during transport happen mainly by accident (Darth, 2014).

The higher likelihood of injuries associated with the use of tranquilizers may be related with the fact that tranquilizers can affect the horse's proprioception and balance, increasing the risk of falling over at loading and during travelling (Waran et al., 2007). The results of this study therefore support studies and recommendation that advocate no use of tranquilizers prior to and during journeys (http://www.animalwelfarestandards.net.au/land-transport/; Kohn, 2000). A higher risk of injuries was also associated with the use of protective equipment. Although this finding seems counterintuitive, the use of leg protections and head bumper guards has previously been discouraged during transportation of horses (Houpt and Wickens, 2014; http://www.animalwelfarestandards.net.au/land-transport/). Protections should be used only on horses completely accustomed to them, they should be properly applied and checked periodically *en route*, and they should not be worn for a long period of time (Houpt and Wickens, 2014). That is, the use of protective equipment should probably be limited and not universally recommended.

The transport vehicle and its internal design has been previously linked to injuries during animal transportation (Marahrens et al., 2011). Our findings show that the two horse straight trailer design appeared to be associated with elevated risk of transport-related injuries compared with truck and gooseneck trailers. This could be related with the reduced stability that trailers have compared with the other vehicles (Houpt and Wickens, 2014). The elevated risk of injury in straight trailers compared with those in which horses are situated at an angle (~45°) was an interesting finding and reflects recommendations made previously that discourage placing horses so that they are facing in the direction of travel (Clark et al., 1993; Padalino et al., 2012). Horses tend to lose their balance more easily when facing the direction of travel, in particular at abrupt stops. Their posture when facing in this direction also tends to be more rigid and less relaxed, potentially making them more susceptible to injuries (Waran et al., 1996). Taking into account that most of the currently used methods and practices for the transportation of horses have been established over a period of time by the demands of the industry, with few governmental or industry standards applicable (Houpt and Wickens, 2014), our findings support the need for a stricter application of standards in horse transport vehicle design (Cregier and Gimenez, 2015).

The link in this study between elevated risk of diarrhoea and amateur status of the respondents was an interesting one. Diarrhoea may be a response to an acute stress (Moberg and Mench, 2000) and equitation science theory suggests that when pressure on the horse is not released correctly, horses tend to be more agitated and show more conflicting behaviours (McGreevy, 2007). Thus, horses managed during transportation by less experienced people may be under more stress and consequently experience diarrhoea more frequently. This hypothesis would need to be investigated in more detail through prospective observational studies comparing handling and management procedures of amateurs and professionals on such potential physiological stress responses, as diarrhoea. Horses transported by amateur respondents were also more likely to experience transport-related heat stroke in their horses. This may have also been due to greater emotional stress when horses were not managed correctly. Agitated horses have been reported to suffer heat stroke even when weather conditions are said to be comfortable (Weeks et al., 2012).

The risk of transport-related heat stroke was elevated when water and hay were restricted before transport. Dehydration can impair equine thermoregulation and lead to heat stroke (Reed et al., 2003). Offering *ad libitum* water and hay prior to the journey, can act to insure good electrolyte balance and hydration status, enabling horses to better handle environmental conditions and stresses that could result in significant dehydration and electrolyte losses during transportation. Due to Australia's climate, horses can at times be transported in extreme heat and high humidity. Therefore ensuring horses are properly hydrated seems a ‘common sense’ strategy to enhance their wellbeing during travel. Therefore, hay and water restrictions prior to transport should be avoided. This finding complements recent published guidelines on watering during transportation of *Equidae* (Anon, 2014).

An elevated risk of muscular problems associated with transport was observed when pre transport health checks were lacking. Electromyography studies have demonstrated that the muscles of the horse are in continuous activity during transport as they adjust posture and balance (Giovagnoli et al., 2002). Elevations in muscle enzymes, such as creatine kinase (CK), have been noted even after trips of relatively modest duration (3 hours) (Tateo et al., 2012), whilst transportation events spanning 300 km produce muscle stress equivalent to a 1,500 m canter (Codazza et al., 1974). The evaluation of the fitness for travel is mandatory and simple guidelines have been recently published by the world organisation for animal health (OIE) (http://www.fve.org/news/index.php?id=208#208, 2016). We therefore recommend that fitness for travel be assessed using these guidelines for all horses before every trip, with assessment by a qualified veterinarian if there is any doubt about an individual horse (http://www.fve.org/news/index.php?id=208#208, 2016) or before journeys longer than 20 hours (Chapter 2).

Laminitis is a pathology which can lead to death or euthanasia of the horse, and it has been associated with a number of factors such as endotoxemia and severe dehydration after colic, placental retention and transport (Reed et al., 2003). In this study, the risk of laminitis associated with transport was significantly elevated when there was a lack of a post transport recovery strategy, such as rehydration and walking. There is a lack of research testing the effects of specific recovery strategies on the risk of transport-related diseases, however rehydration, hand walking and housing in paddocks have been recommended (Kohn, 2000; Marlin, 2004)(Chapter 1).

Transport pneumonia was associated with the respondents’ sector, with racing horses at higher risk than pleasure horses. This could be explained in some way by bred predisposition to transport pneumonia, as reported for the Thoroughbred (Austin et al., 1995), but also may be related to the combined effects of strenuous exercise and transportation, which are both seen as predisposition factors for equine pneumonia (Copas, 2011). Journey duration has been previously associated with increased risk of developing transport pneumonia (Chapter 2), as was reported in the current study. The association between number of horses in care and pneumonia could be interpreted in two ways. The first is just the likelihood of disease increased proportionally with the number of horses, such as for injuries. The second is the typology of horse stable. Horse stables with more horses might have more cases of transport pneumonia because they tend to travel on longer distance or could more easily transmit pathogens among horses. Interestingly, this study showed an association between pneumonia and the monitoring of temperature after the journey. This result may be related to the use of this tool in the early detection of disease, with an elevated temperature a well-recognized early indicator of transport-related respiratory disease (Leadon and Hodgson, 2014). The practice of monitoring the body temperature of horses after transport is commonly recommended within the industry (Leadon et al., 2008; Marlin, 2004), and these results should not discourage this practice but as an evidence in the utility of applying it.

Even though transport-related colic was reported by more than 10% of the respondents, no associations were found with the variables investigated in this study. Colic is a common problem in horses and it may be caused by a variety of factors (e.g. dehydration, abrupt change in diet, ingestion of sand while grazing). Transport has been identified as a risk factor for simple colonic obstruction and distension colic (Hillyer et al., 2002) and for salmonellosis (McClintock and Begg, 1990), but previous colic, age (2-10 years), increased concentrate intake, change in feeding and medical treatment have also been identified as major risk factors in the development of colic (Tinker et al., 1997). We can therefore speculate that transportation may worsen gastrointestinal conditions and contribute to colic development, in particular in association with poor watering (Anon, 2014) and inappropriate diet management (Pagan, 2004). However, more research is required to identify other unknown risk factors in transport-related colic.

3.2.5. Conclusions

This study identified various potential factors contributing to a number of key transport-related health problems in horses. Age, experience and professionalism seemed to influence the risk of injury and other more directly stress related outcomes, such as diarrhoea and heat stroke.

Among the various strategies investigated to prepare the horse for transport, offering *ad libitum* water and hay and an appropriate health check prior to transportation seemed to reduce the risk of heat stroke and muscular problems. The monitoring of the horses after transport, in particular observing their behaviour and recording their body temperature, appeared to increase the risk of disease but such outcomes should not be interpreted as causal but rather are likely to reflect early diagnosis and intervention that would likely benefit the horse’s ability to recover from transport-related problem. Our findings add confirmatory evidence on the importance of best practices during transportation to reduce the negative impacts of transportation on horse health and welfare, confirming previously suggested guidelines and suggesting also some new practices. However, since it is obvious that transport-related problems are multifactorial and this study is only a survey, our findings need to be interpreted with caution. The factors identified in this study warrant future experimental exploration in order to test the efficacy of some strategies and generate more concrete guidelines to safeguard the transported equine.

CHAPTER 3.3

Investigations into equine transport-related problem behaviours: survey results

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3.3.1. Introduction

Transport stress results from a mosaic of stressors which affect the horses' affective and physiological states, leading to behavioural and health problems prior, during and/or after trips (Weeks et al., 2012). The transportation process includes the following stages: preloading handling, loading, transport, unloading and adaptation to a new environment (Chapter 1). Each of these stages can present adaptive challenges to the horse and each is characterized by different stressors which may result in different problem behaviours (Lee et al., 2001).

Preloading handling can result in behavioural anxiety due to enforced separation from familiar physical and social environments. Through associative learning, horses may recognize the pre-transport routine (e.g. handling, grooming, fitting of protective tack, and the noise of the transport vehicle) and associate it with past travel experiences (Weeks et al., 2012). Consequently, animals which have experienced problematic travel in the past, such as falls during transport, tend to exhibit increased behavioural problems during preloading (Leadon et al., 2008). Horses with preloading problem behaviours may show signs of anxiety, such as vocalization, pawing, heightened locomotion and shaking (Padalino, 2015; Waran et al., 2007).

The loading process of leading the horse into an enclosed space which may be poorly lit, often up an unstable and noisy ramp, or a large step, is inherently aversive for the majority of horses (Houpt and Wickens, 2014). Loading horses onto transport vehicles is therefore associated with the majority of transport-related problem behaviours (Houpt, 1986; Lee et al., 2001). Horses may display anxiety when approaching the vehicle or stepping onto the ramp, regardless of the level of experience (Siniscalchi et al., 2014). Behaviours associated with anxiety that may be expressed during loading include rearing, pulling back, head tossing, pawing, standing, bolting, and turning sideways (Lee et al., 2001; Waran and Cuddeford, 1995). Such behaviours may lead to injury to the horse and/or the handler (Mansmann and Woodie, 1995; Riley et al., 2015).

Psychological stress during transportation can be due to confinement, isolation and forced proximity to unfamiliar travelling companions and they are exacerbated by the other physical stressors (noise, poor ventilation and heat) typical of this transport phase (Chapter 1). During transport horses may exhibit a variety of behaviours including pulling back, pawing, kicking out at the vehicle, and biting and kicking directed at travelling companions (Houpt, 1986; Lee et al., 2001). These behaviours can lead to injuries from impacts with vehicle components, such as kicking the vehicle walls or dividers, or due to loss of balance which may result in a fall (Mansmann and Woodie, 1995).

Unloading may be challenging if the ramp is too steep or is slippery, or if the horse is lame or anxious about the environment into which it is being unloaded (Chapter 1). These problems may be exacerbated in vehicles which require horses to be unloaded backwards, preventing them from seeing what is behind (Cregier and Gimenez, 2015). Some horses may freeze inside the vehicle and be difficult to coax from it, whereas others may perform flight responses such as leaping or backing out of the vehicle at speed (Siniscalchi et al., 2014). Messori et al. (2016) reported that when slaughter horses performed such behaviours, handlers intervened using positive punishment which resulted in injuries and poor horse welfare outcomes.

Transport-related problem behaviours (TRPB) have been associated with vehicle characteristics, such as the slope of the loading ramp, position of the horse inside the vehicle, the absence of dividers, and loading density in unstalled group transport (Houpt and Wickens, 2014; Roy et al., 2015). However, minimum design standards for horse transport vehicles do not exist in Australia and the ideal vehicle design in terms of minimizing transport stress and related problem behaviours is still a matter of debate (Cregier and Gimenez, 2015).

A variety of training methods have been developed and proposed to reduce incidence and consequences of TRPB (Ferguson and Rosales-Ruiz, 2001; Houpt and Wickens, 2014; Shanahan, 2003; Slater and Dymond, 2011; Starling et al., 2016). The majority of loading training methods rely on negative reinforcement (McGreevy and McLean, 2011; McLean and MCLean, 2008; Parelli et al., 1993) and a variety of equipment has been used to assist with loading, including winches, whips, various bits and bridles, nose and lip chains, cattle prods, ropes around the hindquarters, fatiguing lunging (Ferguson and Rosales-Ruiz, 2001). Poorly implemented negative reinforcement methods, or methods relying on equipment which apply aversive pressure, may induce anxiety of sufficient intensity that the horse is motivated to escape from the vicinity of the vehicle and/or handler, resulting in the reinforcement of a response which is the opposite of what is intended (McGreevy, 2012). It has been theorised that successful escape from an intensely fear-inducing or aversive stimulus, such as a trailer, is highly reinforcing and may represent a form of "one trial" learning (LeDoux, 1994; McLean, 2003). In situations where the strength of the horse's escape response overpowers the capacities of the handler, the correct application of negative reinforcement to elicit the desired response can become very difficult, often resulting in increased difficulties loading the horse because it has been inadvertently reinforced for escape rather than approach responses (McGreevy and McLean, 2011).

Self-loading (loading on a verbal cue) using target training via positive reinforcement was used to successfully re-train horses which previously refuse to load (Ferguson and Rosales-Ruiz, 2001). Habituation occurs when there is decreased response to a stimulus as a result of the repeated presentation of that stimulus (Thompson and Spencer, 1966). Houpt (1982) recommended training foals to load and exposing them to travel early in their lives. Early positive familiarization with the vehicle and travelling procedures will habituate the horses to these inherently aversive experiences. Habituation practices can include travelling foals with their dam or a familiar older horse, installing leading, backing and parking responses prior to attempting to load the horse for the first time and travelling for short periods before attempting long journeys with naïve horses. Such practices should result in habituation to all aspects of the transport process reducing the likelihood that behaviour problems will develop in these horses (Houpt, 1986).

There is currently little empirical data on how commonly the many methods and equipment recommended by trainers and training schools are used, nor has the effectiveness of these methods been compared on a large scale. The aims of this study were firstly to investigate the perceptions of owners and connections regarding the incidence of TRPB, secondly to identify training methods commonly used to load and travel horses, and thirdly to determine if TRPB were associated with factors such as training method, type of transport vehicle, equine industry sector or transport-related injuries in Australia.

3.3.2. Material and methods

This study was approved by the Human Research Ethics Committee of the University of Sydney *[2015/308]*.

3.3.2.1. Study design and data collection

A cross-sectional online survey was conducted in Australia from June to September 2015. Details of the design and distribution of the cross sectional survey and the description of the demographic characteristic of the study population have been reported previously (Chapter 3.1). Briefly, the survey was digitized using Survey Monkey (SurveyMonkey Inc., California, USA, www.surveymoonkey.com). Participants in the racing, equestrian and recreational equine industry sectors responsible for organizing transportation of horses on a monthly basis or more frequently, were invited to take part in the survey. An invitation letter and the link to the survey were provided to a wide range of Australian horse sports and organisations, and the survey link was also published on various equestrian websites. The link was promoted through several horse magazines, relevant Facebook pages and online horse forums. The survey contained 40 questions (Appendix 1).

This paper considers the findings relating to the following five questions: equine industry sector in which the respondents were involved; whether training in loading and travelling was conducted and a description of the training and the equipment used where applicable; whether TRPB occurred and, via multiple choice options, in what stage of transportation: preloading, at loading, travelling, unloading; the type of vehicle used to move the horses, and whether transport-related traumatic injuries occurred in the last two years.

3.3.2.2. Statistical analysis

Table 1 shows the classifications of the predictive variables.

Table 1. Classification of the studied variables.

Name	Description	Values
Sector	Sector of the horse industry in which the respondent was involved	Endurance, Equestrian sport*, Horse breeding, Recreational non-competitive, Standarbred (SB) racing, Thoroughbred (TB) racing.
Training	If and how the respondent used to train horses to load, travel and unload	Habituation, self-loading, operant conditioning, no identified training method.
Equipment	What equipment the respondent used during loading procedure	No equipment, halter and lead rope, hind quarter rope, whip, lunge, food.
Vehicle	What type of vehicle was used to transport horses	Two-horse straight trailer, two-horse angle trailer, 3-4-horse angle trailer, gooseneck-trailer, truck.
Problem behaviours	What type of problem behaviour was experienced during transportation	Preloading (PLPB), loading (LPB), travelling (TPB), unloading (UPB).

*Dressage, eventing, jumping, reining, driving, vaulting

The respondent replies were classified by one of the researchers (CH) with expertise in animal training into the following training methods: habituation (H), self-loading (SL), no training (NT), operant conditioning (Table 2). To be considered as habituation, the respondent should have specified that the training was applied before the real trips with the aim to desensibilise the horses to the transport procedures. To be considered as self-loading, the respondent should have written “self-loading”.

Based on the respondents’ description, the equipment used to load horses was classified into the following categories by the same expert: no equipment (in self-loading), halter and lead-rope, food, lunge, hindquarter rope and whip. Where the equipment was not specified, including where training method was not used (NT), the equipment “halter and lead rope” was assigned by the expert, as this equipment is uniformly used across all equine industry sectors (Hill, 2000).

Table 2. Definitions and examples of respondents' replies for the training category.

Training category	**Definition**	**Have you used any training to aid in transporting your horses? If so, describe the training tool (i.e. training in loading and unloading the vehicle). Examples of typical responses**
Habituation (H)	The habituation category included techniques used to habituate horses to all aspect of transport prior to travel, such as familiarising young horses (foals and weanlings) and new horses to the transport vehicle, repeated loading and unloading prior to travel and/or taking the horses on short trips and/or using an experienced companion for short trips prior to undertaking longer journeys for specific purposes (Houpt, 1982).	1. The basic training on and off loading. Short trips. "No force policy, no whip etc" All horses are taught to load/unload and tie up prior to travel. 2. Trained to load as foals with mare where possible. Taught to load, tie up and back out when necessary as yearlings or two year olds at the latest.
Self-loading (SL)	Operant conditioning and classical conditioning leading to the horse self-loading onto the vehicle on a verbal, visual or other classically conditioned cue (Bruce, 2009; Ferguson and Rosales-Ruiz, 2001; McLean, 2003).	1. Horses are trained to self-load and unload. 2. Clicker training to self-load and unload horse. 3. Train in self-loading using techniques as per Buck Branaman/ Warwrick Schiller etc
No training (NT)	Respondents did not train their horse to load or travel.	1. No. When I bought my horses they had already tamed. They travel regularly now.
Operant conditioning (OC)	Negative or positive reinforcement, or positive punishment (Baragli et al., 2015).	1. Used various techniques to load. Not sure that is training as such: food incentive (this really is a training aid); bum tape (also linked hands); whip/stick 2. With a horse that is particularly hard to load, I ensure the most uncomfortable place to be is away from the truck, whilst the most comfortable place to be is on the ramp (with a view to being on the truck).

Based on the results of the multiple choice question, the TRPB problems were classified into: preloading (PLPB), loading (LPB), travelling (TPB) and unloading (UPB).

The initial descriptive analysis included creation of frequency tables and was conducted using statulorbeta (http://statulator.com/descriptive.html).

Since initial descriptive analysis showed low numbers of responses for some equipment, including food (< 2%) and lunge ropes (0.8%), food was removed as a factor and lunge ropes were combined with equipment used around the hindquarters variously described as "bumropes", "rump ropes" and "long ropes" as a single term "hindquarter rope". The data were then analysed by univariate and multivariate logistic regression using GenStat® Version 14 (VSNi International, Hemel Hempstead, UK) and two models were derived. The first model had each problem behaviour (PLPB, LPB, TPB, UPB) as the binary outcome (1/0) and the following predictive variables: sector, training, equipment and vehicles. The second model had transport-related traumatic injuries as the binary outcome (1/0) and the following predictive variables: sector, training, equipment, vehicles, PLPB, LPB, TPB, and UPB. For both models, p values were calculated using Wald Test and each predictor variable returning a P value <0.25 from the univariate modelling was considered for inclusion in a multivariate model for that outcome. A step-wise backward elimination procedure was then conducted whereby predictive variables were removed until all variables in the final model had a P value<0.05 indicating significance. The findings are presented as odds ratio (OR) and confidence interval (95% CI) for each predictive variable.

3.3.3. Results

3.3.3.1. Descriptive statistics

The survey generated 797 responses and the distribution of the data after their categorization (including missing values) appears in Table 3. More than one third of the respondents (38.8%, n=309) reported they had experienced problem behaviours with at least one of their horses during transports and some respondents had experienced more than one type of problems. Of the problem behaviours reported, loading problems were the most frequently experienced (157/309, 50.8%), compared to travelling (131/309, 42.4%), preloading (86/309, 27.8%) and unloading (48/309, 15.5%).

Transport relating training was reported by 448/793 (56.5%): OC, H and SL were used by 25.5%, 20.2% and 10.8% of respondents, respectively; whilst 345/793 (43.5%) of respondents did not train their horses (NT). In the OC category, positive reinforcement was employed by only 2% of the respondents. Table 4 shows the distribution of the different training methods by equine industry sector. Of 797 respondents, 359 (45.0%) reported that at least one horse was injured during transportation.

Table 3. Distribution of the data after categorization.

Variable	Category	Number (n)	Percentage (%)
Horse Use	Endurance	51	6.4
	Equestrian sport	389	48.8
	Horse breeding	73	9.2
	Recreational non-competitive riding	192	24.1
	Standardbred racing	34	4.3
	Thoroughbred Racing	58	7.2
	Total	797	100
Vehicle	A 3-4 horse angle load trailer	108	13.6
	A gooseneck-trailer	48	6.0
	A two horse angle load trailer	75	9.4
	A truck	160	20.1
	A two horse straight load trailer	406	50.9
	Total	797	100
Type of training	Habituation	160	20.2
	Self-loading	86	10.8
	No training	345	43.5
	Operant conditioning	202	25.5
	Total	793	100
	Missing Values	4	0.5
Equipment	No equipment	86	11.7
	Halter and rope	581	75.5
	Hindquarter rope	30+7	4.8
	Whip	62	8.1
	Total	770	100
	Missing value	31	3.4
Problem Behaviours	No	488	61.2
	Yes	309	38.8
	Total	797	100
Preloading Problem Behaviours	No	711	89.2
	Yes	86	10.8
	Total	797	100

Loading Problem Behaviours	No	640	80.3
	Yes	157	19.7
	Total	797	100
Travelling Problem Behaviours	No	666	83.6
	Yes	131	16.4
	Total	797	100
Unloading Problem Behaviours	No	749	94.0
	Yes	48	6.0
	Total	797	100
Traumatic Injury	No	438	55.0
	Yes	359	45.0
	Total	797	100

Table 4. Frequency (n (%)) of training method per equine industry sectors.

	No training	**Habituation**	**Self-loading**	**Operant Conditioning**
Recreational	68(35.6)	43(22.5)	28(14.7)	52(27.2)
Endurance	15(29.4)	12(23.5)	9(17.6)	15(29.4)
Equestrian Sport	176(45.5)	70(18.1)	43(11.1)	98(25.36)
Horse Breeding	29(39.7)	26(35.6)	4(5.5)	14(19.2)
SB racing	23(69.7)	3(9.1)	1(3.0)	6(18.2)
TB racing	34(58.2)	6(10.3)	1(1.7)	17(29.3)
Total	345(43.5)	160(20.2)	86(10.8)	202(25.5)

3.3.3.2. Regression analysis: behavioural problems as outcomes

In the univariate regression analysis with PLPB as the outcome, the type of training (P<0.001), equipment (P=0.015), and vehicle (P=0.029) were significantly associated with PLPB. No association was found with industry sector (P=0.470). In the multivariate model, only the type of training (P<0.001) and vehicle (P=0.048) were retained as significant (Appendix 3, Table 1). Compared to habituation, the odds of a horse having a PLPB were 3.43 times greater when given no training, whilst the odds in horses in the OC group having a PLPB was 7.64 times greater than those habituated to transport. The odds of having a PLPB were 3.13 times greater when horses travelled in a two horse trailer than in larger vehicles.

In the univariate regression analysis with LPB as the outcome, no association was found with industry sector (P=0.363), but LPB was associated with the type of training (P<0.001), equipment (P<0.001), and vehicle (P=0.026). In the multivariate model LPB were associated with the type of training (P<0.001) and vehicle (P=0.016) The odds of a horse experiencing loading problems were 16.60 and 3.51 times greater for respondents using OC and NT when compared with respondents using habituation. There was no difference in LPB between habituation and self-loading (Appendix 3, Table 2).

With TPB as the outcome, no association was found with vehicle (P=0.569) or equipment (P=0.792). The type of training was associated with TPB in both the univariate (P<0.001) and multivariate analyses (P=0.002). Industry sector was also associated with TPB in the univariate (P<0.001) and multivariate analyses (P<0.001). Compared to habituation, the odds of a horse exhibiting problem behaviours during travel were 3.52 times greater for those trained by OC and 2.13 times greater for those in the NT group. There was no difference in TPB in horses trained with habituation and self-loading (Appendix 3, Table 3). In comparison to the endurance sector, the odds of horses in the Thoroughbred and Standardbred racing sectors experiencing TPB were 24.92 and 18.60 times greater, respectively.

Using unloading problem behaviours (UPB) as the outcome, no association was found with industry sector (P=0.360), vehicle (P=0.150) or equipment (P=0.303), whereas there was an association between UPB and the type of training (P=0.011). This was true both in the univariate and multivariate analysis. The odds of a horse showing UPB were 3.78 times greater if horses were trained using OC compared with habituation (P=0.007) (Appendix 3, Table 4).

3.3.3.3. Regression analysis: traumatic injury as outcome

In the univariate logistic analysis, horse injury related to transport was associated with the type of training (P=0.002). The odds of experiencing injury in horses trained by OC were 1.72 (CI: 1.12-2.59) times greater compared to horses which had been habituated. However, there was no difference between H, SL and NT. Injuries were also associated with LPB (OR: 2.96; CI: 2.0-4.27, P<0.001), TPB (OR: 3.10; CI: 2.08-4.62, P<0.001) and UPB (OR: 2.18; CI: 1.16-3.87, P=0.014). Injuries were not significantly associated with PLPB (P=0.097), equipment (P=0.900), vehicle (P=0.064), or sector (P=0.120).

In the multivariate logistic regression, injuries were associated only with LPB (P<0.001), TPB (P<0.001) and vehicle (p=0.042). Horses with LPB (OR: 2.72, CI=1.88-3.97) and TPB (OR: 2.85, CI: 1.89-4.29) were more than twice as likely to have experienced an injury in comparison with horses without those problem behaviours. In comparing type of transport vehicle the odds of experiencing a traumatic injury related to transport were higher in two-horse straight load trailers (OR:1.57, CI:1.07-2.33, P=0.022) and in 3-4 horses angle trailers (OR:1.72, CI;1.032-2.88, P=0.038) than in a truck.

3.3.4. Discussion

This survey documented that problem behaviours and injuries associated with equine transportation was common in Australia in agreement with what reported from Lee et al. (2001) in the USA. Only approximately half of the survey respondents reported training horses to load or travel. The type of training was identified as a risk factor for the development of TRPB and horses trained by habituation were at lower risk of developing TRPB then horses received no training or that were trained by negative reinforcement and positive punishment. The importance of TRPB is emphasised in this study through it being identified as a risk factors for transport-related injuries. Although not captured in this study, it is possible that horse related injuries amongst handlers increase with TRPB (Mansmann and Woodie, 1995). Therefore, training of horses for transportation, and the training methods used warrant significant attention both in terms of human safety and horse welfare.

Operant conditioning was employed by one quarter of respondents. The majority (98%) of respondents in the OC category used negative reinforcement to train their horses to load. McGreevy and McLean (2007) have identified that poorly applied negative reinforcement can produce a multitude of undesirable and even dangerous behaviours in horses. Effective negative reinforcement relies on the provision of the reinforcement (reduction in aversive stimulus) immediately after the desired response (Baragli et al., 2015). Delays in providing the reduction or releasing after the performance of an undesired behaviour can result in the horse acquiring unwanted responses to cues (McLean and MCLean, 2008). As previously noted, the inherently aversive characteristics of transport may elicit flight and escape behaviours which mitigate against well timed negative reinforcement. When this is coupled with prior difficulties in loading or travelling, the horse may be strongly motivated to avoid loading (McGreevy, 2012). These reasons could explain why in this study OC was associated with a higher risk of problem behaviours and injuries, compared to habituation and self-loading.

Positive reinforcement training to reinstall loading responses in problem loaders is superior to negative reinforcement (Hendriksen et al., 2011; Slater and Dymond, 2011). However, our data showed that positive reinforcement training is not a widely used training modality in Australia in the context of preparing horses for transport. This could be due to a lack of awareness of the efficacy of the method or because it may not be appropriate in situations in which the horse is in an environment which results in arousal or the environmental stimulus may overshadow the training. These could include shows, or competition, where food rewards may not be sufficiently salient for effective reinforcement (Heidenreich, 2007). Further investigation of the reasons for the low use of positive reinforcement in transport training of horses is warranted.

Our respondents reported that their horses exhibited more problem behaviours during loading and travelling than preloading and unloading. This finding is in agreement with the findings of Lee et al. (2001) who reported that problem behaviours were demonstrated by 53.4% of horses while loading and 51.5% during travelling. Given that loading is nominated as the phase with the highest incidence of problem behaviours, it is not surprising that the focus of advice in many training manuals is on getting the horse to load (McLean and McLean 2008, Parelli 1993). However, simply training the horse to load may not be sufficient to achieve a level of habituation that is protective against the future development of transport-related problem behaviours or health issues. Indeed, as Waran et al. (2007) has noted, transport is a complex activity for animals, comprising a number of phases, all of which have the potential to be aversive. Our findings indicate that habituation training, preparing horses for all phases of travel in advance of undertaking significant journeys, is likely to be important for reducing problem behaviours throughout all phases. Many respondents stated that they exposed their horses to the transport process when they were foals or weanlings. It has been shown that early handling of foals improved their tractability when handled some months later (Henry et al., 2006).

Techniques included in the habituation category all rely on minimizing the aversiveness of the travelling experience by using methods such as shaping (short journeys), social facilitation (the use of a calm con-specific), pre-training the operant responses required for loading (leading from poll pressure, backing, parking) and travelling (parking), unloading, (leading, backing) before exposing the horse to the vehicle (Houpt, 1986). This approach breaks down the travelling process into its components and ensures that the horse is trained to offer correct responses at a sufficiently reliable level prior to the addition of the aversive characteristics of the transport itself (Houpt and Wickens, 2014).

The International Society for Equitation Science training principle 9 advises trainers to dissociate flight responses and reduce aversive pressures to the minimum required to elicit the response (ISES, 2016). The habituation techniques nominated by respondents to our survey are consistent with these principles. By minimizing fear responses through a graduated shaping process, the horse's ability to attend to the relevant stimuli (handler cues) is not impeded. In addition, repeated exposure to the aversive stimulus (transport), whether at loading or during travel, enables the horse to habituate to the stimulus, thus reducing behavioural and physiological reactions.

Self-loading is also associated with fewer problem behaviours during loading. This is unsurprising given that such horses are trained to load without the application of pressure directly to their bodies. In order to train a horse to self-load it is necessary to first install the response via operant conditioning, followed by classical conditioning to associate the loading response with a verbal or non-pressure cue (Ferguson and Rosales-Ruiz, 2001). This method meets the requirement to reduce pressures to their minimum and also reflects a level of habituation to the loading process and being inside the transport vehicle.
Common to both the habitation and self-loading groups is the necessity of allowing sufficient time for individual horses to habituate to the vehicle and the loading process. Both methods require time to implement, however it appears that this time is repaid by increased efficiency in loading and reduced injuries from loading and transport problem behaviours.

Overall, our data suggest that improvements in handler knowledge of the correct application of negative reinforcement, as well as the increased use of classical conditioning to achieve self-loading and the increased use of purposeful habituation activities may lead to a reduction in loading and travelling problem behaviours and reduced injuries.

Our data demonstrated that the likelihood of travelling problem behaviours was lower in horses used for endurance and breeding compared to equestrian sports and racing. The demands of the endurance, racing and equestrian sports sectors generally require horses to be transported on a frequent basis, in some cases daily or weekly. In comparison, breeding horses are usually transported infrequently, often experiencing only a few journeys a year; to and from the stud farm in the case of Thoroughbred broodmares (Leadon et al., 1989). The lower likelihood of travelling problem behaviours in the endurance horses compared to the racing and equestrian sports discipline requires further investigation as the reasons for this difference are not apparent from our data. It has been indeed suggested that horses are able to associate the journey with the previous transport experiences and the activities post-journey, thus short trips for pleasure purpose (e.g. going to grazing) has been recommended to reduce travelling problems (Houpt, 1986). This recommendation requires further investigation.

From our results, it seems that the two-horse straight-load trailer is associated with a higher risk of problem behaviours and injuries. There are a number of potential reasons for this finding. Horse trailers can be driven without a special driving licence, thus it could be possible that the horse trailer drivers had fewer driving skills than the truck drivers; driver's behaviour can indeed affects the behaviours of the transported animals (Cockram and Spence, 2012; Giovagnoli et al., 2002). Horse trailers are less stable than trucks and or goose necks, and maintaining balancing inside a trailer is more challenging (Lee et al., 2001). The effort required to maintain balance has been found to be greater in horses travelling facing forward compared to facing sideways or backwards (Padalino et al., 2012). Our data suggest that the ideal design of a two-horse trailer warrants future research.

Our survey documented that many horses experienced frequently traumatic injuries during transportation, in agreement with a previous survey conducted in Australia (Noble et al., 2013). Transportation appears to be a significant human-related risk to horses (Chapter 2) and a matter of welfare concern (Waran et al., 2007). Our survey documented also that loading and travelling problem behaviours were associated with over a two-fold increase in the risk of horses experiencing an injury during transport. While the inherent risks of injury during loading and transport may be extended to handlers as well as horses (Mansmann and Woodie, 1995; Riley et al., 2015), the wide dissemination of evidence based training techniques which reduce TRPB should be a priority to safeguard horse welfare and improve handler safety (Baragli et al., 2015; Riley et al., 2015; Starling et al., 2016).

Our findings should be interpreted with caution, because our study was a questionnaire, and it had a number of limitations in common with other online questionnaires (Christley, 2016). Firstly, problem behaviours and injuries were self-reported. They relied on accurate recall by the respondents and the respondents may not have accurately identified the causes or symptoms. Secondly, even though the survey was widely publicised to a range of Australian equine industry sectors, the results may not be representative of the industry as a whole due to self-selection bias. Thirdly, the classification of the training methods and the equipment were based on the respondents' descriptions and the authors could not double-check them. Notwithstanding these limitations, our study is the first to report and compare the effects of training methods on problem behaviours and injuries related to transportation in horses. To date, the majority of training advice focuses only on loading and unloading horses (Firestone et al., 2011; Hill, 2000; Shanahan, 2003) rather than taking a more holistic approach which includes purposeful habituation processes (Houpt, 1986). This lack of awareness of the benefits of purposeful habituation is reflected in our results where habituation was applied only by 20.2% of respondents. Conversely, in the light of our findings, advice on preparing horses for trouble free transport should include specific guides to the most appropriate habituation activities that should be undertaken prior to attempting travel. The widespread adoption of purposeful habituation of horses to transport, including training horses to self-load could result in a reduction in transport-related behaviour and associated health problems.

3.3.5. Conclusions

Problem behaviours and injuries related to transport are common in Australia and habituation appeared to be the most effective method to prepare horses for transportation. However, only 20.2% of respondents implemented habituation techniques to prepare their horses for loading and travelling. Our study suggests that the neglect of a holistic approach to familiarizing horses with all aspects of loading, travel and unloading is associated with a higher incidence of problem behaviours and injuries. Based on our results, habituation training methods and the self-loading technique are recommended to safeguard horse and handler wellbeing.

CHAPTER 3.4

Risk factors in equine transport-related health problems: a survey of the Australian equine industry

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3.4.1. Introduction

Transportation is an integral part of many horse related activities, with horses being moved frequently (Rosanowski et al., 2013) and for a wide range of reasons (Leadon, 1994). Horses transported frequently may become habituated to travel (Houpt and Wickens, 2014). However, for other horses, the challenges associated with transport, such as confinement, noise and vibration (Chapter 1) may trigger fear (Grandin and Shivley, 2015). The physical and mental stressors associated with transportation can result in adverse effects on the horses' health (Oikawa et al., 2004). Fear may also trigger behaviours that put horses at risk of injury (Ferguson and Rosales-Ruiz, 2001), ranging from small abrasions to catastrophic fractures (Mansmann and Woodie, 1995). The stress associated with transport, and the way in which horses are managed during transport (Oikawa et al., 2005; Raidal et al., 1997a; Stull and Rodiek, 2002), can contribute also to the development of potentially fatal infections of the respiratory (Austin et al., 1995) or gastrointestinal systems (McClintock and Begg, 1990).

Transport's effects on physiological (Tateo et al., 2012), behavioural (Padalino et al., 2012), endocrine (Fazio et al., 2013a), reproductive (Baucus et al., 1990b), muscular (Giovagnoli et al., 2002), gastric (McClure et al., 2005), inflammatory (Wessely-Szponder et al., 2015) and respiratory parameters (Raidal et al., 1997a) have been described in many observational studies. In contrast, few epidemiological studies have been conducted. The incidence of and risk factors for health problems has been reported in horses being transported for slaughter (Marlin et al., 2011; Roy et al., 2015; Stefancic and Martin, 2005). The only epidemiological study on risk factors for the development of transport-related health problems during long haul transportation of horses for other purposes identified journey duration (more than 20 hours) and season (spring) as risk factors. However, the study reported only 47 cases (Chapter 2).

Consequently, the relationship between journey and horse characteristics, and the risk of developing disease remains poorly defined. We used an online survey to examine associations between transport-related health problems and journey and horse characteristics across a number of Australian equine industry sectors.

3.4.2. Material and methods

3.4.2.1. Study design and data collection

The study was a cross-sectional online survey conducted in Australia from June to September 2015. Detail of the design and distribution of the cross-sectional survey and the description of the demographic characteristics of the study population have been reported previously (Chapter 3.1). Briefly, the survey was digitized using SurveyMonkey (SurveyMonkey Inc., California, USA, www.surveymonkey.com). The target population was people involved in any equine industry sector who had organised or participated in the movement of horses (commercially or non-commercially) at least monthly over the past two years. The respondents classified themselves as either professionals (people who were involved for financial reward, such as trainers, stud/farm managers) or amateurs (people involved as a hobby, such as riders, owners). An invitation letter and the link to the survey (https://www.surveymonkey.com/r/SM9F9SJ) were provided to several Australian horse sport associations and were published on their websites. The link was also promoted through several horse magazines, relevant Facebook pages and online horse forums.

The questionnaire was divided into four parts: respondent details; management strategies pre, during and post-transport; transport-related behavioural and health problems identified during and after transportation in the past two years; details of the most recent case including horse sex, age, breed, the use of the horse, the vehicle in which the horse was transported, the journey duration, and whether the horse was moved by a commercial/non-commercial carrier. The results of the first three parts of the survey are presented elsewhere (Chapter 3.1, 3.2, 3.3). The data collected within the first and fourth part of the questionnaire (Appendix 4, Item 1) are presented in the current report.

3.4.2.2. Risk factors and outcome

Horse-level predictive variables were sex (mare/filly, gelding, stallion/colt), age (8-24 months, 2-5 years, 6-10 years, >10 years), breed (Arabian, Quarter horse, Standardbred, Thoroughbred, Warmblood, use (breeding, recreational non-competitive activities, Standardbred racing, Thoroughbred racing, endurance, equestrian competitive sport), and amateur or professional status.

Journey-level predictive variables were categorized according to the type of vehicle used (truck, horse trailer) and operator (commercial versus non-commercial transporter). The journey duration was categorized as: short (less than 8 hours), intermediate (8-24 hours) and long (more than 24 hours). These cut-offs were chosen on the basis of the European and Australian Code of Animal Transportation, in which the maximum journey durations without watering are of 8 and 24 hours respectively (http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=uriserv:f83007; http://www.animalwelfarestandards.net.au/land-transport/). In Australia a rest stop of 12 hours is recommended after 12 hours of transport and compulsory after 24 hours of transport (http://www.animalwelfarestandards.net.au/land-transport/). Thus all reported multiday trips in this data set included mandatory rest stops**.**

To categorise outcome, there was an open question in which the respondents reported a description of the signs and their veterinarians' diagnosis of any transport-related health problem that affected the horse. Based on the respondent's description, health problems were classified into six categories (injuries, muscular problems, heat stroke, gastrointestinal problems, respiratory problems, and death/euthanasia) by an experienced equine veterinarian (BP; Table 1).

Table 1. Categorisation of health problems observed in transported horses.

Health problem category	Definition
Injuries	Laceration, abrasion, contusion, swelling.
Muscular problems	Tying up, sore muscle, stiffness.
Heat stroke	Rectal temperature >38.5°C, sweating, lethargy.
Gastrointestinal problems	Oesophagal obstruction, gastric ulceration, diarrhoea, colic, enterocolitis.
Respiratory problems	Nasal discharge, coughing, inflammation/infection of the upper or lower respiratory tract, and pneumonia.
Death	Horses found dead or humanely destroyed.

3.4.2.3. Data analysis

Initial descriptive analysis was undertaken using statulor[beta] (http://statulator.com/descriptive.html). Associations between the predictive variables were explored using contingency tables and χ^2 tests using GenStat® Version 14 (VSNi International, Hemel Hempstead, UK).

A multivariable multinomial regression analysis was constructed using SPSS Version 22 (IBM SPSS) with health problem category as outcome with injuries as the reference level for comparisons. Vehicle and operator were excluded as they were found to be collinear with journey duration. Horse age, breed, sex, use, amateur or professional status and journey duration were considered for inclusion in the final multinomial model. A stepwise backward elimination was then conducted to remove the least significant variable one at a time until all variables within the model had P value < 0.05. The data met with all assumptions for multinomial regression including that of IIA. The findings are presented as odds ratio (OR), confidence interval (95% CI) and P value for each predictive variable value.

3.4.3. Results

3.4.3.1. Population

Of the 797 responses to the survey, 214 included details of a transport-related health problem and these 214 records make up the data set. The distribution of the data (including missing values) is reported in Appendix 4 (Table 2).

The frequency of the health outcomes according to the predictive variables is shown in Appendix 4 (Table 3). Ten horses died during transit: 8 were humanely destroyed due to fractures (5 limb, 1 pelvis, and 2 neck), and 2 were found dead. A further 15 horses were humanely destroyed within one week after the journey due to colic (5 cases), colitis (5 cases), and pneumonia (5 cases). Additionally, 7 deaths occurred within 24 hours after arrival, of which 5 underwent post mortem examination, one was diagnosed with water intoxication; no cause of death was identified in the other four cases.

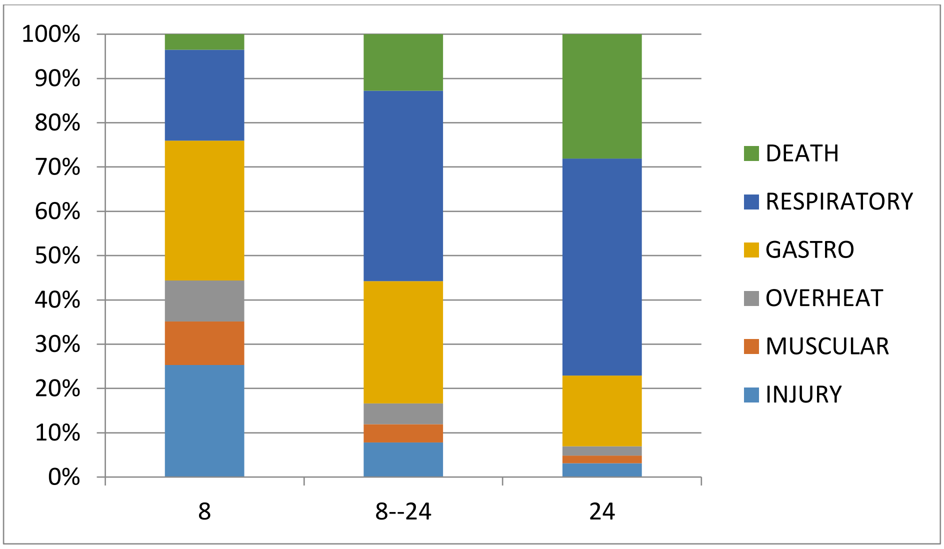
3.4.3.2. Journey variables

Journey duration was associated with vehicle (χ^2: 71.51, df= 2; P<0.001) and transport by a commercial company (χ^2: 78.74, df=2; P<0.001). Companies transported fewer horses on short journeys and more horses on long journeys. The number of horses moved by horse trailer was larger for short journeys, and smaller for long journeys (Appendix 4, Table 4).

3.4.3.3. Factors associated with Health Outcomes

The final multivariate multinomial model for risk factors associated with health problems included journey duration (χ^2: 88.153, df: 10, P<0.001) and breed (χ^2: 46.087, df: 20, P=0.001). None of the other predictive variables considered for inclusion reached significance. Figure 1 shows the distribution of the different transport-related illness according to the journey duration category. Using injuries as reference, death/euthanasia (OR: 101.6, CI: 10.2-1010.5, P<0.001), gastrointestinal (OR: 14.2, CI: 1.5-133.8, P=0.02) and respiratory (OR: 113.9, CI: 12.2-1060.7, P<0.001) problems were more likely to occur on long journeys than on short journeys. Respiratory problems were also more likely (OR: 15.7, CI: 4.3-56.7, P<0.001) to occur on intermediate journeys than on short journeys. Using the injury group as the reference, muscular problems were more likely to occur on an intermediate journey than on a short one (OR: 5.8, CI: 1.1-29.5, P=0.03). There was no significant difference among the journey duration categories comparing injuries versus heat stroke (Appendix 4, Table 5).

Figure 1. Frequencies of illness and injury in 214 of 797 horses in transportation by journey time.



Using injuries as reference group, gastrointestinal problems were more likely to occur in Arabians (OR: 95.8, CI: 4.6-1990.3, P=0.003) and Warmbloods (OR: 43.0, CI: 3.8-485.9, P=0.002) compared with Standardbreds. Respiratory problems were more likely to occur in Arabians (OR: 20.8, CI: 1.2-345.2, P=0.034), Warmbloods (OR: 18.5, CI: 2.5-136.89, P= 0.004), and Thoroughbreds (OR: 7.4, CI: 1.2-45.7, P=0.031) compared with Standardbreds. Death/euthanasia was more likely to occur in Thoroughbreds than in Standardbreds (OR: 7.5, CI: 1.0-56.0, P=0.048) (Appendix 4, Table 5).

3.4.4. Discussion

This is the first study to investigate whether journey and horse characteristics were associated with transport-related health problems across a diverse range of Australian horses used for various activities undertaking different journeys. Journey duration and breed were identified as risk factors for the development of transport-related health problems, while horse sex, age, use and amateur or professional status were not predictors. The main finding of this study was the association between journey-duration and the nature of transport-related health problems, confirming that journeys longer than 24 hours pose the greatest risk of horses having serious health outcomes (Nielsen et al., 2011)(Chapter 2). The association between health problem category and breed should be considered preliminary, and warrants future research using a larger dataset.

The observation that shorter trips are associated with a higher risk of injury is in agreement with previous reports. In an epidemiological study conducted in Australia, injuries occurred more often at the beginning of a 3.5 day journey and that they were often related to behavioural problems (Chapter 2). Previous studies have also identified that behavioural problems and movement of the horse within the vehicle are greatest during the first hour of transportation, and that horses become habituated after 5 hours of transport (Baucus et al., 1990b; Fazio et al., 2008a; Schmidt et al., 2010; Tateo et al., 2012). The higher risk of injuries during short trips is likely to be associated with behavioural problems and lack of habituation.
In this study muscular problems were reported to occur more often during non-commercial transport of non-racing horses, and the risk of muscular problem was greater for intermediate journeys compared with short journeys. The reasons for this cannot be ascertained from the available data, although it could be speculated that limitations in driver ability and in horse fitness could have contributed. In an electromyographic study on the effects of transportation on muscle, horses transported by less experienced drivers required more muscular effort to maintain balance compared with horses transported by expert drivers (Giovagnoli et al., 2002). In another study it was found that the effort required to maintain balance during a 300 km journey had the same impact on muscles as a 1,500 meters canter, and caused a comparable increase in serum muscle enzyme activities (Codazza et al., 1974). The effects of journey duration on muscular problems warrant future research.

Respiratory diseases were the most commonly identified problem in this study, a finding that agrees with previous studies (Leadon et al., 1989; Leadon and Hodgson, 2014). In our study the likelihood of respiratory problems was approximately 15 times greater on intermediate journeys and over 100 times greater on long journeys than on short journeys. The relationship between duration of transport and incidence of respiratory disease has been previously reported (Austin et al., 1995; Kohn, 2000; Marlin, 2004; Oikawa et al., 2004) and our data supports it. This relationship is likely to relate to the head position of the transported horses, vehicle ventilation or air quality. If horses are restrained in a way that prevents them lowering their head, mucociliary clearance will be adversely affected (Raidal et al., 1997a). Ventilation may be inadequate in many types of vehicles (Purswell et al., 2006) resulting in an accumulation of dust, bacteria and noxious gases in the vehicle as journey length increases (Oikawa et al., 2005). The ongoing high incidence of respiratory diseases suggest that more research is needed to identify how ventilation systems can be improved and how any periods of enforced head elevation can be shortened.

In agreement with our previous study (Chapter 2), the risk of gastrointestinal and respiratory disorders and death/euthanasia was greater than the risk of injury for journeys longer than 24 hours. In livestock the association between adverse outcomes and journey duration is influenced by the physiological and clinical state of the animal before and during journey, the management of feeding and watering, the opportunities animals have to rest and the thermal environment rather than journey length *per se* (Nielsen et al., 2011). These factors may also be important in determining whether horses experience adverse outcomes as a result of transport. However, the reasons why some horses develop fatal diseases during and after a multi-day journey, while others remain healthy under the same conditions are unknown. Protracted transport stress may compromise the immune system and lead to psychological and physical exhaustion and death (Marlin, 2004). The use of immunostimulants before shipping has been found to be useful in reducing the incidence of transport-related pneumonia in horses transported for more than 24 hours (Nestved, 1996). Thus, the higher risk of severe diseases in horses transported for longer than 24 hours might be related to immunosuppression, and the relationship between long journeys and the immune system requires further investigation.

Compared with Standardbreds, Thoroughbred, Arabian and Warmblood horses were more likely to develop gastrointestinal and respiratory diseases than to be injured during transportation. Thoroughbreds were found to be at higher risk of transport pleuropneumonia in a previous study (Austin et al., 1995). Arabians have been found at higher risk of colic compared with other breeds (Cohen and Peloso, 1996). There might therefore be a breed-predisposition for developing a particular type of transport-related diseases. However, our data should be considered preliminary and a larger data set would be required to determine the actual effects of breed on different types of transport-related illnesses.

This study has a number of limitations that must be considered in interpreting the results. The problems of bias associated with self-selected participation in the study could not be addressed, nor could the possibility of response bias in the answers provided. It was not possible to check the diagnoses reported, nor was there any standardisation of the processes by which the diagnoses were made. The target population was not estimated and it was not possible to calculate a response rate, a common problem with online surveys (Christley, 2016). Notwithstanding these limitations, this is the first study in which 214 transport-related equine health cases were analysed using a novel approach. The study findings may be important in helping reduce the negative impact of transportation on horse health.

3.4.5. Conclusions

There is an association between transport-related health problems and journey duration and the likelihood of developing a more severe illness (i.e. respiratory and gastrointestinal problem or death/euthanasia) was higher on journeys over 24 hours than on journeys of less than 8 hours, suggesting the need to decrease the maximum journey time in Australia. This study also highlights the need for further research into the effects of long haul transport on the respiratory, gastrointestinal and immune systems to assist in proposing improved management practices to safeguard horse health and welfare during travel, particularly over long distances.

CHAPTER 4

Immunological, clinical, haematological and oxidative responses to long distance transportation in horses

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4.1. Introduction

Transportation is generally regarded as an exceptionally stressful episode in the life of the animal (Knowles and Warriss, 2000), and there is an increasing public interest in and concern for the welfare of livestock during transportation (Grandin and Shivley, 2015). A number of important diseases have been associated with animal transportation (Knowles and Warriss, 2000)(Chapter 3.4), and links between transport and health are well documented in human medicine (Cohen et al., 2014). Horses are transported for a variety of reasons, including shows, competitions, slaughter, breeding, hospitalization and leisure. Consequently they travel frequently and over long distances, and are estimated to be the second most-travelled species after humans (Leadon et al., 1989). Journey duration has been identified as a risk factor for the development of severe transport-related diseases, such as pleuropneumonia and enterocolitis, because the risk of their occurrence increases in journeys longer than 20 hours (Chapter 2). Hence, the need for research on the implications of long distance transportation for horse health and welfare has been raised (Messori et al., 2016)(Chapter 3.2).

Transport-induced immune-suppression has been identified as a possible cause for the development of disease during and after long journeys (Hines, 2000; Marlin, 2004; Stull et al., 2004). In horses transported for 12 hours, peripheral blood neutrophilia and a reduction in neutrophil phagocytic function were evident for at least 36 hours (Raidal et al., 1997a). A decrease in both B and T lymphocyte numbers was found after 38 hours of road transport, with a greater effect on T cells (Oikawa and Jones, 2000). Decreased lymphocyte numbers and variation in the distribution of lymphocyte subpopulations were also observed after a 24 hour road journey by Stull et al. (2008), who attributed the decline in the $CD8\alpha^+$, $CD21^+$, $CD3^+$, $CD4^+$, $CD8\beta^+$ populations to a cortisol mediated stress response (Stull et al., 2008; Stull et al., 2004).

Impaired cell-mediated immunity and release of cortisol have been identified as two components of the acute phase response (Kushner, 1982) which is an immune based reaction to non-specific stimuli characterized by systemic, metabolic and physiological alterations including oxidative stress and the release of acute phase proteins (Cray et al., 2009; Fallon et al., 2001; Kushner, 1982). The nexus between transportation and acute phase responses has been investigated in animals, including pigs (Murata, 2007), camels (Baghshani et al., 2010), and horses (Casella et al., 2012). In performance horses, increased fibrinogen was documented after long journeys (37 hours of transportation by plane), and a plasma concentration greater than 3.2 g/L was proposed as a reliable marker for horses at risk of pleuropneumonia (a.k.a. shipping fever) (Leadon, 2000). In slaughter horses, 6 hours of road transportation induced an acute phase response, characterized by increased fibrinogen and oxidative products (Wessely-Szponder et al., 2015; Wessely-Szponder et al., 2014). Increased plasma total antioxidant status (PTAS) was detected in slaughter mares after an 8 hour journey (Niedźwiedź et al., 2013) and this finding was suggested to be a homeostatic mechanism to balance the production of free radicals and the acute phase response induced by transport stress (Ishida et al., 1999; Niedźwiedź et al., 2013). Transport associated alterations of oxidative balance may induce oxidative stress with cellular damage (Kirschvink et al., 2008) and increase susceptibility to disease (McCord, 2000), if not adequately mitigated by anti-oxidant responses. Thus, oxidative stress might be involved in the development of transport-related diseases, and monitoring of the redox balance by assessment of reactive oxygen metabolites (ROMs) and PTAS could be a useful tool to assess stress and disease-susceptibility, and consequently the welfare of transported horses, as already proposed for transported ewes (Piccione et al., 2013).

A holistic, multidisciplinary approach has been advocated for research on animal welfare (Veissier and Miele, 2014). The current multidisciplinary study was conducted to assess immunological, clinical, haematological, inflammatory and oxidative responses and recovery in transported horses by comparison with a group of similar horses that had not undergone transportation and to explore potential diagnostic relationships between observed responses. It was hypothesised that clinical and haematological responses reported in previous studies of transportation in horses would be recognized in transported horses in this study, but not in control horses. It was further hypothesized that transportation would be a physiological stressor able to activate an acute phase response and decreasing transported horses' immunological capacity to react to a mitogen, and that the severity of such changes might be related to clinical examination or other laboratory findings.

4.2. Materials and methods

The ethical aspects of this study have been approved by the University of Sydney Animal Ethics Committee (AEC) (Project Number 2015/950).

4.2.1Animals

Sixteen show jumping horses of different breeds (Thoroughbred, Warm blood, Australian Stock Horse) were selected for this study. The experimental group (EG) comprised ten horses (7 geldings, 3 mares), aged from 5 to 15 years (10.3 ± 3.2), with body condition score of 3.0 ± 0.1 (Carroll and Huntington, 1988). These horses had travelled from Perth to Glossodia (New South Wales, Australia), a distance of 4,000 kilometers, as described in session 4.2.2. Six horses (5 geldings, 1 mare), aged from 6 to 15 years (9.7 ± 3.6), with body condition score of 3.4 ± 0.1 (Carroll and Huntington, 1988) formed the control group (CG). They were resident at the horse stable in Glossodia for more than two years and had not travelled in the previous three months. They were at a similar stage of fitness and competition as the transported group and there were no statistical differences between groups for age or body condition score. The health (and hence fitness for travel) of the EG horses was assessed by an experienced equine veterinarian (not a member of the study group) before the journey in their private stalls following criteria listed in the Australian code of animal transportation (http://www.animalwelfarestandards.net.au/land-transport/). The health of the CG horses was assessed by another experienced equine veterinarian (BP) in the horse stall in Glossodia (New South Wales, Australia), following the same criteria. Clinical variables for EG horses prior to transportation and CG horses were within normal ranges (Reed et al., 2003).

4.2.2. Journey

The EG animals left Perth at 8:44 am and reached Glossodia four days later at 6:00 am. The trip consisted of four stages: Perth-Kalgoorlie (six hours), Kalgoorlie-Adelaide (24 hours), Adelaide-Melbourne (nine hours) and Melbourne-Glossodia (12 hours). Horses were given 12 hour rest periods both at Kalgoorlie and Adelaide, and a 19 hour rest stop in Melbourne. The total duration was approximately 94 hours with approximately 51 hours in transit and 43 hours for rest stops. Horses were fed and watered on route every 6 hours, during the travel section from Kalgoorlie to Adelaide. In the other travel sections, water and food were offered at the rest stops. At each rest stop the fitness for travel of each horse was assessed by trained personnel following the Australian code for animal transportation, including assessment of rectal temperature, heart and respiratory rate (http://www.animalwelfarestandards.net.au/land-transport/).

At the collection stable and rest points, horses were individually housed in walk in-walk out rubber lined stables and paddocks that were used only for horses in transit. The animals travelled on a semi-trailer (Mega Ark Trailers, MAN®, Munich, Germany). The ventilation system comprised venturi vents, louvres and electric fans generating an airflow which the manufacturer verified was compliant with the Australian code of animal transportation (http://www.animalwelfarestandards.net.au/land-transport/) throughout the trailer. When the vehicle was moving, fresh air entered through the louvres and was extracted by the venturi vents. The fans were used when the truck was stationary (e.g. feeding and watering times, fuel stops) to ensure constant air flow. The horses travelled in individual stalls, restrained by rubber cords which would break under extreme pressure. The two biggest horses were allocated 1½ stall spaces to allow them a more comfortable journey. Two drivers were used for the journey and both were licensed to drive heavy combination vehicles and were experienced horse handlers with many years' experience in commercial horse enterprises. At the start of the journey the temperature in Perth was 14.0°C with humidity at 69%. The temperature and humidity in Glossodia at the end of the journey were 17°C and 42% respectively. The journey complied with the standards and the guidelines for the transport of horses required by the Australian code and all horses passed the assessment of fitness for travel at each rest stop before continuing the journey (http://www.animalwelfarestandards.net.au/land-transport/).

4.2.3. Experimental protocol

A case-control study design was selected to allow comparison between transported horses and a similar cohort of horses that had not been travelled. Each animal was assessed at 6:00 am within 5 minutes of unloading for the EG and at rest for the CG (day 1), and one week later at the same time of day (day 7), at rest conditions for both groups. Clinical assessment was conducted by an experienced equine veterinarian (BP) according to the methodology suggested by Reed et al. (Reed et al., 2003) and consisted of the following parameters: demeanour, mucous membrane (colour, status), capillary refilling time (CRT), heart rate (HR), respiratory rate (RR), rectal temperature (RT) and pulmonary auscultation. CRT was assessed three times in different parts of the oral mucosa (right, middle and left side of the upper jaw, above the incisors). Gastrointestinal tract motility was assessed by auscultation of gut sounds as described previously (Sundra et al., 2012). Subjective gastrointestinal motility scores were assigned for each quadrant: score 2 indicated regular and ongoing peristaltic activity, score 1 was assigned when the period of no borborygmi was longer than the period of peristaltic sound and a score 0 was recorded when there were no gut sounds. Results from each quadrant were summed to give a total score. A score of 7-8 was deemed normal, 1-6 was classified as reduced and 0 was absent. Body weight (BW) was assessed by the same veterinarian using a horse weight tape (Strategy, Virbac, New South Wales, Australia) positioned around the horse's girth just behind the wither (Carroll and Huntington, 1988). The EG and CG horses were clinically examined daily in this manner for a further five days (day 2 - day 6) after the journey. None developed clinical signs of disease.

Blood was taken from the jugular vein of all horses into five Vacutainer tubes (Becton Dickinson, Franklin Lakes, New Jersey, USA), three with heparin, one with EDTA and one without an anticoagulant on day 1 and day 7. Blood samples were collected while horses were at rest and standing in a tethered stall, restrained only by lead rope, at 6:00 am on day 1 and day 7. After collection, blood samples were kept at 4°C and analyses started within 4 hours.

During the study (day 1 to day 7), all horses (EG and CG) were kept on pasture during the day and stabled overnight. They were fed at the ground level with lucerne hay and commercial horse feed (EasiRide, Prydes®, Gunnedah, New South Wales, Australia) twice daily (7:00 am, 6:00 pm) and had access to water *ad libitum*. The diet was calculated individually to meet maintenance requirements (Waldron, 2012). During the trial all horses were on the same training plan consisting of three days of complete rest, followed by four days of easy work (20 minute walk and 20 minute trot/day).

4.2.4. Haematological and biochemical parameters

Haematology was performed using the Sysmex, XT-2000i cell counter analyzer. The following parameters were recorded: red blood cells (RBC) ($x10^{12}$/L), haemoglobin (Hb) (g/L), hematocrit (Hct) (%), platelets (PLT) ($x10^9$/L), white blood cells (WBC) ($x10^9$/L), neutrophils (N) ($x10^9$/L), lymphocytes (L) ($x10^9$/L), monocytes (M) ($x10^9$/L), eosinophils (E) ($x10^9$/L) and basophils (B) ($x10^9$/L). Fibrinogen was calculated by heat precipitation (Millar et al., 1971). Serum biochemistry parameters (chlorine (Cl, mmol/L), potassium (K, mmol/L), sodium (Na, mmol/L), creatine kinase (CK, U/L), total calcium (Ca, mmol/L), albumin (Alb, g/L), aminotransferase (AST, U/L) and total serum proteins (TP, g/L)) were assessed with Thermo Scientific reagents and the Konelab 20XT photometer (Thermo Fisher Scientific, Finland) with interferential filters.

4.2.5. Oxidative stress parameters

Plasma was obtained by centrifugation of heparin blood tubes at 1600 x *g* for 15 min. Reactive oxygen metabolites (ROMs) and plasma total antioxidant status (PTAS) were determined in plasma by commercial kits (d-ROMs test and PAT test, respectively, H&D srl, Parma, Italy) following manufacturer's instructions using a dedicated photometer (Free Radical Analytical System 4 Evolvo, H&D srl, Parma, Italy, Europe). The intra-assay co-efficient of variations (CVs) were 3.7% and 6.4 % for the d-ROMs and PAT tests respectively, while the inter-assay CVs were 1.9% and 8.1% for d-ROMs and PAT tests respectively. The concentration of ROMs was expressed as U. Carr, where 1 U. Carr = 0.08 mg H_2O_2/dl, PTAS was calculated using the ferric reducibility ability of plasma (FRAP) method (Benzie and Strain, 1996), with results expressed as U.Cor, where 1 U.Cor = 1.4 µMol/L of ascorbic acid. The degree of oxidative stress (oxidative stress index, OSI) was estimated using the ratio of ROMs/PTAS multiplied by 100 (Crowley et al., 2013).

4.2.6. Immunological parameters

4.2.6.1 Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation. Briefly, lithium heparin blood tubes were centrifuged at 1455 x *g* for 20 min, buffy coats were harvested, diluted 1:3 in phosphate buffered saline (PBS), layered over Ficoll-Paque Plus (GE Healthcare) (2:4) and centrifuged at 754 x *g* for 30 min. Harvested PBMC were washed twice in PBS (255 x *g*, 10 min) and resuspended in warm culture medium (RPMI 1640/10% FBS/penicillin streptomycin). Cells were counted on a flow cytometer (0500-4008 Guava® easyCyte 8HT, Merck Millipore, MilliporeSigma, Merck Millipore, MilliporeSigma, Billerica, Massachusetts, USA) using a viability dye (4000-0040 Guava ViaCount Reagent for Flow Cytometry, Merck Millipore, MilliporeSigma, Merck Millipore, MilliporeSigma, Billerica, Massachusetts, USA).

4.2.6.2 Lymphocyte proliferation

A non-radioactive method was used to assess lymphocyte proliferation (Parish, 1999). PBMC were labelled by incubating 1x10^6 cells in warm buffer (PBS/5% newborn calf serum) for 5 min at 37°C with the fluorescent tracking dye carboxyfluorescein diacetate succinimidyl ester (CFSE) (5 μM) (de Silva et al., 2010) followed by two washes with cold buffer, and resuspended in warm culture medium. The cells were then plated into a 96-well plate (final concentration of 2.5x10^6/mL) and cultured in medium alone, 10 μg/mL concanavalin A (Con A) or 5 μg/mL pokeweed mitogen (PWM) for 4 days at 37°C in 5% CO_2. At the end of the culture period, samples were acquired on a flow cytometer (Guava EasyCyte 8HT, Merck Millipore, MilliporeSigma, Merck Millipore, MilliporeSigma, Billerica, Massachusetts, USA) to determine total cell proliferation. The lipophilic dye, CSFE, is membrane permeable but is trapped within cells following cleavage into an impermeable form which binds covalently to intracellular amine groups thus increasing its intracellular retention. Fluorescence intensity is halved with each cell division of labeled cells. Thus proliferation is detected as the loss of fluorescence intensity. Lymphocyte proliferation was measured based on the lower fluorescence of cells labelled with CFSE (using GuavaSoft^TIM 3.1.1, Merck Millipore, MilliporeSigma, Merck Millipore, MilliporeSigma, Billerica, Massachusetts, USA) and data are presented as a percentage (% CFSE).

4.2.6.3 Interferon gamma (IFNγ)

Whole blood collected into lithium heparin (500 μL) was plated into 48-well flat-bottom plates containing an equal volume of either culture medium alone or 5 μg/mL PWM, and incubated for 2 days at 37°C and 5% CO_2. At the end of the culture period supernatants were harvested and stored at -80°C. The Equine IFN-γ VetSet Elisa kit (Kingfisher, Biotech, Inc, St. Paul, Minnesota, USA) was used to measure IFN-γ concentration in culture supernatants following manufacturer's instructions. The absorbance was read on a plate reader (Multiskan, Thermo Electron Corporation, ThermoFisher Scientific, Waltham, Massachusetts, USA) at 450 nm. Intra-and inter assay coefficients of variation were 4.2% and 5.5%, respectively. IFN-γ was expressed in ng/mL and was calculated by subtracting the concentration in medium alone from the concentration in PWM.

4.2.6.4. Cortisol

Cortisol concentration was assessed in serum samples by radioimmuneassay (RIA) using the ImmunChem^{TM} Cortisol 125 kit following manufacturer's instructions (MP Biomedicals, LLC, Orangeburg, New York, USA). Sensitivity was 0.17 µg/dL and intra-and inter assay coefficients of variation were 5.3% and 7.5% respectively. Cortisol concentration was expressed in µg/dL.

4.2.7. Statistical analysis

Descriptive statistics of the data were obtained using Statulator^{beta} (http://statulator.com/descriptive.html). Normal distribution of all quantitative data was checked using the Anderson-Darling test, and all data were normally distributed. All data were analysed by mixed linear model using PROC mixed procedure (SAS, version 9, 1999); in the model, horse was used as random factor, to account for multiple observations. Group (EG, CG), day (day1, day7) and their interaction (group*day) were specified as fixed factors. When the interaction was significant, the Tukey-Kramer post hoc test was used for multiple pairwise comparisons. Results are presented as least square mean ± standard error (SE). Pearson correlations were calculated for dependent variables using PROC Corr (SAS, version 9, 1999). Significance was defined as P<0.05.

4.3. Results

4.3.1. Clinical parameters

Table 1 shows the summary statistics of the clinical examination of the CG and EG horses at day 1. Soon after unloading EG horses were quiet and less responsive than normal (Reed et al., 2003). Heart rates, respiratory rates and CRT were higher than normal (Reed et al., 2003). Six of ten horses evidenced decreased borborygmi (score 1 and 0) in one or more quadrants on abdominal auscultation. Three of the horses coughed during the examinations and coarse airway sounds were audible during thoracic auscultation of these horses. Coughing resolved within 24 hours after the journey and none of the three affected horses showed hyperthermia during the study.

On day 7, BW was significantly increased relative to results obtained immediately following transportation (day 1: 508.9±24.8 kg vs day 7: 519.6±24.8 kg, P<0.001) and all physiological and clinical variables were within normal ranges. Clinical variables from CG horses were in the normal range on both day 1 and day 7. None of them showed clinical signs during the trial and no variation in BW was observed (day1: 622.1±32.1 kg vs day7: 622.1±32.1 kg, P=1.000). Comparison of RT, HR, RR and CRT between groups and on days 1 and 7 is shown in Table 2. Significant differences were observed between EC and CG horses for HR, RR, RT and CRT on day 1, but not on day 7, and significant differences were observed in EG horses between day 1 and day 7.

Table 1. Summary statistics for the clinical parameters in Control Group (non-transported horses) and in Experimental group (transported horses) at day1 (soon after unloading for EG and at rest CG).

Variable	Control Group (CG)				Experimental group (EG)				Normal range**
	Mean	Median	Standard deviation	Range (min-max)	Mean	Median	Standard deviation	Range (min-max)	
RT (°C)	37.3	37.4	0.2	37.1-37.6	37.9	37.8	0.2	14-24	37.0-38.5
HR (bpm)	34	36	3.3	28-36	45.2	45	4.6	40-54	30-40
RR (bpm)	10.6	12	2.0	8-12	19.4	18	3.4	14-24	8-12
CRT (sec)	2	2	0	2	3.5	4	0.6	2.5-4	1-2
GITM* left dorsal q		2	2	2		1	1.0	0-2	2
GITM* left ventral q		2	2	2		2	0.8	0-2	2
GITM* right dorsal q		2	2	2		1	0.5	1-2	2
GITM* right ventral q		2	2	2		1.5	0.5	1-2	2
GITM* total score		8	8	8		5.5	2.3	2-8	7/8
	Summary of non-quantitative variables				**Summary of non-quantitative variables**				
Membrane colour	All horses had pink membranes				7 pink/ 2 pale pink/1dark				pink
Lung Sound	All horses had normal lung sounds				3 abnormal/7 normal				normal
Membrane status	All horses had moist mucous membranes				All horses had dry mucous membranes				moist
Demeanour	All horses were alert				All horses were quiet				alert

H: horse; RT: rectal temperature; HR: heart rate; RR: respiratory rate; CRT: capillary refilling time; GIT: gastrointestinal tract. *Gastrointestinal tract motility (GITM) was assessed by auscultation of all four quadrants (q) and scored as described previously (0 = no intestinal sounds, 1 = decreased borborygmi, 2 = normal borborygmi) (Sundra et al., 2012);** (Reed et al. 2003).

Table 2. Physiological parameters in Control Group (non-transported horses) and Experimental Group (transported horses) at day 1 (soon after unloading for EG and at rest CG) and day 7 (7 days after the journey for EG and at rest for CG). Data are expressed as least square mean ± standard error (SE). Differing superscripts within rows indicate significant difference (A,B: P<0.01; a,b: P<0.05) (Tukey-Kramer test).

Parameter	**Control group (n=6)**		**Experimental group (n=10)**		**P values**			**Normal range***
	Day 1	**Day 7**	**Day 1**	**Day 7**	**Group**	**Day**	**Group*day**	
RT (°C)	37.4 ± 0.1^{a}	37.5 ± 0.1^{a}	37.9 ± 0.1^{Bb}	37.5 ± 0.1^{Aa}	0.144	0.039	0.0109	37.0-38.5
HR (bpm)	34.0 ± 1.4^{A}	34.6 ± 1.4^{A}	45.2 ± 1.1^{B}	35.0 ± 1.1^{A}	0.002	0.002	<0.001	30-40
RR (bpm)	10.6 ± 0.9^{A}	10.6 ± 0.9^{A}	19.4 ± 0.7^{B}	11.8 ± 0.7^{A}	<0.001	0.004	0.004	8-12
CRT (sec)	2.0 ± 0.1^{A}	2.0 ± 0.2^{A}	3.5 ± 0.1^{B}	2.2 ± 0.1^{A}	<0.001	<0.001	<0.001	1-2

RT: rectal temperature; HR: heart rate; RR: respiratory rate; CRT: capillary refilling time
*(Reed at al., 2003)

4.3.2. Haematological and biochemical parameters

Haematological and biochemical parameters for CG and EG on day 1 and day 7 are shown in Table 3. CG haematological parameters were within the reference range (Reed et al., 2003) and there was no variation between day 1 and day 7. There was no significant interaction (group*day) for red blood cell count, haemoglobin, or hematocrit. There was no effect of transportation on total leukocyte counts, but there was significant variation related to transportation in leukocyte sub-populations. Specifically, EG horses demonstrated increased neutrophil counts, with lower lymphocytes and eosinophil counts at day 1, and a higher number of neutrophils and basophils at day 7, relative to CG horses.

A significant effect of the interaction (day*group) was observed for total protein (P=0.013), albumin (P=0.001) and globulins (P<0.001). At day 7 the mean total protein concentration observed in EG horses was significantly greater than that seen in these horses on unloading (day 1) or in CG horses (day 1 and 7). At day 1 EG horses showed a higher mean serum concentration of albumin than what was observed at this time in CG horses, or in the EG group at day 7. At day 7 EG horses showed a higher mean serum concentration of globulins than that was observed at both times in CG horses, or in the EG group at day 1.

A significant interaction (group*day) was observed for potassium (P=0.038), with EG horses demonstrating a significantly lower serum potassium concentration following transportation (day 1), relative to values obtained for this group on day 7 or values obtained from CG horses at either time. EG horses demonstrated a significant increase in AST activity (P=0.008) in samples obtained immediately after arrival (day 1) relative to results obtained on day 7 or from CG horses at either times. There were no significant effects of the interaction (group*day) on Cl (P=0.441), Na (P=0.969), CK (P=0.648), Ca (P=0.600) or fibrinogen (P=0.494).

Table 3. Haematology and serum biochemistry parameters in Control Group (non-transported horses) and Experimental Group (transported horses) at day 1 (soon after unloading for EG and at rest CG) and day 7 (7 days after the journey for EG and at rest for CG). Data are expressed as least square mean ± standard error (SE). Differing superscripts within rows indicate significant difference (A, B: P<0.01; a, b: P<0.05) (Tukey-Kramer test).

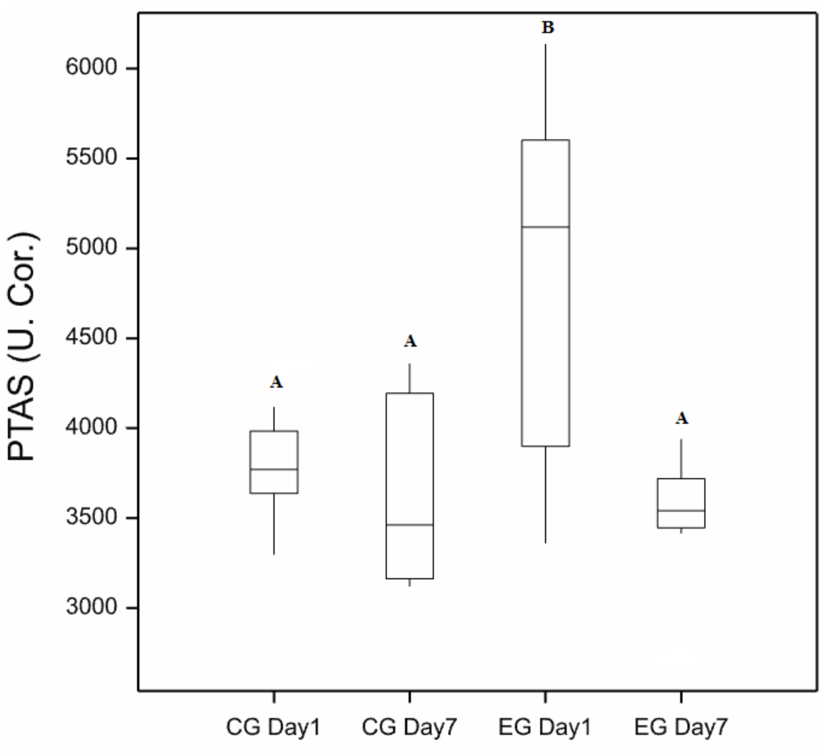
Parameters	Control Group (n=6)		Transported Group (n=10)		P values			Normal range*
	Day 1	Day 7	Day 1	Day 7	Group	Day	Group*day	
RBC (10^{12}/L)	6.9±0.3	7.4±0.3	7.6±0.2	7.6±0.2	0.137	0.225	0.163	6.5-12.5
Hb (g/L)	118.3±5.2	126.1±5.2	127.5±4.0	127.3±4.0	0.402	0.189	0.148	110-190
HCT (%)	32.5±1.3	34.4±1.3	34.2±1.0	34.2±1.0	0.625	0.160	0.155	32-52
WBC(10^9/L)	6.6±0.5	6.3±0.5	7.2±0.4	7.3±0.4	0.188	0.658	0.453	6.0-13.0
Neutrophils (10^9/L)	3.7±0.4^{Aa}	3.4±0.4^{A}	5.4±0.3^{B}	4.8±0.3^{Bb}	0.002	0.074	0.0483	2.4-6.9
Lymphocytes(10^9/L)	2.2±0.2^{A}	2.3±0.2^{A}	1.3±0.1^{B}	1.8±0.1^{A}	0.006	0.042	0.028	1.6-3.4
Monocytes (10^9/L)	0.3±0.0	0.3±0.0	0.3±0.0	0.4±0.0	0.496	0.246	0.178	0.0-0.7
Eosinophils (10^9/L)	0.3±0.0^{Aa}	0.3±0.0^{Aa}	0.0±0.00^{B}	0.1±0.0^{ACb}	0.008	0.005	0.015	0.0-0.9
Basophils (10^9/L)	0.01±0.0^{A}	0.01±0.0^{A}	0.01±0.0^{A}	0.03±0.0^{B}	0.300	0.007	0.027	0.0-0.3
PLT (10^9/L)	179.5±19.8	174.5±19.8	158.9±15.4	153.0±15.4	0.403	0.347	0.937	80.0-300.0
Fibrinogen (g/L)	2.7±0.3	2.5±0.3	3.4±0.2	2.9±0.2	0.111	0.182	0.494	2.0-4.0
Total protein (g/L)	62.0±1.6^{a}	62.0±1.6^{a}	65.8±1.3^{a}	67.9±1.3^{b}	0.026	0.166	0.013	60.0-76.0
Albumin (g/L)	35.0±1.0^{A}	35.3±1.0^{A}	37.1±0.8^{B}	32.7±0.8^{A}	0.821	0.004	0.001	29.0-38.0
Globulins (g/L)	27.0±1.5^{A}	26.6±1.5^{A}	26.6±1.2^{A}	35.2±1.2^{B}	0.017	0.000	<0.001	26.0-40.0
Cl (mmol/L)	99.8±0.8	101.5±0.8	100.6±0.6	103.2±0.6	0.146	0.002	0.444	99-110
K (mmol/L)	3.8±0.1^{a}	3.7±0.1^{a}	3.4±0.1^{b}	3.7±0.1^{a}	0.105	0.474	0.038	2.8-5.0
Na (mmol/L)	141.1±0.5	142.0±0.5	142.0±0.4	142.8±0.4	0.117	0.077	0.969	132.0-150.0
Ca (mmol/L)	3.0±0.0	3.0±0.0	3.0±0.0	3.0±0.0	0.684	0.121	0.600	2.78-3.32
CK (U/L)	176.2±16.2	193.7±16.2	175.1±12.5	205.5±12.5	0.737	0.108	0.648	< 400.0
AST (U/L)	227.1±27.4^{a}	246.8±27.4^{a}	322.8±21.2^{Bb}	283.7±21.5^{A}	0.068	0.325	0.008	< 400

RBC: Erythrocytes, Hb: hemogoblin, HCT: hematocrit, WBC: white blood cells; Cl: Chlorine, K: potassium, Na: sodium, CK: creatine kinase, Ca: total calcium, AST: aminotransferase. * Normal range of the laboratory that performed the analysis

4.3.3. Oxidative stress parameters

The effect of interaction (group*day) was not significant for ROMs (CG day 1: 167.1±13.9; CG day 7: 161.3±12.0; EG day1: 186.8±9.3; EG day 7: 171.4±9.3 U.Carr; P=0.558) or OSI (CG day 1: 4.2±0.5; CG day 7: 4.1±0.4; EG day 1: 4.0±0.3; EG day 7: 4.8±0.3; P=0.111). However there was a significant effect of the interaction (group*day) on PTAS, with an increased value registered at unloading (Figure 1).

Figure 1. PTAS in Control Group (CG) (non-transported) and Experimental Group (EG) (transported horses) at day 1 (soon after unloading for EG and at rest CG) and day 7 (7 days after the journey for EG and at rest for CG). (A, B: P<0.01).



4.3.4. Immunological parameters

The effect of interaction (group*day) was significant for lymphocyte proliferation in medium alone (P=0.038), PWM (P=0.014) and ConA (P=0.029) (Figure 2). In unstimulated cultures, lymphocyte proliferation was significantly increased in EG horses on day 7 in comparison to day 1. Following stimulation with either PWM or ConA, proliferation was significantly lower on day 1 in relation to day 7 and CG at either time point. IFNγ production was significantly increased in EG horses on day 1 relative to day 7 (Figure 3). In the CG, there were no significant differences between lymphocyte proliferation responses (Figure 2) or IFNγ (Figure 3).

Figure 2. Proliferation of lymphocytes in culture medium alone (1), ConA (2), PWM (3) in Control Group (CG) (non-transported) and Experimental Group (EG) (transported horses) at day 1 (soon after unloading for EG and at rest CG) and day 7 (7 days after the journey at rest for EG and at rest for CG). Different subscript shows statistical differences. (A, B: P<0.01; a, b: P<0.05).

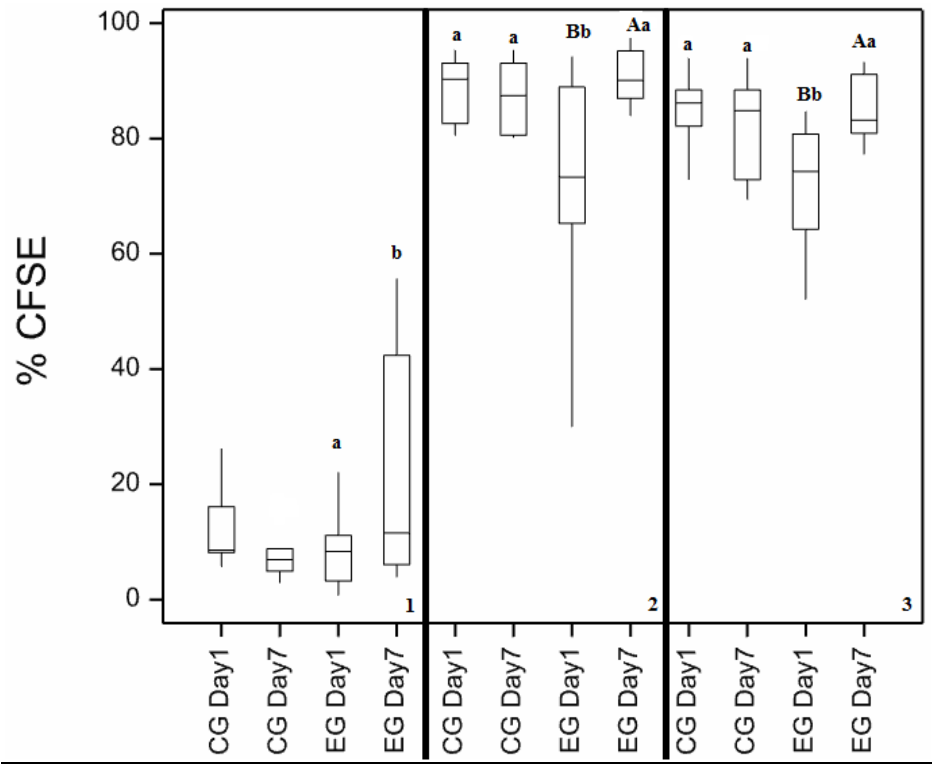
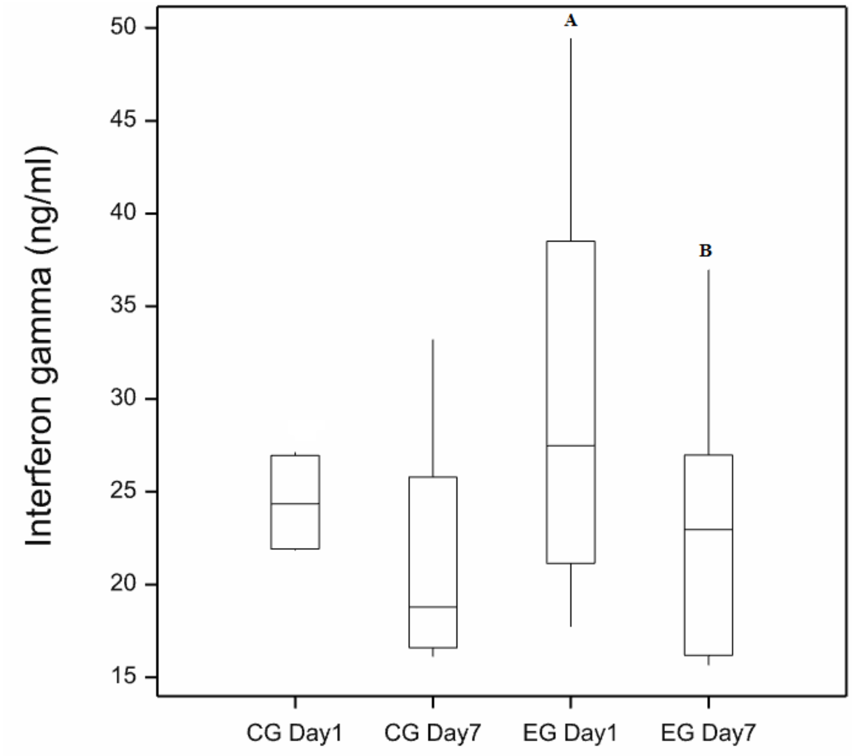


Figure 3. PWM-induced IFNγ response in Control Group (CG) (non-transported) and Experimental Group (EG) (transported horses) at day 1 (soon after unloading for EG and at rest CG) and day 7 (7 days after the journey for EG and at rest for CG). (A, B: P<0.01).



4.3.5. Cortisol

There was no significant interaction (group*day) for cortisol (CG day 1: 4.8±0.6; CG day 7: 4.2±0.6; EG day 1: 3.9±0.4; EG day 7: 4.7±0.4 µg/dl; P=0.0753).

4.3.6. Pearson correlations

Table 4 shows the results of significant Pearson correlations. Clinical variables (RT, HR, RR, CRT) were relatively strongly correlated (r from 0.5 to 0.73) and were also positively correlated with PTAS, neutrophil count and AST. Lymphocyte count and the proliferation of lymphocytes both in ConA and PWM were negatively correlated with the clinical variables, neutrophil count and AST.

Table 4. Pearson correlation (expressed as value of r) between the dependent variables which reported at least one significant correlation (P<0.05).

	RT	HR	RR	CRT	PTAS	N	L	TP	K	ALB	Glo	AST	IFNγ	Media	PWM	ConA
RT	*															
HR	0.503 ***	*														
RR	0.630 ***	0.733 ***	*													
CRT	0.622 ***	0.742 ***	0.767 ***	*												
PTAS	0.344 *	0.527 ***	0.679 ***	0.420 **	*											
N	0.213 ns	0.566 ***	0.542 ***	0.468 ***	0.277 ns	*										
L	-0.352 *	-0.370 *	-0.486 ***	-0.602 ***	-0.300 ns	-0.352 *	*									
TP	-0.039 ns	0.238 ns	0.142 ns	0.105 ns	0.047 ns	0.649 ***	-0.207 ns	*								
K	-0.243 ns	-0.316 ns	-0.440 **	-0.431 **	-0.460 ***	-0.269 ns	0.403 *	-0.232 ns	*							
Alb	0.235 ns	0.439 **	0.408 *	0.346 *	0.408 *	0.174 ns	-0.030 ns	0.124 ns	-0.440 **	*						
Glo	-0.171 ns	-0.041 ns	-0.109 ns	-0.287 ns	-0.199 ns	0.478 ***	-0.167 ns	0.819 ***	0.047 ns	-0.466 ***	*					
AST	0.564 ***	0.487 ***	0.669 ***	0.508 ***	0.615 ***	0.397 *	-0.333 ns	-0.036 ns	-0.287 ns	-0.021 ns	-0.019 ns	*				
IFNγ	0.217 ns	0.303 ns	0.284 ns	0.146 ns	0.373 *	0.109 ns	-0.034 ns	-0.489 **	-0.027 ns	-0.037 ns	-0.228 ns	0.404 *	*			
Medium	-0.417 *	-0.166 ns	-0.249 ns	-0.169 ns	-0.135 ns	0.077 ns	0.020 ns	0.223 ns	0.201 ns	-0.198 ns	0.313 ns	-0.262 ns	-0.155 ns	*		
PWM	-0.577 ***	-0.546 ***	-0.615 ***	-0.571 ***	-0.241 ns	-0.371 *	0.239 ns	-0.090 ns	0.411 *	-0.223 ns	0.048 ns	-0.442 *	-0.108 ns	0.4935 **	*	
ConA	-0.466 **	-0.524 **	-0.615 ***	-0.551 ***	-0.193 ns	-0.525 **	0.285 ns	-0.138 ns	0.449 **	-0.350 *	0.079 ns	-0.364 *	-0.042 ns	0.423 *	0.876 ***	*

* P<0.05; ** P<0.01; ***P<0.001

RT: rectal temperature; HR: heart rate; RR: respiratory rate; CRT: capillary refilling time; PTAS: plasma total antioxidant status; N: Neutrophils; L: Lymphocytes; TP: total proteins; K: potassium; Alb: albumin; Glo: globulins, IFNγ: PWM-induced IFNγ concentration; Medium: proliferation of lymphocytes in culture medium alone**;** ConA: proliferation of Lymphocytes in culture with ConA; PWM: proliferation of lymphocytes in culture with PWM.

4.4. Discussion

This multidisciplinary case-control study assessed immunological, clinical, haematological, inflammatory, and oxidative parameters in performance horses, immediately after transport and after a 7-day recovery period, and by comparison with a non-transported group of horses at the same time points. The long distance journey was associated with an acute phase response, characterized by abnormal clinical variables, neutrophilia, impaired lymphocyte responsiveness and increased PTAS and IFN**γ**. In particular, the decreased lymphocyte proliferative response at unloading supports the hypothesis that a horse's immunological capacity might be decreased after a long journey. A number of clinical examination findings (RT, HR, RR, CRT) were above the normal range, as has been previously reported (Padalino et al., 2012; Stull and Rodiek, 2000)(Chapter 1). As evident in Table 4, these parameters were positively correlated with each other, and negatively correlated with lymphocyte proliferation suggesting that the degree of insult to homeostatic mechanisms might influence the magnitude of clinical response and severity of immunological impairment. Although correlation is a weak measure of association, these preliminary findings emphasize the importance of clinical examination in assessing an individual horse's response to transportation. Thus, in agreement with the available literature, a clinical examination after arrival is recommended as a best practice to identify horses at increased risk for disease and to plan a recovery period (Leadon and Hodgson, 2014).

In this study, the decreased proliferative response to mitogen suggests that long distance transportation impairs the ability of lymphocytes to react to a stimulus (de Silva et al., 2010; Julia and Felippe, 2016). Lymphocyte proliferation is an essential feature of the adaptive immune response to antigenic stimulation as the host responds to an infectious challenge by clonal expansion of pathogen-specific cells (Felippe, 2016). ConA and PWM are plant-based mitogens that are commonly used to assess lymphoproliferative capacity (Crotty et al., 2004; Dwyer and Johnson, 1981; Kruisbeek et al., 2004). In many species, ConA is essentially a T cell mitogen and PWM stimulates proliferation of both T and B cells. While both mitogens induce a proliferative response in equine lymphocytes (Robbin et al., 2011; Sanada et al., 1992), the specific lymphocyte subtypes that respond are not known in this species. Therefore the findings relate to the overall lymphocyte response.

As was observed in the current study, impairment of mitogen induced lymphocyte proliferation has been reported in calves after a 4-hour journey (Murata et al., 1987), in steers after a 10-hour journey

(Blecha et al., 1984), in pigs transported to a fattening farm (Artursson et al., 1989), in horses transported for 38 hours (Oikawa and Jones, 2000), and only in the more stress susceptible horses after 24 hour journey (Stull et al., 2004). The observed reduction was transient, with complete restoration of the proliferative response observed following a 7-day recovery period. Interestingly and as previously observed by Oikawa and Jones (2000), lymphocytes from the transported horses demonstrated increased spontaneous proliferation on day 7 (Artursson et al., 1989; Oikawa and Jones, 2000). The increased mitogen-induced IFNγ response observed in the EG group on day 1 compared to day 7, was unexpected and might be related to the immunosuppressive effect of this cytokine. IFNγ can have a suppressive effect on lymphocyte proliferative responses via activation of other T cell subsets (Gajewski and Fitch, 1988; Sheng et al., 2008) or antigen presenting cells (Shimabukuro et al., 1992). As IFNγ is produced by both innate and adaptive immune cells, the cell type responsible for the observed increase warrants further investigation.

The changes in leucocyte counts observed in the current study are in line with previous studies. In horses that did not develop disease, transportation induced neutrophilia and lymphopenia without increasing the total leucocyte count (Oikawa et al., 2005; Stull et al., 2008). Such changes in leucocyte populations, and impaired leucocyte function, have been related to the transport-induced increase in cortisol (Hines, 2000; McGlone et al., 1993; Stull et al., 2004). However, no change in cortisol concentrations was found in the current study. This finding is in disagreement with the literature, as increased cortisol has been reported in many previous studies including after short journeys (Fazio et al., 2013a; Tateo et al., 2012), or transportation for 12-hours (Baucus et al., 1990b) or 24-hours (Stull and Rodiek, 2000; Stull et al., 2008; Stull et al., 2004). However limited data are available for transportation lasting several days. Schmidt et al. (2010) evaluated salivary cortisol in response to road transport over 1370 km and 2 day transport eight days later, and showed that cortisol peaked at mid-transport and then tended to decrease with each day of transport. The authors suggested that this response was likely due to a degree of adaptation. In our study, cortisol was not determined prior to or during the journey, so it is not possible to know whether the normal values observed on arrival represent an adaptive response (due to negative feedback inhibition after prolonged stress, or due to habituation of the animal to the stressor (Moberg and Mench, 2000)) or whether there was no observable increase in cortisol attributable to transportation in this study, as has been reported in some previous studies (Söder et al., 2012). In pigs transported for 24 and 48 hours, no differences in cortisol were found at unloading, and the authors commented that cortisol concentrations may return to baseline levels before the end of the journey if the transport is of long duration (Pineiro et al., 2007).

In contrast with previous studies (Stull and Rodiek, 2000; Tadich et al., 2015), no variations in RBC, hematocrit or haemoglobin were observed in the current study. Increased red cell mass following journeys of shorter duration has been attributed to transport-induced catecholamine release and/or dehydration (Stull and Rodiek, 2000; Tadich et al., 2015). At unloading, our horses were very quiet and less responsive, suggesting that the acute catecholamine response to transportation had waned, due to depletion of catecholamine or habituation to external stimuli for coping with the long journey (Alexander and Irvine, 1998; Moberg and Mench, 2000).

Changes on clinical examination and serum biochemistry parameters were expected based on previous studies. It is reported that transportation causes increased rectal temperature, heart rate and respiratory rate, decreased intestinal peristalsis (Oikawa et al., 2005), and variation in plasma concentrations of AST and potassium (Codazza et al., 1974). These changes have been interpreted as an inevitable response to transportation associated with catecholamine release, muscle work and thermoregulation (Leadon et al., 1991; Padalino et al., 2012). In EG horses, CRT, which has previously been suggested as the most reliable welfare indicator for adequate water intake or absence of prolonged thirst (Dalla Costa et al., 2014), was much higher at unloading than in CG. This suggests that animals suffered from thirst, thus watering on route every 4.5 hours might be recommended in Australia as already applied in Europe (Anon, 2014). The EG animals increased their body weight (BW) at day 7 by approximately 2%. Dehydration and weight loss have been considered as the most common consequences of transportation, with transport-related weight loss ranging from 0.10 to 0.63% BW per hour of journey and recovery time usually within three days of arrival (Marlin, 2004). As serum total proteins and albumin were still within a normal range, a severe degree of dehydration was not present (Reed et al., 2003); thus, the observed changes in CRT and body weight in this study might be considered as common and mild reactions to transit stress and limitations to water intake. The monitoring of CRT and assessment of body weight by tape following transportation are easy practices which all owners and agents could perform before and after journey, and are therefore recommended as readily implemented measures to assess the recovery of horses following transportation.

Our preliminary results on the effects of a long journey on the immune system are interesting because transport-related diseases usually become clinically evident in the week after the transport. In our study, transport-induced changes reversed by day 7 and no animals became unwell. Our data suggest that a rest period is fundamental for the immune system to recover and to react to the pathogens to which animals may be exposed during the journey (Raidal et al., 1997a) and in the new environment after arrival (Herholz et al., 2008). It is therefore better to avoid adding further stress such as a strenuous exercise (e.g. racing or competition), soon after unloading. The ideal rest duration after long hauling warrants future research, as interim data were not collected between day 1 and day 7 in the current study.

The reported changes in albumin, fibrinogen and globulins might be interpreted as a transport induced inflammatory response (Cray et al., 2009; Crisman et al., 2008). The higher value of albumin recorded at unloading might be associated with dehydration or haemoconcentration (Stull and Rodiek, 2000). The lower value registered at day 7 might be explained because homeostatic mechanisms have recovered post-transportation hydration indices, or because albumin is considered a negative acute phase response (APR) protein (Crisman et al., 2008). Globulin concentration was increased on day 7 in the EG group, consistent with increased positive APPs (Cray et al., 2009; Crisman et al., 2008). In our study, there was no significant variation in fibrinogen, but it is worth noting that the average value for EG horses at unloading was higher than 3.2 g/L, which was proposed by Leadon as an indicator of shipping fever (Leadon et al., 1991); in particular the three horses with abnormal pulmonary auscultation at unloading had a fibrinogen concentration of 4 g/L. Pre-transportation fibrinogen values were not determined in the current study. Further studies including serum protein electrophoresis and repeated determination of specific APPs might enhance the understanding of the link between transit stress and APPs in horses.

The reported changes in the oxidative/antioxidative parameters, PTAS and ROMs, should also be interpreted as components of the acute phase response inducted by transport stress (Onmaz et al., 2011; Wessely-Szponder et al., 2015). We did not find a significant increase in ROMs, as reported in transported ewes (Piccione et al., 2013), however, peaks in oxidative products may be observed one or two days after transportation (Nazifi et al., 2009; Piccione et al., 2013; Wessely-Szponder et al., 2015), suggesting serial sampling during recovery might be considered in future studies. The highest ROMs values were observed on day 1 in EG horses in the current study, with a simultaneous and marked increase of PTAS, which was almost double the normal range (Kusano et al., 2016). The latter could be interpreted as an antioxidant response to the increased production of free radicals for avoiding oxidative stress (Celi, 2011; Niedźwiedź et al., 2013). This extremely high value of PTAS could be related also to the marked increase in serum albumin concentrations on day 1 in EG horses. Albumin is indeed assessed within PTAS as the sulfhydryl (SH) groups of albumin are considered a significant element of extracellular antioxidant defense system (Celi, 2011). However, it has been estimated that proteins contribute only 10% of PTAS values, while uric acid, ascorbic acid and α-tocopherol contribute for 60, 5 and 5%, respectively (Benzie and Strain, 1996). Thus, our data are in agreement with the literature suggesting that transportation induces an increase in PTAS, likely due to mobilization of antioxidants (Niedźwiedź et al., 2013). It has been reported that animals under stress mobilize antioxidants to balance the increased production of free radicals (Sconberg et al., 1993), thereby avoiding oxidative damage to the cells (Ralston and Stives, 2012). Oxidative stress has been implicated in numerous disease processes in human and veterinary medicine and oxidative parameters have been proposed as biomarkers to identify animals at risk of diseases (Kirschvink et al., 2002; Kirschvink et al., 2008; Soffler, 2007). In particular, PTAS was proposed as useful indicator of stress in transported calves, since it may be more sensitive and reliable than the measure of single antioxidant parameters (Pregel et al., 2005). Consequently, monitoring of PTAS and ROMs pre and after journey might be recommended as best practices to identify animals at risk of oxidative stress, and consequently poor health and welfare outcomes. In further studies, it would be interesting to determine if depletion of antioxidants might be a cause of the development of oxidative stress and transport-related diseases.

Contrary to previous reports (Leadon, 2000; Marlin et al., 2011; Messori et al., 2016; Oikawa et al., 2005), no horse in the current study developed pyrexia or other clinical complications, demonstrating that transportation over long distances without adverse health complications is possible when management is consistent with the Australian code of animal transportation (http://www.animalwelfarestandards.net.au/land-transport/) and current best practice recommendations (Kohn, 2000)(Chapter 1). Since we did not have any sick horses, our results need to be confirmed in future studies comparing healthy and unhealthy horses after being transported for long distance, with all parameters measured before and after transportation.

Our results are preliminary and should be interpreted with caution because this study has a number of limitations. As in other studies (Pineiro et al., 2007), the lack of sampling prior to and during the journey means that the effects of transportation on measured variables can only be inferred. However, as animals are often not assessed prior to transport also in a ‘real-world’ setting, reliable welfare indicators were recently proposed only at unloading (Messori et al., 2016), and the current study was designed to allow comparison with a non-transported control population and to assess recovery following arrival. Determinations of other important APPs, such as C-reactive protein, haptoblobulin or serum amiloid A, or other stress hormones, such as catecholamine, were also not included in the current study. Assessing a greater number of horses would also be beneficial. Notwithstanding these limitations, this study has enhanced the understanding of the implications of long transportation for horse health and welfare using a multidisciplinary approach.

4.5. Conclusions

Overall, this multidisciplinary case-control study suggests that long distance transportation was associated with an acute phase response characterized by neutrophilia, hyperglobulinemia, increased IFNγ and PTAS and an impairment of the immune system evidenced by reduced lymphocyte responsiveness. Recovery was evident by 7 days after arrival, by which time most values were not different to non-transported control horses. Transport was also associated with clinical changes, including prolonged CRT and mild weight loss. Clinical examination, including assessing dehydration by CRT and body weight by tape, and the monitoring of redox balance are proposed as useful means to evaluate the effect of transport on horse health and welfare and to ensure optimal recovery of horses following transportation. Further studies are needed to investigate the duration of transport-induced immunosuppression and to ascertain whether this impairment of the immune system and antioxidant depletion after transportation may be linked with the development of transport-related diseases.

CHAPTER 5

Behavioural, clinical, haematological, oxidative and respiratory responses to 8 hour transportation in horses

5.1. Introduction

Respiratory disease is a common consequence of equine transportation (Leadon et al., 1989; Oikawa et al., 2005; Oikawa and Jones, 2000; Raidal et al., 1997a; Stull and Rodiek, 2002)(Chapter 3.4). The risk of transport associated respiratory disease in the horse may be influenced by pre-existing inflammation of the airways (Leadon, 1994; Leadon and Hodgson, 2014), journey duration (Austin et al., 1995)(Chapters 2, 3.4), head position (Raidal et al., 1995, 1996; Stull and Rodiek, 2002), poor air quality inside the truck (increased concentration of ammonia and carbon monoxide gas (Katayama Y, 1995; Stull, 1999), dust and bacteria (Leadon et al., 1991; Smith et al., 1996)), inability to cope with transport stress (Marlin, 2004; Oikawa et al., 2004), stress-induced immunosuppression(Hines, 2000; Stull et al., 2008; Stull et al., 2004)(Chapter 4), thermal stress *en route* (Purswell et al., 2010) and transport-related dehydration (Hobo et al., 1997; Raidal et al., 1996).Whether food offered during transport influences transport related disease risk is still uncertain. The presence of dusty and dry hay at the level of the nostrils during transportation was identified as a risk factor for respiratory disease (Hobo et al., 1997; Hotchkiss et al., 2007). However, it has been recently shown that horses travelling for 2 ½ hours with hay at the level of the carpus showed fewer neutrophils in their tracheal washes in comparison to horses which travelled without hay; the authors' explanation was that the hay motivated the horses to lower their head during the journey (Allano et al., 2016).

Transportation causes an increase in oxidative products in horses (Niedźwiedź et al., 2013; Onmaz et al., 2011; Wessely-Szponder et al., 2015). Oxidative stress has been associated with health problems in horses (Kirschvink et al., 2002; Kirschvink et al., 2008; Soffler, 2007), farm animals (Bordignon et al., 2014; Lykkesfeldt and Svendsen, 2007), and dogs (Mongillo et al., 2015).While oxidative stress has been identified as a possible contributor to the development of *Rhodococcus equi* pneumonia (Crowley et al., 2013), it may also be involved in the pathogenesis of equine transport pneumonia.

Equine transport pneumonia is not linked to a specific pathogen of note, rather a mixture of different bacterial species have been linked to transport related lung infections with varying involvement of the pleural space (Racklyeft et al., 2000). *Streptococcus* spp., in particular *Streptococcus equi* subsp. *zooepidemicus* and *Streptococcus equi* subsp. *equisimilis*, have frequently been isolated from pneumonic lesions in cases of transport pneumonia (Mair and Lane, 1989). However, other bacteria, including *Pasteurella caballi*, *Escherichia coli*, *Streptococcus suis*, *Bacillus* spp., *Staphylococcus* spp., *Enterococcus* spp. and *Clostridium* spp. have been associated with cases of equine transport pneumonia (Austin et al., 1995; Hayakawa et al., 1993; Johns et al., 2016; Oikawa and Jones, 2000). The possible reactivation or spread among travelling horses of equine respiratory viruses, in particular those in the *herpesviridae* family (EHV-1 and EHV-4) has been identified as a possible contributor to the development of equine transport pneumonia (Austin et al., 1995). However, it is not clear yet why after the same type of journey some horses develop transport pneumonia and others do not (Kohn, 2000).

It was hypothesised that the stress related behavioural and physiological responses to an 8 hours transportation event would be different among horses and would be associated with changes in redox balance, herpes virus shedding, and increased mucus and changes in bacterial flora of the lower respiratory tract. The aim of this current multidisciplinary study was therefore to document the effects of an 8 hour transportation event, devoid of water and feed, on behavioural, clinical, haematological, oxidative and respiratory parameters including microbiological flora, and explore possible associations between these factors.

5. 2. Material and Methods

The study was approved by the Charles Stuart University Animal Care and Ethics Committee (Project Number 14/037).

5.2.1. Animals

Twelve horses (7 gelding, 5 mares; 6 Standarbred and 6 Thoroughbred), aged from 3 to 8 years (mean 4.9 ± 1.9 years), with mean body condition score (BCS) of 2.2 ± 0.4 (Carroll and Huntington, 1988), were recruited into this study (Appendix 5, Table 1). All were well accustomed to handling, of good temperament (determined by reaction to human handling test (Olsson, 2010)), and had previous experience of transportation.

5.2.2. Experimental Protocol

The trial was conducted from the 24th of September to 28th of October 2014 at the Equine Centre of the School of Animal and Veterinary Sciences, Charles Sturt University, Wagga (NSW, Australia).

On day 1, all animals were clinically assessed, weighed, and dewormed by two research team members (BP and SR). Blood samples were collected for haematological and biochemical screening, with results within the normal range (data not shown).

The animals then had a two week acclimation period. During the first week, the horses were kept on pasture. During the second week, the horses were kept in single boxes (4x4 m), with wood shavings as bedding. The front door, back and side walls of the box were half grid, so the horses could see and interact with each other (Appendix 5, Figure 1). They were stabled for the rest of the experiment, spending at least one hour a day in a yard to meet their social and locomotory behavioural needs (Mills and Clarke, 2007).They were fed lucerne hay and oats twice a day (08.00;18.00 h), and had water *ad libitum*. The diet was calculated individually to meet maintenance requirements, assuring that the horses with lower BCS would recover some condition before the experiment (Waldron, 2012). During the acclimation period, all horses were handled by one of the researchers (BP) and underwent training for saliva and exhaled breath condensate (EBC) sampling procedures once daily using positive reinforcement.

The horses were transported in two identically managed transport events on different days, 48 hours apart (6 horses on each day) in a commercial truck without water and feed for the duration of travel. The horses travelled in the same 6-horse commercial truck (Freighter, Fuso, Mitsubishi, Japan), with a ventilation system of venturi vents and louvres (Appendix 5, Figure 2). The truck was driven by the same driver, who was licensed to drive heavy combination vehicles and was an experienced horse handler with many years' experience in commercial horse enterprises. The horses travelled in individual stalls (0.80 m width x 2.30 m length), in a sideways position, restrained by rubber cords (Appendix 5, Figure 3). The cord was attached to the low ring of the head collar, so animals were able to turn their head and lower their head below wither height, such that they could touch their carpus, but not the floor, with their nose.

The horses travelled for 8 hours around the Riverina district of NSW, starting from and arriving back at Charles Sturt University (Wagga, NSW, Australia)(Appendix 5, Figure 4). To comply with the occupational health and safety act (OHS, 2011)(OHS, 2011), a rest stop for the driver, from 12.35 to 13.00, was included. During the rest stop, the horses were not unloaded and the truck was parked in the shade.

Horse behaviour and environmental parameters were recorded when the horses were at rest in their stables (the day before the journey), and *en route*. Air samples were collected in the stable (the day before and one day after the journey) and in the truck at preloading and unloading on both journeys.

During the trial all experimental procedures were sequenced according to invasiveness and possible impact on animal welfare. Experimental procedures were conducted in the following order at each sampling point: clinical examination, venous blood collection, saliva sampling, EBC sampling, arterial blood collection, nasal swabbing, thoracic ultrasound and upper respiratory endoscopic examination and tracheal wash collection. This order was chosen to minimise the effects of the procedure used to restrain animals (e.g. rope restraint, application of twitch, sedation) during the different sampling collections on physiological parameters and serum cortisol concentration, which were used as stress indicators, and to also minimise horse discomfort. All sampling protocols are summarized in Table 1.

Table 1. Detailed experimental protocol. Same procedures were undertaken on each group (n=6) 48 hours apart.

	Day	1	2-8	9-15	16	17	18	19			20		21			22		23	24	25	26	33	35
	Hours							6.00		17.00	6.00	17.00	6.00			6.00		6.00	6.00			6.00	
Group 1	CA	✓	First habituation week	Second habituation week	✓			✓	Travel	✓	✓	✓	✓			✓		✓	✓				
	Saliva							✓		✓	✓	✓							✓				
	Ven. B	✓						✓		✓	✓	✓							✓			✓	
	Art. B							✓		✓		✓							✓				
	EBC							✓		✓	✓	✓							✓				
	Weight	✓						✓		✓		✓							✓				
	Nasal sw				✓					✓		✓							✓				
	Scop+TW				✓					✓		✓							✓				
	Thor. US				✓					✓		✓							✓				
	Hours												6.00		17.00	6.00	17.00	6.00	6.00	6.00	6.00		6.00
Group 2	CA	✓	First habituation week	Second habituation week									✓	Travel	✓	✓	✓	✓	✓	✓	✓		
	Saliva												✓		✓	✓	✓				✓		
	Ven. B	✓											✓		✓	✓	✓				✓		✓
	Art. B												✓		✓	✓	✓				✓		
	EBC												✓		✓		✓				✓		
	Weight	✓											✓		✓		✓				✓		
	Nasal sw						✓								✓		✓				✓		
	Scop+TW						✓								✓		✓				✓		
	Thor. US						✓								✓		✓				✓		

CA: Clinical assessment; Ven: Venous; Art: Arterial; EBC: Exhaled breath condensate; sw: Swabbing; Scop: Scoping; TW: Tracheal wash; Thor. US: Thoracic ultrasound.

5.2.3. Clinical Assessment (CA)

The clinical assessment was conducted on day 1, before the horses underwent any other procedures and on the morning before the journey prior to departure (preloading), at unloading, 12 hours after journey (AJ), 24 hours AJ, 2 days AJ, 3 days AJ, 4 days AJ and 5 days AJ. Clinical assessment was performed by an experienced equine veterinarian (BP). It consisted of the assessment and recording of the following parameters: demeanour, mucous membrane (colour, status), capillary refilling time (CRT), heart rate (HR), respiratory rate (RR), rectal temperature (RT) and pulmonary and gastrointestinal tract auscultation. Gastrointestinal tract motility was assessed by auscultation of gut sounds as described previously (Sundra et al., 2012)(Chapter 4). In addition, palpable lymph nodes, presence of nostril discharge and coughing were also recorded (Table 2). All clinical assessments were performed without knowledge of endoscopic or laboratory findings for each horse. All horses were weighed at preloading, unloading, 24 hours AJ and 5 days AJ using digital scales (Ibeef®,Farm, Weigh System, Ruddweigh™ 200, Tauranga, New Zealand).

Table 2. Clinical assessment sheet used to record the clinical data modified from Reed (2003).

Demeanour:_______ Temperature: ____________ Mucus Membrane: ________________ CRT:____

Heart Rate: _____________ Respiratory Rate: _____________ Gut sounds:
LD:___LV:___RD:___RV:___

Nostrils: Bilateral air movement **Discharge:** Unilateral Bilateral None

Nostril Discharge Type: Serous Mucus Mucopurulent Serosanguineous Mal-odorous

Percuss Sinus: Normal Abnormal ______________ **Facial Symmetry:** Yes No- explain____________

Palpate Lymph nodes: Sub Mandibular ☐ Thyroid ☐

- Lymphadenopathy: Not evident Min Moderate Severe
- Comments: ___________________________________

Auscultate:

- Trachea ☐ Normal Comments: ____
- Left lung field ☐ Normal Comments: ____
- Right lung field ☐ Normal Comments: ____

Cough during physical exam: Yes No

LD: left dorsal; LV: left ventral, RD: right dorsal, RV: right ventral

5.2.4. Environmental parameters and air quality

A weather tracker (Kestrel: 4000 Pocket Weather Tracker, Nielsen-Kellerman, USA) recorded the following climate parameters: temperature (T, °C), humidity (H, %), wind speed (W, miles per hour (mph)). An additional parameter, heat-index (HI, °C), was also calculated. The HI combines air temperature and relative humidity in order to determine the animal perceived equivalent temperature (Atiyah et al., 1973). A gas detector (Dräger X-am 5000, Serial Number: ARFB0560, Dräger Safety AG & Co., Lübeck, Germany) monitored the concentration of the following gases: oxygen (O_2, vol%), ammonia (NH_3, ppm), hydrogen sulphide (H_2S, ppm), carbon monoxide (CO, ppm), methane (CH_4, % of lower explosive limit (LEL)). The weather tracker and the gas detector were placed inside a stable box at horse head height the day before the journey (day 18 and 20, respectively), and were placed in the last bay of the truck at horse head height during the two trips (day 19 and 21, respectively). The weather tracker recorded parameters every 5 minutes during the 8 hours. The gas detector continuously monitored the parameters and was programmed to signal the researcher/driver if limit values were reached. Air samples were collected using an air sampler (Coriolis µ, Bertin Instrument, CNIM Group, Montigny-le-Bretonneux, France) to assess the concentration of bacteria in the air inside the truck and the box stable. Air samples were collected in the middle of the truck before loading and immediately after unloading, with the ramp opened and without animals. Air samples were collected in the middle of the box stable the day before and the day after the journey. Air was aspirated at 300L/min for three minutes into a sterile sampling cone into 0.1% peptone water. The samples were put in a sterile container and kept on ice until analysis within 4 hours of collection. The air samples underwent the same bacteriological evaluation as described for the tracheal wash samples below (section 5.2.10).

5.2.5. Behavioural parameters

The horses were recorded in their stables by a security camera system (TechView DVR Kit, Model Number QV-3034) placed in each box. The day before transport, behaviour was recorded in the boxes for 1 hour in the middle of the day (from 12.00 to 13.00), while the horses were devoid of water and feed. A camera was placed in each single box of the trailer pointing toward the horse's head and the horse's behaviour was recorded during the journey, including the rest stop. A behaviour sampling ethogram (Table 3) was developed based on those used previously to study behaviour during transportation (Padalino et al., 2012; Siniscalchi et al., 2014; Tateo et al., 2012).Videos were analysed by an experienced ethologist (BP) based on 25 minute time window during the first 25 minutes of each hour of the journey, during the rest stop, and the last 25 minutes of recording whilst stabled. This time window was selected to permit direct comparison with behaviours observed during the rest stop. Behavioural assessment was performed independently to clinical and laboratory assessment for each horse.

Table 3. Behaviour sampling ethogram used. Each animal was observed for 25 minutes for recording the occurrence and duration of the following choose behavioural events and states.

Behaviour	Description
Behavioural events (Expressed as frequency) (n/25 min)	
Behavioural events related to stress (Expressed as frequency) (n/25 min)	
Evasive behaviour/Pulling back	The horse tries to escape from the truck, he pulls back trying to break the rope
Explorative behaviour/Sniffing	The horse sniffs around, it sniffs some area of the truck/box
Licking/chewing	Opening of mouth with extension and retraction of tongue, lip smacking without tongue extension, lateral jaw movements involving partial opening of the lips (McGreevy, 2012)
Licking the truck/wall	The horse licks part of the truck/ box (wall, stall rails)
Nose outside	The horse puts his nose between the bars of the truck/box
Pawing	One front leg is lifted from the ground slightly, then extended quickly in a forward direction, followed by a movement backward, dragging the toe against the floor in a digging motion (McDonnell and Haviland, 1995)
Touching rubber tie cord	The horse touches the rubber cord which he is tied with
Turning the head	The horse turns his head and neck to the right or to the left appearing to look at his flank
Total stress related behaviours	Sum of the behavioural events related to stress
Behavioural Events related to balance (Expressed as frequency) (n/25 min)	
Backward movements	The horse steps backward
Forward movement	The horse steps forward
Lateral movements	The horse steps sideways
Leaning on stall rails	The horse gently leans laterally against one of the two stall rails
Loss of balance/dashing on the partitions	The horse losses his balance and crashes/bumps on one stall rails
Total balance related behaviours	Sum of the behavioural events related to balance
Other behavioural Events (Expressed as frequency) (n/25 min)	
Head tossing/shaking	The horse shakes its head suddenly, violently and frequently
Interaction with neighbours	The horse interacts with one of his neighbour trough the stall rails, they sniff each other
Biting neighbour	The horse bites the neighbour

Look outside	The horse looks outside, head and ears pointing outside
Lowering the head	The horse lows his head below the withers height
Shaking head	The horse shakes its head
Yawning	An involuntary sequence consisting of mouth opening, deep inspiration, brief apnea, and slow expiration (Waring, 2003)
Total behavioural events	Sum of all behavioural events
Behavioural States (Expressed in duration) (s/25 min)	
Head down duration	The horse stays his head at the level or below the withers height

5.2.6. Venous blood, saliva and EBC collection

Venous blood, saliva and EBC were collected by the same veterinarian (BP) at preloading, unloading, 12 hours AJ, 24 hours AJ, and 5 days AJ. Blood samples were collected from the external jugular vein into three Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ), one with heparin, one with EDTA, and one without an anticoagulant. After collection, blood samples were kept at 4^{o}C and analysed within 4 hours of collection in the haematology laboratory of Charles Sturt University. Saliva was sampled using a cotton swab on a surgical forceps, which was maintained in the horse's mouth for 30–40 s, over and under the tongue. The cotton swab was then squeezed into a 20 mL syringe, and the saliva was transferred to an Eppendorf tube (Tarantola et al., 2016). After collection, saliva samples were kept at 4^{o}C and analysed within 4 hours of collection. EBC collection was performed using a modified ERA mask (ERA Equine Mask, Biomedtech, Moorabbin, Victoria, Australia) connected to the Turbo 14 DECCS breath condensation collection system (Medivac, Parma, Italy). The mask used to collect respiratory condensate was placed over the muzzle and held by two operators positioned on either side of the horse (Appendix 5, Figure 5). The horse was approached quietly and gently the mask was placed over the muzzle. Collection time was approximately 20 minutes per horse. At least one mL of EBC was collected, transferred into a cryo tube and stored for six months at -80°C until analysis.

5.2.6.1. Haematological parameters

Blood cell counts were performed using the Abbott cell counter analyser (Cell Dyn 3700; Abbott, Chicago, IL), and the following parameters were recorded: red blood cells (RBC, x10^{12}/L), haemoglobin (Hb, g/L), haematocrit (Hct, %), white blood cells (WBC, x10^{9}/L), neutrophils (N, x10^{9}/L), lymphocytes (L, x10^{9}/L), monocytes (M, x10^{9}/L), eosinophils (E, x10^{9}/L) and basophils (B, x10^{9}/L).

Serum was obtained by centrifugation (1600 x g for 15 min). Serum biochemistry parameters (total serum proteins (TP, g/L), albumin (Alb, g/L), globulins (Glob, g/L), creatine kinase (CK, U/L), aminotransferase (AST, U/L) were assessed with Thermo Scientific reagents and the Konelab 20XT photometer (Thermo Fisher Scientific, Finland, EU) with interferential filters. Fibrinogen concentration was calculated by heat precipitation (Millar et al., 1971). Serum Amyloid A was determined by commercial kit (Equinostic, DN, Equibnostic, Copenhagen, Denmark) following manufacturer's instruction using a dedicated spectrophotometer (EVA, Equinostic, Copenhagen, Denmark) (Hillström et al., 2010). Serum Amyloid A was expressed in mg/L. Cortisol concentration was assessed in serum samples by radioimmune Assay (RIA) using the ImmunChem™Cortisol 125 kit following manufacturer's instructions (MP Biomedicals, LLC, Orangenburb, NY, USA). Cortisol was expressed in µg/dl.

5.2.6.2 Oxidative stress parameters

Heparinised plasma was obtained by centrifugation (1600 x g for 15 min). Reactive oxygen metabolites (ROMs) and plasma total antioxidant status (PTAS) were determined in plasma as described previously (section 4.2.5). The degree of oxidative stress (oxidative stress index, OSI) was estimated using the ratio of ROMs/PTAS multiplied by 100 (Crowley et al., 2013). Advanced oxidation protein product (AOPP) concentrations were measured according to the methods of Witko-sarsat et al. (Witko-Sarsat et al., 1998). Ceruloplasmin concentration was determined according to the method described by Sunderman and Nomoto (1970) except that absorbance was read at 510 nm with a plate reader (FLUROstar Optima, BGM Labtech, Melbourne, Australia). Plasma Glutathione (GSH) concentration was measured by an enzymatic recycling method (chemicals from Sigma Aldrich Pty Ltd, Castle Hill, NSW, Australia) adapted for a microtitre plate reader (FLUROstar Optima, BMG Labtech, Melbourne, Australia) as previously described (Baker et al., 1990).

Saliva total antioxidant status (STAS) level was determined by commercial kits (SAT test) following manufacturer's instructions (H&D srl, Parma, Italy) using a dedicated photometer (Free Radical Analytical System 4 Evolvo, H&D srl, Parma, Italy). STAS levels were also expressed in U.Cor.

Hydrogen peroxide (H_2O_2) concentration in EBC was measured using the d-ROMs exhalation kit (Diacron International, Grosseto, Italy) by means of a dedicated spectrophotometer, the FREE system (Diacron International, Grosseto, Italy). H_2O_2 levels in EBC were expressed in µEq/L.

5.2.7. Arterial blood collection

Arterial blood samples were collected anaerobically from the supraorbital artery by an experienced equine veterinarian at preloading, unloading and 24 hours AJ. They were collected with a 3 mL heparinized syringe. Samples were kept at 4ºC and analysed within 2 hours of collection using a blood gas analyser (Gem Premier 3500; Diamond Diagnostic, Holliston, MA, USA) and the following parameters were recorded: pH, PCO_2 (mmHg), PO_2 (mmHg), sodium (Na, mmol/L), potassium (K, mmol/L), total calcium (Ca, mmol/L), glucose (mmol/L), lactate (mmol/L), HCO_3 (mmol/L).

5.2.8. Thoracic Ultrasound

Ultrasound was performed by an experienced veterinarian (SR) using an ultrasound machine (LOGIQ E9, GE Healthcare, ESAOTE) with a linear probe (ML 6-15 MHz). The area of the chest wall (4th, 5th and 6th intercostal space) to be examined was cleaned and then wet with alcohol. Videos and images were saved for later interpretation. The ultrasound images were deidentified and evaluated by one member of the research team (SR) blinded to the time of examination and clinical findings for each horse. The images were interpreted based on the method suggested by Morresey (2014). Briefly, the presence of any ventral pleural fluid accumulation at each site imaged was recorded as the distance between parietal and visceral pleura measured by electronic calipers. Decreased pulmonary aeration was determined subjectively (present or absent) by loss of reverberation artefact. Pleural thickening was identified subjectively as mild (+) or moderate (++) by the presence of comet tail artefact, apparent irregularity or hyperechogenicity of the pleural interface.

5.2.9. Respiratory endoscopy and Tracheal wash (TW) collection

Respiratory endoscopy and tracheal wash (TW) collection were performed as previously described (Hodgson and Hodgson, 2007) two days prior to the journey, at unloading, 24 hours AJ and 5 days AJ. All horses were sedated using xylazine and acepromazine (0.4 mg/kg BW and 0.02 mg/kg BW, respectively) prior to the procedure. Briefly, a 1.35m, 9.2mm outer diameter, fibre-optic video endoscope (Olympus, Gif Q165, Ausvet Endoscopy, Melbourne, Australia) connected to a video processor (Olympus CV-160 Exerm, Ausvet Endoscopy, Melbourne, Australia) was passed through the right or left ventral nasal meatus and advanced into the trachea to a point just proximal to the thoracic inlet. TW was obtained trans-endoscopically using a guarded polyethylene catheter (EMAC800, Mila International, Ausvet Endoscopy, Melbourne, Australia) passed through the endoscope instrument channel immediately following instillation of 30 mL of sterile, room temperature 0.9% saline. Endoscopic observations and gross characteristics of TW samples were recorded using a clinical sheet (Table 4). The endoscope and the instrument channel were disinfected with alcohol between each horse examination.

Table 4. Clinical sheet recording observations during respiratory endoscopy.

Pharyngeal Lymphoid Hyperplasia (Burrell, 1985):

0	No follicles
1	Few small white lymphoid follicles over dorsal pharyngeal wall – inactive & shrunken
2	Mainly small white follicles, occasional larger pink follicles over dorsal pharyngeal wall, extends laterally to level of guttural pouch ostia
3	Pink and white follicles covering entire dorsal and lateral pharyngeal walls, can involve dorsal surface of soft palate
4	Larger pink, oedematous follicles covering all visible mucosa of pharynx and sometimes including polyps

Tracheal Inflammation(Gerber et al., 2004): 0 No **1** Mild **2** Moderate **3** Extreme

Tracheal Mucus (Gerber et al., 2004):

Quantity

0	None present – clean
1	Little – multiple small blobs
2	Moderate – larger blobs
3	Marked – Confluent stream-forming
4	Large – pool forming
5	Extreme – profuse amounts

TW Turbidity:

0	Transparent
1	Clouded
2	Smoked Glass
3	Opaque

TW Colour:

1	Clear
2	White
3	Yellow
4	Blood

5.2.9.1. Tracheal wash (TW) cytology

Two smears were prepared from each TW sample using a cytospin centrifuge (Shandon, Cytospin III, GMI, Minnesota, USA). For viscid samples a TW smear was obtained by squash preparation. The smears were stained using Wright-Giemsa stain (Hema-tek stain pack, Sigma-Aldrich Pty Ltd, Castle Hill, NSW, Australia). Differential inflammatory cells counts were performed, using a modification of the method suggested by Tee et al. (2012). Briefly, four differential cell counts (DCC) of 100 cells (neutrophils, lymphocytes, macrophages, eosinophils and mast cells) were made using high-power (100X) light microscopy. Differential cell counts of all samples were performed (by BP) blinded to the horse identification. They are expressed as percentage.

5.2.10. Bacterial parameters

5.2.10.1. Quantitative and phenotypic bacteriology

One millilitre of air sample and TW was 10-fold serially diluted with sterile saline and 100 µL of each diluent inoculated onto 5% sheep blood agar (SBA) plates and incubated under aerobic conditions at 37^{o}C and 6% CO_2 (HF212 UV, Heal Force, Burwood, Victoria, Australia). Bacterial growth was assessed after 24 hours and 48 hours and colony-forming unit (CFU) were totally counted at 48h and expressed in log_{10} CFU/ml. Bacterial colonies were further identified based on colony morphology (shape, size, elevation, surface, edge, colour, opacity, consistency, odour), presence of haemolysis (nil, complete or incomplete haemolysis), Gram staining and basic bacterial morphology record (i.e. Gram +/-, coccus, cocci in chain, cocci in clusters, coccobacillus, bacillus, filamentous rod, or pleomorphism). Anaerobic culture of isolates were attempted and catalase tests (for Gram positive bacteria) or oxidase tests (for Gram negative bacteria) (Markey et al., 2013) were performed and recorded on isolated subcultures of predominate isolate types recovered from TW.

5.2.10.2 Genotypic bacteriology

Bacterial DNA extraction was performed using 1 mL of TW with the addition of 0.5 mL of dithiothreitol (150µg/mL) and a commercial kit designed to extract DNA from human sputum (Sputum DNA isolation kit, NorgenBiotek Corp., Thorold, ON, Canada). Similarly Bacterial DNA was extracted from air samples using a commercial kit designed to extract DNA from soil (Mobio Soil Powerlyzer kit; Qiagen, Carlsband, Ca, USA). Both purified DNA samples were sent to the Australian Genome Research Facility (AGRF) were microbial diversity profiling was conducted on these samples. Essential this process involved PCR amplification and sequencing of the resulting partial 16s rRNA (V1-V3 region) amplicons (i.e. products). Briefly, PCR amplicons were generated using the primers and conditions outlined in Table 5 and 6, using AmpliTaq Gold 360 mastermix (Life Technologies, Australia) for the primary PCR.

Table 5. PCR procedure used before sequencing

Target	Cycle	Initial	Disassociate	Anneal	Extension	Finish
16S rRNA: V1- V3	29	95 °C for 7 min	94°C for 45s	50°C for 60S	72°C for 60S	72°C for 7 min

Table 6. V1-V3 primers and relevant region of the 16srRNA gene.

V1-V3 region	27F – 519R
V1 Forward Primer(27F)	AGAGTTTGATCMTGGCTCAG
V3: Reverse Primer(519R)	GWATTACCGCGGCKGCTG

A secondary PCR to index the amplicons was performed with TaKaRaTaq DNA Polymerase (Clontech). The resulting amplicons were measured by fluorometry (Invitrogen Picogreen) and normalised. The equimolar pool was then measured by qPCR (KAPA) followed by sequencing on the Illumina MiSeq (San Diego, CA, USA) with 2 x 300 base pairs paired-end chemistry. Paired-ends reads were assembled by aligning the forward and reverse reads using PEAR (version 0.9.5) (Zhang et al., 2014). Primers were trimmed using Seqtk (version 1.0) (https://github.com/lh3/seqtk). Trimmed sequences were processed using Quantitative Insights into Microbial Ecology (QIIME 1.8) (Caporaso et al., 2010) USEARCH (version 8.0.1623) (Edgar, 2010; Edgar et al., 2011) and UPARSE (Edgar et al., 2011) software. Sequences were clustered followed by chimera filtered using "rdp_gold" database as the reference. To obtain the number of reads in each operational taxonomic unit (OTU), reads were mapped back to OTUs with a minimum identity of 97% using Qiime taxonomy and Greengenes database (version 13_8, Aug 2013) (DeSantis et al., 2006).

5.2.11. Viral parameters

All analysis were conducted at the Centre for Equine Infectious Diseases of the University of Melbourne.

5.2.11.1. Detection of EHV-1 and -4 specific antibodies

Blood samples were collected by jugular venepuncture for antibody testing at preloading and two weeks after the journey. Serum was obtained by centrifugation and sent for analysis. Levels of EHV-1 and -4 antibodies present BJ and 2 weeks AJ were determined using an ELISA (Crabb et al., 1995). Antibody levels were considered to be negative if the mean optical density of the 3 test wells was less than 0.1, questionable if between 0.1 and 0.2, and positive if more than 0.2, as has been validated in field studies (Crabb et al., 1995; Gilkerson et al., 1999). Seroconversion in the horses was defined as an increase in optical density of more than 0.2 above the previous absorbance reading (Gilkerson et al., 1999).

5.2.11.2. Nasal swab collection

Nasal swabs were collected for virus identification and isolation two days before the journey (baseline measurement), at unloading, 24 hours AJ and 5 days AJ. A sterile rayon dry swab (Sarstedt, Adelaide, Australia) was passed into the ventral nasal concha and the swab was moved at least 5 times up and down close to the nasal septum. This was repeated for both nostrils. Each swab was placed in saline solution and stored in ice. Within 4 hours of collection the swabs were transferred to -80°C storage until analysis.

5.2.11.3. Detection of viral shedding by quantitative PCR (qPCR)

Nucleic acid was extracted from 200 μL of nasal swab sample using an automated nucleic acid extraction system (QIAxtractor, QIAGEN) and a QIAxtractor Vx kit according to the manufacturer's protocol. Quantitative PCR assays specific for EHV-1, -2, -4 and -5 were performed using the GOTaq mastermix system (Promega) according to manufacturer's instructions using GOTaq Flexi Buffer, 1.5 mM $MgCl_2$, 200 µM dNTP, 0.8 µM Syto 9 (Life Technologies), 1 U GOtaq Flexi DNA polymerase and 170 nM of each of the forward and reverse primers. Primers used for detection of each of the viruses are shown in Table 1. The final 25 µL reaction volume included 2 μL of the extracted DNA template. DNA extracted from cell cultures infected with EHV-1 (strain EHV1.438/77(Studdert and Blackney, 1979)), EHV-2 (strain EHV2.86/67 (Studdert et al., 1970)), EHV-4 (strain EHV4.405/76 (Studdert and Blackney, 1979)) and EHV-5 (strain EHV5.2-141 (Turner et al., 1970)) were used as positive controls. Dilutions of nucleic acid extracted from these reference virus cultures were included on each plate along with nuclease free water as a negative control. The qPCR was performed using the Stratagene Mx3000P instrument. Thermocycling conditions were at 94°C for 15 minutes, then 40 cycles of 94°C for 15 sec, 60°C annealing for 30 sec and 72°C extension for 30 sec. The melting curve analysis of each amplicon was analysed after one cycle of 95°C for 1 min, 55°C for 30 sec and 95°C for 30 sec. The fluorescence threshold value used was the default value set by the analysis software. Samples were considered positive based on the linear range of detection of positive controls and the melting temperature of the amplicon. Using these factors, the positive cycle threshold cut off (C_t) value for the EHV-1, -4, -2 and -5 specific assays were determined to be 35.01, 34.07, 35.76 and 37.5 respectively.

5.2.11.4. Virus isolation

Virus isolation through cell culture was attempted on all nasal swab samples. Briefly, the nasal swab infused saline sample (500 µL) was diluted with 1mL of maintenance media (Dulbecco's minimal essential medium (DMEM), 1% v/v foetal bovine serum (FBS), 10mM4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 50 µg/mL gentamicin, 5 µg/mL amphoterin B, and filtered through a 0.45µm syringe filter. Semi-confluent monolayers of rabbit kidney (RK13), Vero, and equine foetal kidney (EFK) monolayers in 12-well tissue culture plates were inoculated with 200µl of filtrate and incubated for up to 10 days at 37°C, 5% v/v CO_2 in air. Cultures were considered positive based on the presence of cytopathic effect and viruses were identified by qPCR as described above.

5.2.12. Statistical analysis

All data were explored initially using raw and summary descriptive statistics Statulator[beta] (http://statulator.com/descriptive.html). Normal distribution of all quantitative data was checked using the Anderson-Darling test, with abnormal data being transformed accordingly. SAS (SAS, version 9, 1999) was used to analyse data requiring ANOVA, mixed linear model, and Spearman correlation. Gen Stat®Version 14 (VSNi International) was used for χ^2, and linear regression. R-3.3.1 (R Core Team) was used to analyse data required ordinal logistic analysis.

For all statistical analyses, a P value of <0.05 was used to indicate significance.

5.2.12.1. Environmental and air quality parameters inside the truck and the boxes

The environmental data were analysed using ANOVA to determine the effect of the situation (box 1, box 2, trip 1, trip 2) on the following variables: temperature (T), humidity (H), heat index (HI) and wind speed (W). A least significant difference (LSD) test was used to perform multiple comparisons in all models. Results are presented as least square mean ± standard error (SE).

5.2.12.2. Behavioural parameters

All behavioural data (frequency and duration) were analysed by mixed linear model using PROC mixed procedure with random factors utilised to account for multiple records per animal. Firstly, to identify variation in behavioural parameters over the 8 hour journey duration, models were developed using the hour (from 1st to 8th) as a fixed factor, with horse and trip (trip 1, trip 2) as random factors. A separate model was developed with each of the behavioural parameters as the outcome. Secondly, to identify behavioural differences during travelling, during the rest stop and when inside the box, models were developed using the situation (travelling, rest-stop, box) as a fixed factor, with horse and trip as random factors. A separate model was developed with each of the behavioural parameters as the outcome. A LSD test was used to perform multiple comparisons in all models. Results are presented as least square mean ± standard error (SE).

5.2.12.3. Clinical, haematological, oxidative and blood-gas parameters

Based on the clinical examination, particularly lung auscultation, at unloading the horses were split into two groups: abnormal lung sounds (A) and normal (B). To explore whether the journey, the group and their interaction affected these parameters, all data were analysed by mixed linear model using PROC mixed procedure using the time (preloading, unloading, AJ), the group (A, B) and their interaction as fixed factor. In the model, horse and trip were used as random factors, to account for multiple observations. A separate model was developed with each of the parameters as the outcome. LSD was used as a *post-hoc* test. Results are presented as least square mean ± SE.

5.2.12.4. Respiratory parameters

5.2.14.4.1. Thoracic Ultrasound

The effect of the time (preloading, unloading, 24 hours AJ, 5 days AJ) and group (A, B) on the quantity of fluid was evaluated by mixed linear model using PROC mixed procedure, using horse and trip as random factors. χ^2 tests were conducted to determine the association between time (preloading, unloading, 24 hours AJ, 5 days AJ) and group (A, B) and loss of aeration and pleura changes.

5.2.12.4.2. Scoping and TW score

The results of the observations made during endoscopy and TW scores were analysed by ordinal logistic regression analysis with the time (preloading (T1), unloading (T2), 24 hours AJ (T3), 5 days AJ (T4)), the group (A, B) and their interaction as fixed factors. The horse and trip were used as random factors. The null hypothesis was that the distribution of the scores was the same. The data were expressed as proportion of the distribution of the score.

5.2.12.4.3. TW cytology and bacterial concentration

TW data (differential cells count and concentrations of bacteria in $\log_{10}$ CFU/ml) were analysed by mixed linear model using time (preloading, unloading, 24 hours AJ and 5 days AJ), group (A, B) and their interaction as fixed factors. Horse and trip were used as random factors. A separate model was developed with each of the TW parameters as the outcome. LSD was used as a *post-hoc* test. Results are presented as least square mean ± SE.

5.2.12.4.4. Statistical evaluation of microbial diversity in TW

Statistical analysis was undertaken using the programming language R (R Core Team), specifically the *phyloseq* (McMurdie and Holmes, 2013) and *edgeR* (Robinson et al., 2010) packages available through Bioconductor (Huber et al., 2015). The biom file, the OTU table, the taxonomic assignments and associated sample data were imported into R to create a phyloseq object. OTUs for which the variance across all samples was very low were filtered out. In order to test for bacterial diversity changes the Shannon diversity index was calculated and compared using Welch two sample t-test between the different times (preloading, unloading, 24 hours AJ, 5 days AJ) and between groups (A, B). Exact tests for differences in the means between two times (preloading, unloading, 24 hours AJ, 5 days AJ) and the two groups of negative-binomially distributed counts were computed. Data was normalized using the RLE scaling factor method and dispersions estimated. The counts were extracted and ranked by p-value.

The percentage of bacteria reported in the OTU table were also classified into 5 groups: *Pasteurellacae*, *Streptococcacae*, *Enterobacteriacae*, anaerobes (based on the genera of strict anaerobes associated with equine respiratory disease: *Fusobacterium* spp, *Clostridium* spp, *Peptostreptococcus* spp, *Bacteroides* spp, *Porphorymonas* spp, *Prevotella* spp)(McGorum, 2007), saprophytes (based on the most common environmental organisms: Plactomycetes, Gammatimondetes, Cyanobacteria, Chloroflexi, Acidobacteria, Verrucromicrobia)(Markey et al., 2013). Those bacteria unable to be classified into one of these 5 groups were placed in a group designated 'others'. Those data were analysed by mixed linear model using time (preloading, unloading, 24 hours AJ and 5 days AJ), group (A, B) and their interaction as fixed factors. Horse and trip were used as random factors. A separate model was developed with each of the grouped bacteria percentage within floral population as the outcome. LSD was used as a *post-hoc* test. Results are presented as least square mean ± SE.

5.2.14.5 Viral shedding, transport and clinical status analysis

To explore whether the journey affected viral shedding, the qPCR Ct values for EHV-2 and EHV-5 were analysed by mixed linear model using PROC mixed procedure using the time (preloading, unloading, 24 hours AJ and 5 days AJ), as fixed factor and horse and trip as random factors. Association between the positivity pre and post journey to EHV-2 and EHV-5 and the clinical status (group A: abnormal auscultation; group B: normal) were explored using χ^2 test.

5.2.14.6 Associations between behavioural, clinical, haematological, oxidative and respiratory parameters

Pearson correlations were performed to identify possible associations between behavioural, clinical, haematological, oxidative and respiratory parameters. All behaviours measured during the journey (while travelling and during the rest stop) were used. HR, RR, and T, haematological, and oxidative parameters recorded at unloading were used. The delta between preloading and unloading of the scoping scores and the TW bacterial concentration (log_{10} CFU/ml) was used to account for differing preloading status between horses. Associations with a significant Pearson correlation ($P<0.05$) were further investigated using linear regression analysis. Data were expressed as r and R^2.

5.3. Results

5.3.1. Clinical parameters

All horses had clinical parameters within the normal range (Reed et al., 2003) before travelling. Table 7 shows the results of the clinical examination of the horses immediately after unloading. Briefly, they were quiet and less responsive than before transport. Their heart rates, respiratory rates and CRT were higher than normal (Reed et al., 2003).

Ten of twelve horses had decreased borborygmi in one or more quadrants on abdominal auscultation. Coarse airway sounds were audible during thoracic auscultation for six horses (i.e. H1, H2, H3, H4, H11 and H12, these six horses define group A), and three of these horses coughed during the examination. Coughing and coarse airway sounds persisted in these horses until 5 days AJ. None of the horses showed pyrexia AJ.

One horse (H10) was injured during transportation, sustaining a full thickness laceration to the caudal aspect of the hock. The horse was treated with procaine penicillin, phenylbutazone and butorphanol for 5 days. Raw data from this horse has been retained but results from this individual were excluded from statistical analysis due to potential confounding effects of the injury or treatment on parameters of interest.

The effect of time was significant on the clinical parameters, while group and the time*group interaction were not significant ($P>0.05$). Comparison of heart rate (HR), respiratory rate (RR) and rectal temperature (RT) between the different times is shown in Table 8. Significant differences were observed between unloading and the other time points. HR, RR and RT peaked at unloading and returned to normal within 12 hours. All horses lost body weight (BW) due to transportation. A significant decrease in BW was found at unloading and 24 hours AJ in comparison with preloading and 5 days AJ (Preloading: mean= 444.0, unloading: 431.2, 24 hours AJ: 437.4, 5 days AJ: 442.9 Kg; SE: 15.8 Kg; $P<0.001$).

Table 7. Results of the clinical exam of the horses at unloading. Clinical assessment was conducted according to the methodology described by Reed et al. (2003). Gastrointestinal tract motility was assessed by auscultation of all four quadrants and scored as described previously (0 = no intestinal sounds, 1 = decreased borborygmi, 2 = normal borborygmi)(Sundra et al., 2012).

	H1	**H2**	**H3**	**H4**	**H5**	**H6**	**H7**	**H8**	**H9**	**H10**	**H11**	**H12**	**Normal value**
RT (°C)	38.0	38.2	38.2	38.5	38.1	38.1	37.4	37.7	37.9	38.3	37.6	38.1	37.0-38.5
HR(bpm)	40	46	42	44	40	36	36	44	40	76	52	52	30-40
RR (bpm)	12	24	12	16	12	16	16	28	20	28	40	12	8-12
Loss in BW (%/h)	0.31	0.26	0.34	0.56	0.40	0.25	0.08	0.29	0.72	0.30	0.22	0.32	0.09-0.63*
Lung Sound	Abn	Abn	Abn	Abn	Nor	Nor	Nor	Nor	Nor	Nor	Abn	Abn	
Left dorsal flank	1	0	0	0	1	1	1	2	2	1	0	1	2
Left ventral flank	2	2	2	2	1	1	2	2	2	1	1	1	2
Right dorsal flank	1	0	1	1	1	1	1	2	2	1	0	1	2
Right ventral flank	2	1	2	1	1	1	2	2	2	1	2	1	2
GIT total score	6	3	5	4	4	4	6	8	8	4	3	4	7/8
Membrane colour	pink	pink	pink	pink	pink	pink	pink	pink	pink	pink	pink	pink	pink
Membrane status	dry	dry	dry	dry	dry	dry	dry	dry	dry	dry	dry	dry	wet
CRT (sec)	3	3	3	2.5	3	3	3	3	3	3	3	3	1-2
Demeanour	quiet	quiet	quiet	quiet	quiet	quiet	quiet	quiet	quiet	agitated	quiet	quiet	alert
Coughing	+	+	+	-	-	-	-	-	-	-	-	-	
Lymph nodes	Nor	Nor	Nor	Nor	Nor	Nor	Nor	Nor	Nor	Nor	Nor	Nor	

H: horse; RT: rectal temperature; HR: heart rate; RR: respiratory rate; CRT: capillary refilling time; GIT: gastro intestinal tract; Nor: Normal; Abn: Abnormal; +: positive; -: Negative.
*(Marlin, 2004)

Table 8. Effect of transportation on heart rate (HR), respiratory rate (RR) and rectal temperature (RT) at preloading, unloading, 12 and 24 hours after journey (AJ), and at 5 days AJ. Data are expressed as square mean and standard error (SE). P value of the time of examination.

Parameter	Preloading	Unloading	12h AJ	24h AJ	5d AJ	SE	P value
HR (Beat/min)	34.7^{A}	43.3^{B}	36.4^{A}	36.4^{A}	34.7^{A}	1.1	<0.0001
RR (breath/min)	13.2^{a}	18.9^{Bb}	13.5^{a}	12.1^{A}	11.1^{A}	1.6	0.0045
RT (˚C)	37.5^{A}	38.0^{B}	37.5^{A}	37.5^{A}	37.3^{A}	0.1	<0.0001

Means with different superscript differ significantly (A, B: P<0.001; a, b: P<0.05)

5.3.2. Environmental parameters and air quality inside the truck and the boxes

The internal truck temperature ranged from 10°C to 20°C during travelling and from 10°C to 27°C in the boxes, with the highest values recorded during the afternoon (Appendix 5, Fig 6). Humidity ranged from 21% to 82% inside the boxes and from 28% to 85% during travelling; it was higher in the morning and decreased in the afternoon (Appendix 5, Fig 7). The heat index ranged from 10°C to 25°C inside the boxes and from 10°C to 20°C during travelling; it increased progressively with the duration of travelling (Figure 1). The wind speed ranged from 0 mph to 3.2 mph inside the truck, 0 mph was recorded at the truck stops and consequently no air movement or any type of ventilation was present during the rest stop. No wind was registered inside the box (Figure 2). The effect of the situation (Box 1, Box 2, Trip 1, Trip 2) was significant on all the environmental parameters, except for the wind speed (Table 9).

Figure 1. Heat index (°C) inside the truck (Trip 1, Trip 2) and in the stable (Box 1 and 2).

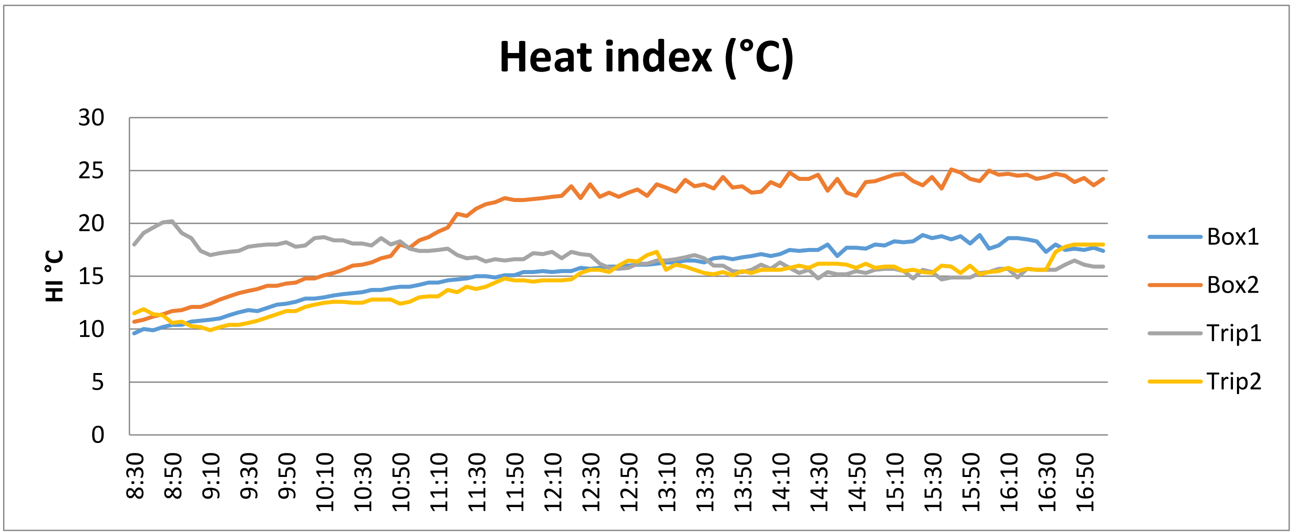


Figure 2. Changes of wind speed (mph) inside the truck (Trip 1, Trip 2). The wind speed in the boxes (Box 1 and 2) was 0 mph.

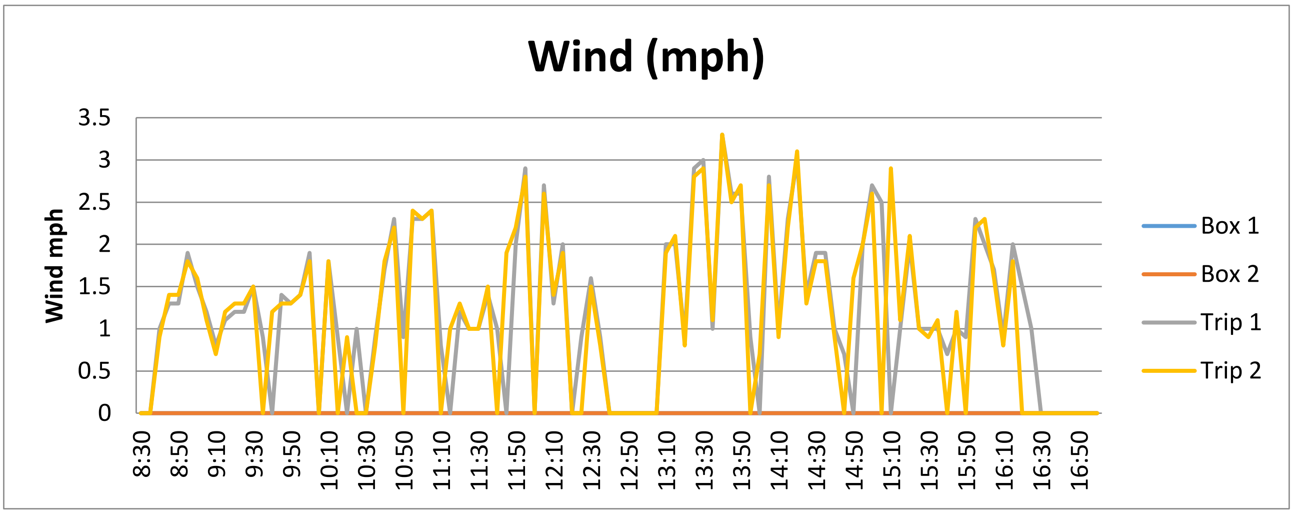


Table 9. Difference in the environmental parameters monitored inside the box and the truck. Data are expressed as square mean and standard error (SE).

Parameter	**Box 1**	**Box 2**	**Trip 1**	**Trip 2**	**SE**	**P**
Temperature (°C)	15.3^{Aa}	22.2^{B}	18.1^{C}	16.1^{Ab}	0.3	<0.001
Humidity (%)	49.8^{A}	39.2^{B}	56.3^{C}	46.4^{A}	1.4	<0.001
Heat index (°C)	15.3^{Aa}	20.6^{B}	16.7^{C}	14.4^{Ab}	0.3	<0.001
Wind speed (mph)	0.0^{A}	0.0^{A}	1.2^{B}	1.1^{B}	0.1	<0.001

Means with different superscript differ significantly (A, B, C: $P<0.001$; a, b: $P<0.05$)

Air composition did not vary by day (Trip 1, Trip 2), location (Box 1, Box 2), or between boxes and the truck. In all cases, oxygen was 20.9 vol%, and ammonia, hydrogen sulphide, carbon monoxide and methane were not detected (0 ppm or 0 %LEL), suggesting that pollutant gases did not accumulate in the transport environment. The bacterial concentrations in the truck were 3.1 $log_{10}CFU/m^3$ at preloading and 3.2 $log_{10}CFU/m^3$ unloading in trip 1; they were instead 6.2 $log_{10}CFU/m^3$ at preloading and 4.0 $log_{10}CFU/m^3$at unloading in trip 2. The bacterial load inside the stable box were 5.9 $log_{10}CFU/m^3$ pre journey and 6.1 $log_{10}CFU/m^3$AJ (Figure 3).

From the microbial diversity profiling data, the populations of bacteria in the air samples collected in the box stable were similar before and after the journey. Table 10 show the percentage of the more predominant bacterial families detected. *Enterobacteriaceae* were not detected.

Table 10. Percentage of the predominant bacterial family of the total genome in the air samples collected in the stables and in the truck.

Bacterial Family (% of the total genome)	Box BJ	Box AJ	Trip1 preloading	Trip1 unloading	Trip2 preloading	Trip2 unloading
Staphylococcaceae	41.7	41.4	11.1	11.0	32.2	25.3
Corynebacteriaceaea	20.5	23.6	5.1	8.0	33.0	18.9
Yaniellaceae	14.1	14.9	3.7	4.4	17.5	13.7
Aerococcaceae	7.2	7.7	7.6	3.3	4.7	6.6

5.3.3. Behavioural parameters

H3 showed a locomotory stereotypy (head tossing) after the second hour of trip. H2 which was on the right side of H3 started showing the same stereotypy in the last two hours of trip. H3 was very agitated during the rest stop (i.e. high frequency of pawing, evasive behaviours) and he recorded the highest frequency of movements during the journey. H11 and H9 recorded the highest frequency of biting neighbours. During the seventh hour of the journey H11 showed many aggressive behaviours toward H10, which started kicking as a response and got injured.

5.3.3.1. Effect of the journey hour (from 1st to 8th)

Table 11 shows the average values of the studied behavioural parameters in each hour of the journey and the significant differences between them. During the first hour of the journey the frequency of stress related behaviours, including licking, nose outside, touching rubber tie cord, peaked (221 in 25 min; one every 6 s). The frequency of these behaviours then decreased progressively. In the first hour of the journey horses lowered their head less frequently and lost their balance more frequently in comparison to the rest of the journey. The total frequency of balance related behaviours decreased until the 5th hour of the trip after which it began to increase.

The effect of the journey hour was significant for the total head down duration. The shortest duration in a head down position was registered during the first hour of the journey, this increased as the journey progressed and reached a peak during the eighth hour of journey.

Table 11. Effect of the journey hour (from the first to the eighth) on frequency of the measured behavioural events (n/25 min) and total head down duration (s/25 min). Data are expressed as square mean and standard error (SE). P value of the journey hour.

Parameter	**1st h**	**2nd h**	**3rd h**	**4th h**	**5th h**	**6th h**	**7th h**	**8th h**	**SE**	**P**
Backward movements	1.6	1.8	1.2	1.6	1.4	1.1	0.9	0.7	0.8	0.773
Biting neighbours	2.6	3.2	1.4	1.3	2.4	0.9	6.1	2.0	1.9	0.451
Evasive behaviour/Pulling back	2.7	2.2	1.0	1.2	0.3	1.4	0.9	0.6	1.0	0.054
Explorative behaviour/Sniffing	34.9	27.4	25.5	22.9	26.7	17.6	25.6	21.9	12.4	0.228
Forward movements	1.8	1.03	0.5	0.9	0.8	0.7	0.4	0.6	0.6	0.113
Head tossing/shaking	13.6	43.4	46.0	69.9	53.6	77.9	71.7	42.8	49.1	0.428
Interaction with neighbours	43.7	35.9	32.3	31.1	35.4	35.4	32.4	28.9	6.7	0.361
Lateral movements	16.1	18.4	14.0	15.3	16.3	15.9	16.5	22.3	3.9	0.343
Leaning on stall rails	34.0^{ac}	23.4^{ab}	26.3^{ab}	31.5	22.7^{b}	30.0^{ab}	30.1	40.1^{c}	5.5	0.023
Licking/chewing	42.9^{A}	22.6^{Ba}	17.5^{B}	12.3^{B}	15.5^{B}	10.1^{Bb}	12.3^{Bb}	10.7^{Bb}	7.4	<.0001
Licking the truck	7.0	3.5	2.4	2.8	2.7	2.4	2.3	1.2	1.4	0.067
Look outside	38.8	35.0	34.7	38.6	35.1	34.1	38.0	33.4	5.9	0.933
Loss of balance/ dashing on the partitions	8.8^{Aa}	5.6^{b}	2.3^{BC}	3.6^{BC}	4.2^{BC}	4.7^{BC}	5.5^{b}	6.5^{AC}	2.1	0.004
Lowering the head	12.1^{A}	39.7^{B}	43.1^{Ba}	44.6^{Ba}	31.4^{Bb}	38.4^{B}	42.3^{Ba}	49.3^{Ba}	5.0	<.0001
Nose outside	41.8^{A}	24.9^{Ba}	10.2^{BCb}	15.5^{BC}	9.9^{BCb}	9.4^{BCb}	6.2^{C}	2.7^{C}	6.7	<.0001
Pawing	5.5	1.9	1.3	3.4	0.8	1.2	1.8	1.1	1.4	0.284
Shaking head	7.0	6.4	3.5	6.4	3.1	3.4	5.7	3.5	2.9	0.779
Touching rubber tie cord	35.5^{Aa}	30.0^{ab}	28.3	21.2^{bc}	21.2^{bc}	20.8^{bc}	18.7^{B}	16.9^{Bc}	5.2	0.032
Turning the head	12.8	8.4	8.4	5.4	5.0	1.9	4.5	5.9	3.1	0.089
Yawning	1.3	0.7	1.0	0.8	0.1	0.2	1.0	0.1	0.6	0.672
Total movement	362.3	333.1	298.6	328.2	286.6	305.1	320.7	289.9	71.2	0.216
Total stress-behaviours	221.8^{A}	155.7^{Ba}	129.1^{B}	123.3^{B}	117.2^{B}	98.7^{B}	110.0^{B}	94.4^{Bb}	26.8	<.0001
Total balance related behaviour	62.2^{ac}	49.9^{A}	44.1^{Ab}	52.7^{ab}	45.2^{Ab}	52.2^{ab}	53.3^{ab}	70.8^{Bc}	6.8	0.006
Total head down duration	55.1^{A}	245.4^{B}	318.9^{BC}	268.3^{BCa}	249.7^{B}	363.9^{BC}	265.9^{Ba}	429.5^{Cb}	81.6	<.0001

Means with different superscript differ significantly (A, B, C: $P<0.001$; a, b: $P<0.05$)

5.3.3.2. Effect of the situation (travelling, rest-stop, stable box)

Table 12 shows the average values of the studied behavioural parameters during the journey, whilst the vehicle was stopped (rest stop) and whilst horses were inside the stable box, and the significant differences between them. Backward and forward movements were more frequent inside the box compared to during the journey or during the rest stop. Lateral movements were more frequent during the journey. Whist travelling the horses performed more sniffing of partitions and walls, looking outside, interaction with neighbours compared to the other two situations. They pawed, yawned and licked the wall more during the rest stop than in the other two situations. Horses during transport showed the highest frequency of stress related behaviours and balance related behaviours compared with when the vehicle was at rest (i.e. rest stop) (P=0.044 and P<0.001, respectively) or when the horses were in the stable box (P<0.001 and P=0.004). During the rest stop, the horses demonstrated more stressed-related behaviours (P=0.011), but moved less (P=0.016) in comparison with being into their stable boxes.

The effect of the situation was significant on the total head down duration. Whilst travelling it was approximatively two and four time shorter in comparison with the rest stop and the stable box, respectively.

Table 12. Effect of the situation (journey, rest stop, stable box) on frequency of the measured behavioural events (n/25 min) and total head down duration (s/25 min). All data are expressed as square means± standard error (SE).

Parameter	**Journey**	**Rest stop**	**Stable box**	**P**
Backward movements	1.3 ± 0.7^{Aa}	0.3 ± 0.8^{Ab}	3.1 ± 0.8^{B}	<.0001
Biting neighbours	2.4±1.02	0.3±1.8	0.0±1.77	0.167
Evasive behaviour/Pulling back	1.3±0.8	0.5±0.9	0.4±0.9	0.163
Explorative behaviour/Sniffing	25.4 ± 10.1^{A}	12.9 ± 10.9^{Ba}	0.9 ± 10.9^{Bb}	<.0001
Forward movements	0.9 ± 1.2^{A}	0.6 ± 1.9^{A}	21.2 ± 1.9^{B}	<.0001
Head tossing/shaking	52.4±39.1	30.3±44.7	0.1±44.7	0.089
Interaction with neighbours	34.4 ± 4.6^{A}	20.7 ± 6.1^{B}	3.4 ± 6.1^{C}	<.0001
Lateral movements	16.8 ± 2.7^{A}	8.1 ± 3.6^{B}	8.2 ± 3.7^{B}	0.0003
Leaning on stall rails	29.8 ± 3.4^{A}	4.2 ± 5.0^{B}	na	<.0001
Licking/chewing	18.1 ± 5.6^{A}	12.8±7.0	4.1 ± 7.0^{B}	0.0118
Licking the truck/wall of the box	3.1 ± 0.9^{a}	3.8 ± 1.5^{a}	0.1 ± 1.4^{b}	0.0493
Look outside	35.9 ± 4.0^{A}	19.8 ± 5.3^{B}	11.8 ± 5.3^{B}	<.0001
Loss of balance/dashing on the partitions	5.1 ± 1.5^{A}	0.2 ± 1.8^{B}	na	<.0001
Lowering the head	37.6 ± 3.4^{Aa}	31.8 ± 5.4^{b}	16.4 ± 5.4^{B}	0.0001

Nose outside	15.1±4.8^{A}	6.6±6.7	0.0±6.7^{B}	0.0097
Pawing	2.1±0.9^{a}	6.5±2.0^{b}	0.1±2.0^{a}	0.0481
Shaking head	4.9±1.8	3.3±2.6	1.5±2.6	0.2748
Touching rubber tie cord	24.1±3.1	18.1±4.9	na	0.1814
Turning the head	6.5±1.7	2.3±2.8	3.8±2.8	0.1658
Yawning	0.6±0.3^{A}	4.0±0.7^{B}	0.1±0.7^{A}	<.0001
Total movements	316.3±58.1^{A}	185.3±62.5^{B}	73.3±62.5^{C}	<.0001
Total stress-behaviours	95.6±25.5^{Aa}	63.3±25.4^{b}	9.0±25.4^{Bc}	<.0001
Total balance related behaviour	53.9±4.4^{A}	13.3±6.8^{Ba}	32.8±6.8^{Bb}	<.0001
Total head down duration	273.9±63.5^{A}	644.9±80.9^{B}	1204.7±80.9^{C}	<.0001

Means with different superscript differ significantly (A, B, C P<0.001; a,b P<0.05)

5.3.4. Haematological parameters

There was a significant effect of time on red blood cell (RBC), haemoglobin (Hb), haematocrit (Hct), and neutrophil (N), lymphocyte (L), monocyte (M), and eosinophil (E) counts, and total serum proteins (TP), albumin (Alb), globulins (Glob), creatine kinase (CK), fibrinogen and cortisol concentrations (Table 13). The effects of group and the time*group interaction were not significant for all haematological parameters (P>0.05). RBC, Hb and Hct were lower at unloading than at 24 hours AJ. The concentration of N peaked at unloading, whist L, M and E registered their minimal value at that point. The concentration of TP and Alb increased from preloading to unloading, remained elevated until 24 hours AJ, and restored to normal by 5 days AJ. Globulins increased progressively from preloading to 12 hours AJ, and started decreasing to restore to normal by 5 days AJ. CK increased from preloading to unloading, stayed elevated until 24 hours AJ and returned to normal by 5 days AJ. At unloading, two horses in group A had a fibrinogenaemia with fibrinogen concentrations (8 g/L) higher than normal , and the average fibrinogen concentration of group A was higher than B: 4.6 g/L *vs* 2.8 g/L. Fibrinogen concentrations was higher at 5 days AJ in comparison with all the other times. The highest level of cortisol was registered at unloading, the minimum level at 24 hours AJ.

Table 13. Effect of the time (Preloading, Unloading, 12h AJ, 24h AJ, 5d AJ) on the haematological parameters. All data are expressed as square means± standard error (SE).

Parameter	Preloading	Un loading	12h AJ	24h AJ	5 d AJ	SE	P value	Normal Laboratory range
RBC ($x10^{12}$/L)	7.6^{a}	7.2^{A}	8.0	8.5^{Bb}	7.9	0.2	0.0006	6.5-12.5
Hb (g/L)	116.3^{A}	113.6^{A}	121.5	130.8^{B}	121.6	3.7	0.0009	110-190
Hct (%),	33.4^{a}	32.2^{A}	35.1	37.4^{Bb}	35.1	1.0	0.0010	32-52
WBC ($x10^{9}$/L)	7.4	8.1	7.6	8.0	6.8	0.5	0.0768	5.5-12.5
N ($x10^{9}$/L)	4.2^{A}	6.5^{B}	4.3^{A}	4.4^{A}	3.4^{A}	0.4	<.0001	2.5-8.0
L ($x10^{9}$/L)	2.7^{A}	1.4^{B}	2.9^{A}	3.2^{A}	3.2^{A}	0.3	<.0001	1.5-5.5
M ($x10^{9}$/L)	0.22	0.07^{a}	0.24^{b}	0.24^{b}	0.15	0.04	0.0149	0.0-0.9
E ($x10^{9}$/L)	0.23^{A}	0.01^{B}	0.05^{B}	0.12	0.10	0.03	0.0003	0.0-0.8
B ($x10^{9}$/L)	0.04	0.00	0.02	0.06	0.02	0.02	0.2310	0.0-0.3
TP (g/L)	65.1^{A}	69.6^{B}	69.4^{B}	68.3^{B}	64.3^{A}	1.2	<.0001	58-76
Alb (g/L)	28.2^{A}	30.3^{B}	29.9^{B}	29.7^{B}	28.5^{A}	0.7	<.0001	28-38
Glob (g/L)	36.9^{ACa}	39.3^{ABb}	39.5^{B}	38.5^{AB}	35.8^{C}	1.5	<.0001	26-40
Alb/Glob	0.8	0.8	0.8	0.8	0.8	0.1	0.3751	0.8-1.9
CK (U/L)	248.8^{a}	291.4^{b}	294.5^{b}	292.5^{b}	268.5^{ab}	9.9	0.0072	50-400
AST (U/L)	271.0	272.5	287.8	270.2	245.2	29.8	0.8725	150-400
Fibrinogen (g/L)	3.8^{a}	3.8^{a}	3.5^{a}	3.7^{a}	5.4^{b}	0.4	0.0087	1.0-4.0
SAA(mg/L)	1.6	2.8	2.2	2.9	2.8	0.9	0.7537	< 7
Cortisol (µg/dl)	4.4^{A}	6.6^{B}	5.1^{A}	2.9^{Cc}	4.2^{Aa}	0.3	<0.0001	n.a.

AJ: after transport; RBC: red blood cells; Hb: haemoglobin; Hct: hematocrit; WBC: white blood cells N: neutrophils; L: lymphocytes; M: monocytes; E: eosinophils; B: basophils; TP: total proteins; Alb: albumin; Glob: globulins; Alb/Glob: albumin globulins ratio; CK: creatine kinase; AST: aminotransferase; SAA: Serum Amyloid A.
Means with different subscript differ significantly (A, B, C P<0.001; a, b, c P>0.05)

5.3.4. Oxidative stress parameters

The effect of time was significant for ROMs, PTAS, AOPP, ceruloplasmin (CP), and STAS (Table 14). The effects of group and the time*group interaction were not significant for any of the oxidative parameters (P>0.05). ROMs increased from preloading to unloading, peaking at unloading, and they progressively decreased, registering the minimum value at 5 days AJ. PTAS and STAS had a similar trend, increasing from preloading to unloading and then slowly decreasing; PTAS reached its minimum value at 5 days AJ. Transportation caused an increase in

AOPP and CP at unloading and at 24 hours AJ; these values returned to normal 5 days AJ. There was no effect of time on H_2O_2 levels detected in EBC.

Table 14. Effect of the time (Preloading, Unloading, 12 hours AJ, 24 hours AJ, 5 AJ) on the oxidative parameters. All data are expressed as square means± standard error (SE).

Parameter	**Preloading**	**Unloading**	**12h AJ**	**24hAJ**	**5 d AJ**	**SE**	**P value**
ROMs (U.Carr)	167.9	180.7^{Aa}	155.18^{b}	166.3	146.1^{B}	18.9	0.0001
PTAS (U.Corr)	2474.3	2732.9^{Aa}	2615.2^{ab}	2422.4^{b}	2294.4^{Bc}	69.9	0.0007
OSI	6.7	6.6	5.9	6.9	6.4	0.7	0.1078
AOPP (µmol/L)	16.1^{Aa}	19.6^{Bb}	18.0	19.2^{bc}	16.7^{ac}	1.07	0.0017
Ceruloplasmin (g/L)	0.38^{AC}	0.50^{Bb}	0.39^{a}	0.44^{BC}	0.32^{A}	0.02	0.0001
GSH (µM/ml)	9.3	9.5	10.2	10.2	10.8	0.7	0.5401
STAS (UCorr)	969.7	1519.1^{a}	1438.4	852.8^{b}	926.3^{b}	155.9	0.0047
EBC H_2O_2 (µEq/L)	161.0	111.30	143.8	113.35	161.8	18.4	0.0889

ROMs: Reactive oxygen metabolites; PTAS: plasma total antioxidant status; OSI: Oxidative stress index; AOPP: Advanced oxidation protein product; GSH: plasma glutathione; STAS: saliva total antioxidant status; H_2O_2 in EBC: hydrogen peroxide in the exhaled breath condensate. Means with different subscript differ significantly (A, B, C P<0.001; a, b P<0.05).

5.3.6. Blood-gas parameters

Table 15 shows the effect of transportation on blood gas parameters. Transportation was associated with small, transient, but significant, decreases in Ca++, K+ and HCO3-. Glucose and lactate increased after transportation and remained elevated the day AJ. There was no effect on arterial blood gas pressures, pH and Na+. The effects of group and time*group interaction were not significant for any of the blood-gas parameters.

Table 15. Changes of the examined blood gas parameters at preloading, unloading and 24 hours AJ. Data are expressed as square mean and standard error (SE). A, B: P< 0.01: a, b: P<0.05.

Parameter	Preloading	Unloading	24 h AJ	SE	P value
pH	7.43	7.42	7.45	0.01	0.054
PCO_2 (mmHg)	47.93	45.93	46.39	1.34	0.479
pO_2 (mmHg)	117.00	113.00	106.73	5.06	0.371
Na+ (mmol/L)	133.20	134.20	133.11	0.65	0.211
K+ (mmol/L)	3.94^{Aa}	3.48^{B}	4.23^{Ab}	0.08	<.0001
Ca++ (mmol/L)	1.51^{Aa}	1.42^{B}	1.58^{Ab}	0.02	<.0001
glucose (mmol/L)	5.24^{A}	6.71^{B}	6.10^{C}	0.30	<.0001
lactate (mmol/L)	0.51^{Aa}	0.77^{b}	0.81^{B}	0.09	0.002
HCO3-(mmol/L)	31.68^{a}	29.51^{b}	31.25^{a}	0.51	0.012

5.3.7. Respiratory parameters

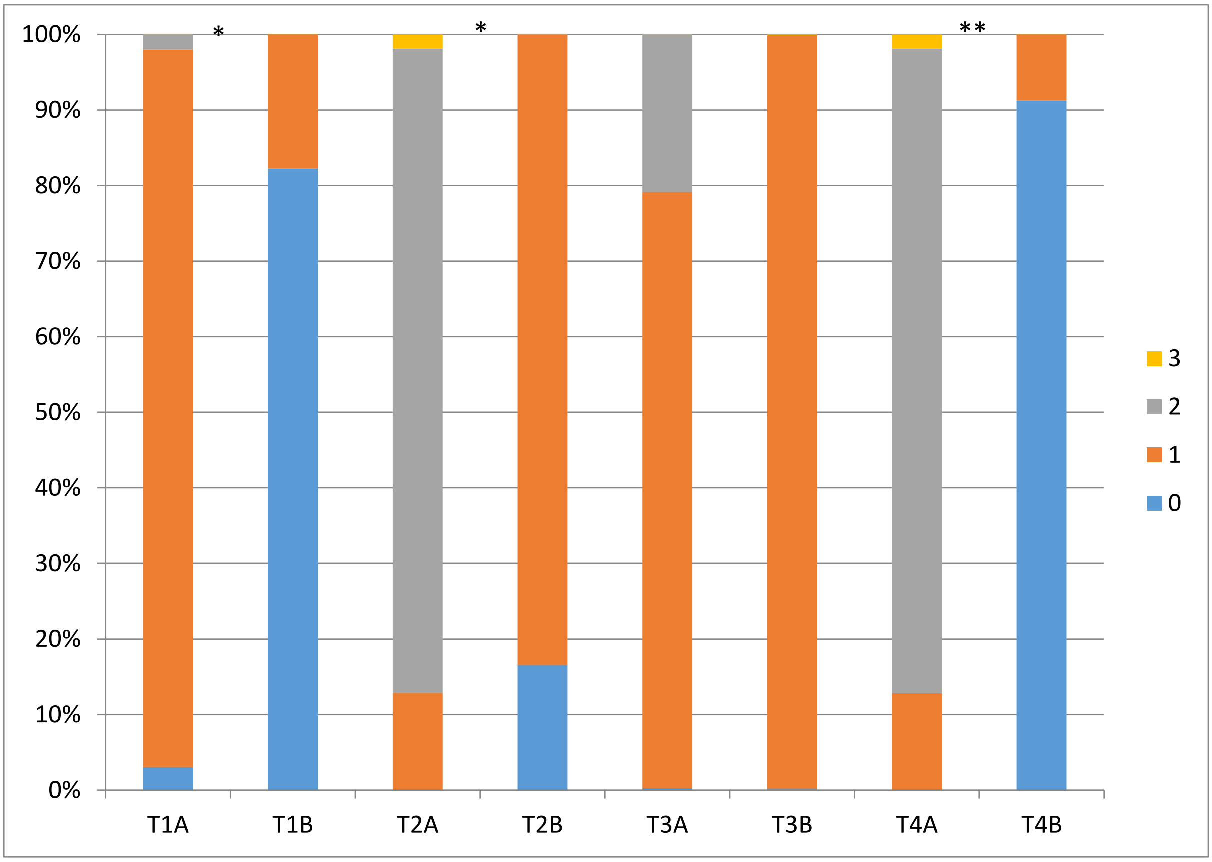
5.3.7.1. Pharyngeal Lymphoid Hyperplasia

The effect of time was not significant (P=0.979); the group was not significant (A vs B: P=0.303).

5.3.7.2 Tracheal inflammation score:

The time*group interaction was significant (P<0.001) (Figure 3). In particular, group A had significantly higher tracheal inflammation scores in comparison with group B at preloading (P=0.017), unloading (P=0.019) and 5 days AJ (P=0.003). A general trend is apparent whereby scores are significantly greater (P<0.001) at unloading compared with preloading.

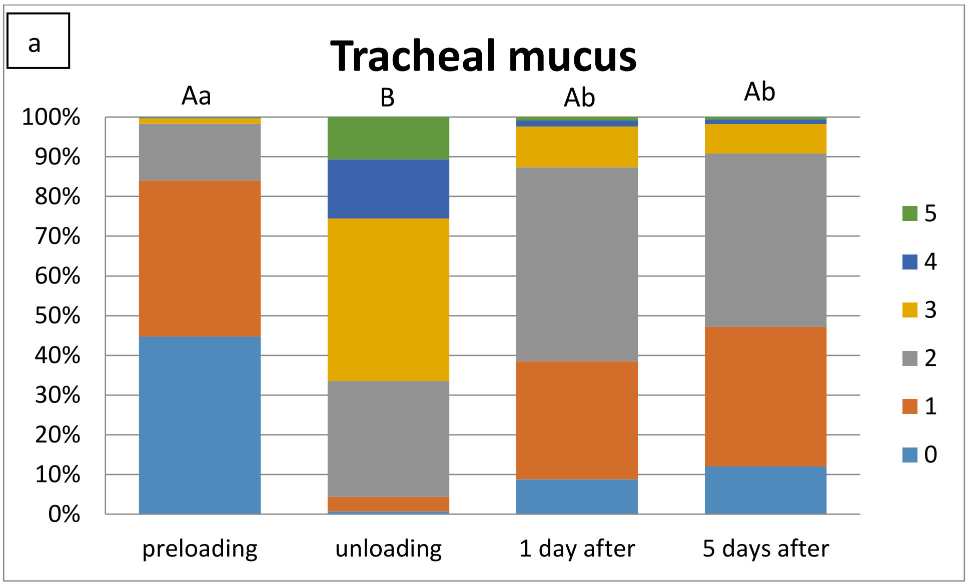
Figure 3. Effect of the time*group interaction on the distribution of the Tracheal inflammation score (0= no, 1= mild, 2= moderate, 3= extreme) (*=P<0.05; **=P<0.01). (Time: T1: preloading; T2: unloading; T3: 24 h AJ; T4: 5days AJ). (Group: A: abnormal lung sounds at unloading; B: normal lung sound at unloading).

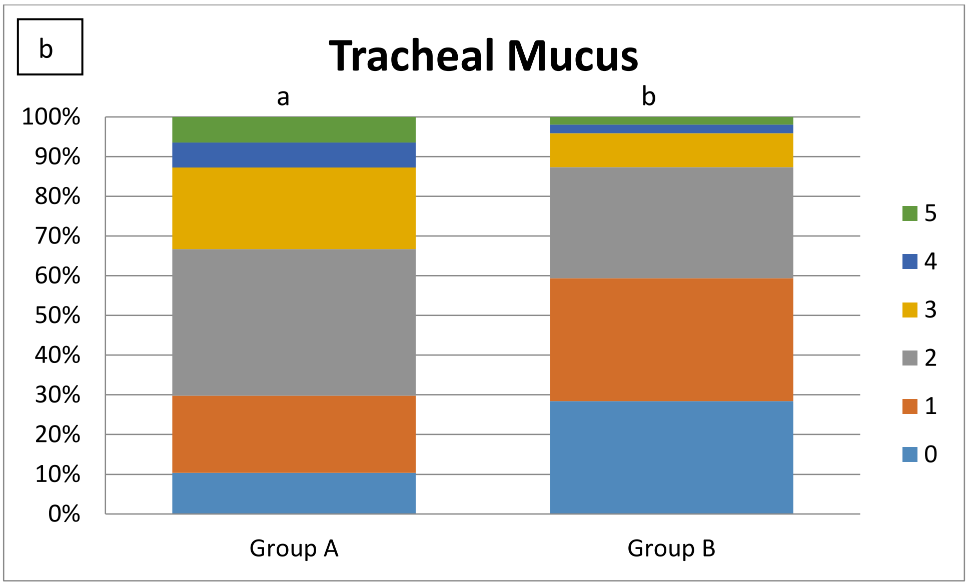


5.3.7.3 Tracheal mucus score

The effect of time was significant (time 1 vs 2: <0.001; time 1 vs 3: 0.014; time 1 vs 4: 0.035; time 2 vs 3 P=0.004; time 2 vs 4: P= 0.002; time 3 vs 4: P=0.632) (Figure 4a); the effect of group was significant (P=0.022) (Figure 4b), and the interaction was not significant (P=0.131). The time effect illustrates scores being highest at unloading compared with preloading (P<0.001), with scores progressively lowering from 1 day and 5 days after transport, but not reaching initial preloading distribution (P=0.035). The effect of group indicates that animals in group A had significantly higher tracheal mucus scores (in general) compared with Group B animals.

Figure 4. Effect of the Time (a) and Group (b) on the distribution of the Tracheal Mucus score (0=none, 1=little, 2=moderate, 3=marked, 4= large, 5=extreme). (Group: A: abnormal lung sounds at unloading; B: normal lung sound at unloading). Columns with different superscripts are significantly different: A, B P<0.01; a, b P<0.05.

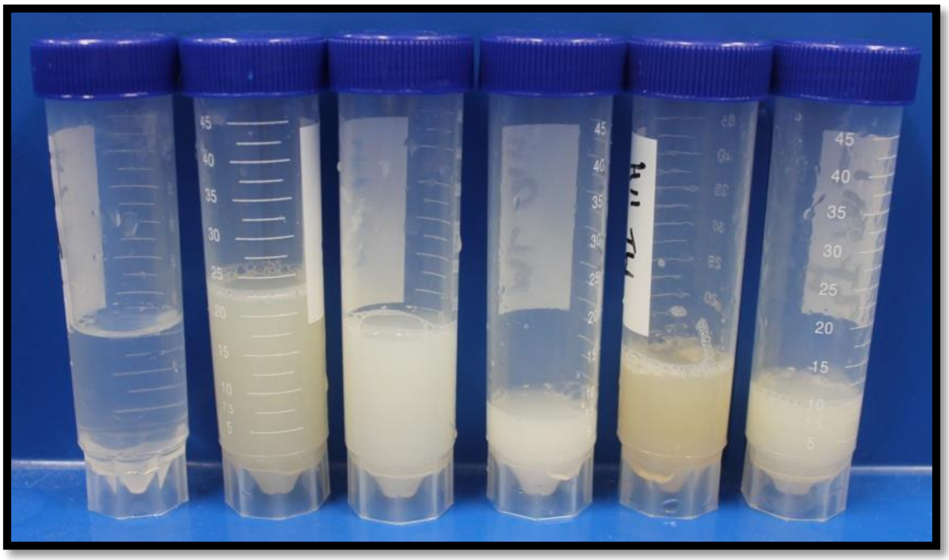




5.3.7.4. TW Colour (from 0: clear to 4: blood)

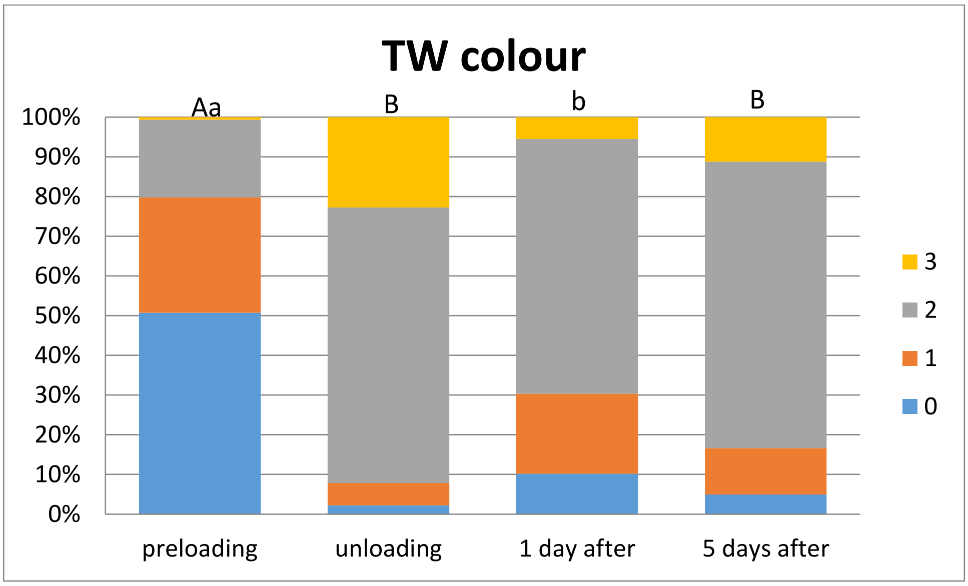
At unloading, the TW colour ranged from clear to yellow (Figure 5).

Figure 5. TW collected at unloading of trip 1 from the 6 horses. One TW was transparent, three were white and two yellow.



The effect of time was significant (time 1 vs 2: 0.0003; time 1 vs 3: 0.011; time 1 vs 4: 0.002; time 2 vs 3 P=0.068; time 2 vs 4: P= 0.355; time 3 vs 4: P=0.373) (Figure 6); the group and the time*group interaction were not significant (P=0.241 and P=0.131, respectively). The time effect is seen only at preloading were 50% of the TW were clear (0), while the predominant colour was white (2) at the other time points.

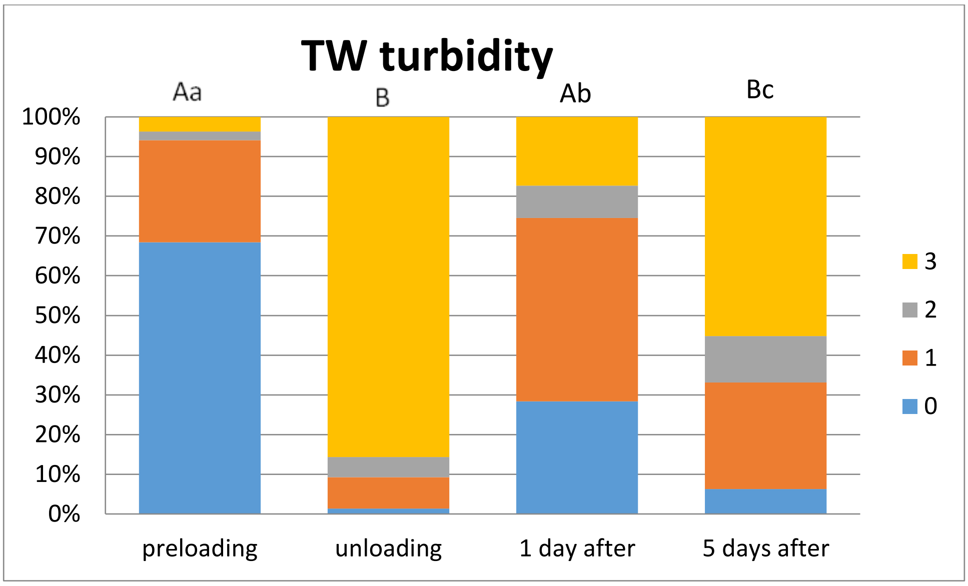
Figure 6. Effect of the Time on the distribution of the TW colour score (1=clear, 2=white, 3=yellow, 4=blood). Columns with different superscripts are significantly different: A, B P<0.01; a, b, c P<0.05.



5.3.7.5 TW Turbidity score

The effect of time was significant (time 1 vs 2: <0.001; time 1 vs 3: 0.052; time 1 vs 4: <0.001; time 2 vs 3 P=0.002; time 2 vs 4: P= 0.120; time 3 vs 4: P=0.030), the group was not significant (A vs B: P=0.272) and the time*group interaction was also not significant (P=0.080).The time effect was illustrated with higher turbidity scores (mainly opaque) at unloading compared with preloading (mainly transparent) (P<0.001) and at 1 day AJ (P=0.002) and with turbidity being similar at 5 days AJ (P=0.124). The scores did not reach initial preloading distribution (preloading vs 5 days AJ, P<0.001) (Figure 7).

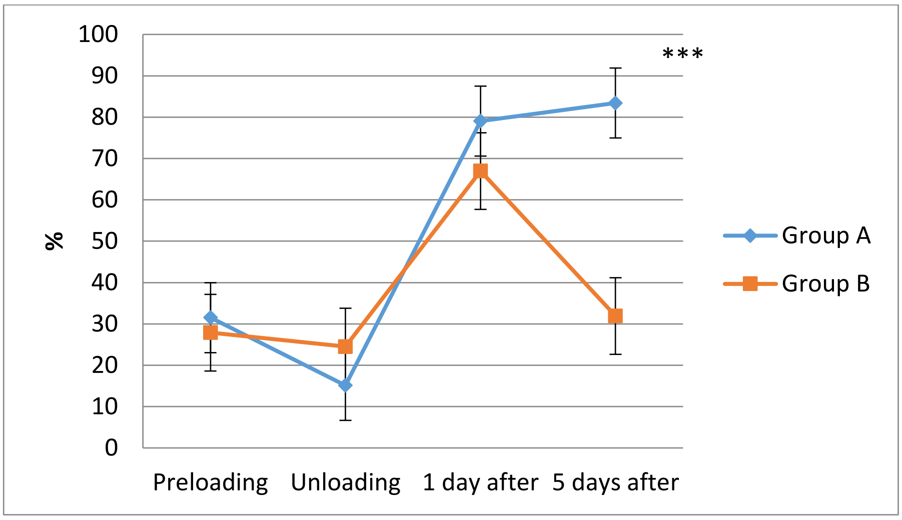
Figure 7. Effect of the Time on the distribution of the TW turbidity score (0= transparent, 1= clouded, 2= smoked glassed, 3= opaque). Columns with different superscripts are significantly different: A, B P<0.01; a, b, c P<0.05.



5.3.7.6. TW cytology

The effect of time (P<0.001), group (P=0.030) and time*group interaction (P=0.0121) were significant on the % of neutrophils and macrophages observed in TW cytology. The general trend was an increase in the proportion of neutrophils, with consequent decrease in the proportion of macrophages, at one day AJ compared with preloading and unloading. Whilst Group B recovered, group A showed a protracted high proportion of neutrophils in TW at 5 days AJ (Figure 8).

Figure 8. Changes in the % of neutrophils in TW over the four time points between the two groups. (*** P<0.001). (Group: A: abnormal lung sounds at unloading; B: normal lung sound at unloading).



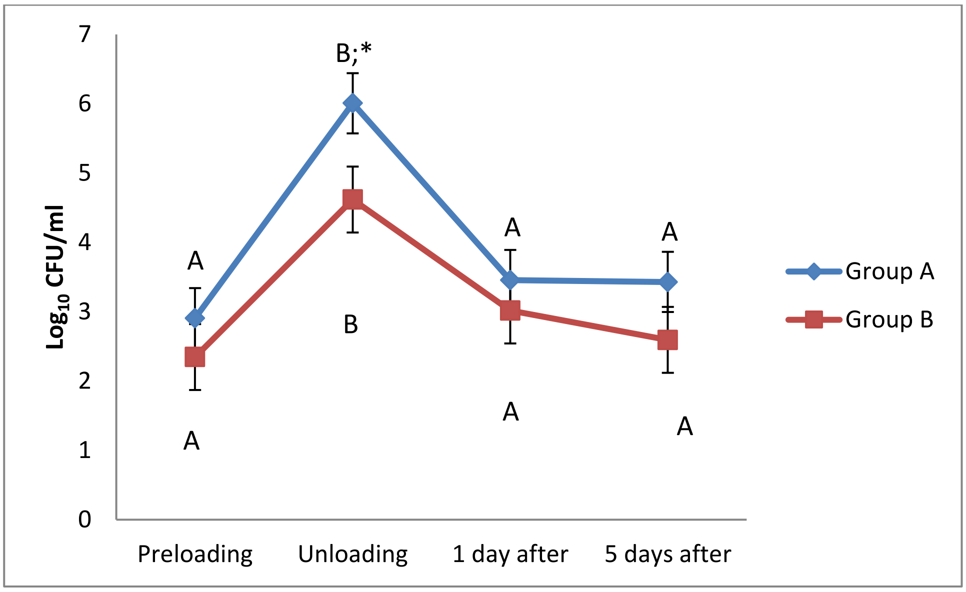
The effect of time was significant on the prevalence (%) of lymphocytes (P<0.001) but group and the time*group interaction were not significant. There was a decrease in the prevalence of lymphocytes at one and 5 days AJ in comparison with preloading and unloading (12.08 ±1.27 vs 8.62 ± 1.27 vs 4.06 ± 1.27 vs 3.42 ± 1.27 %).

There was no significant effect of time, group and the time*group interaction on the proportion of eosinophil and mast cells in TW.

5.3.7.8. Preliminary quantitative and qualitative bacteriological analysis of TW samples

The time had a significant effect on the concentration of bacteria recovered from TW (P<0.001). At unloading there was an increase of approximately 3 log in both groups, but Group A had an overall higher concentration of bacteria in the TW than group B (P=0.013) (Figure 9). The effect of the time*group interaction was not significant (P=0.701).

Figure 9. Bacterial concentration recovered in TW (log_{10} CFU/ml) in group A and B from preloading to 5 days after journey. (Group: A: abnormal lung sounds at unloading; B: normal lung sound at unloading).



The following major groups of bacteria were identified:

α-haemolyotic *Streptococcus* spp: Gram positive facultative anaerobic cocci in pairs or chains with obvious α-haemolysis (incomplete) on SBA; catalase negative.

β-haemolytic *Streptococcus* spp : Gram positive facultative anaerobic cocci in pairs or chains with obvious β-haemolysis (complete) on SBA; catalase negative.

non-haemolytic *Streptococcus* spp: Gram positive facultative anaerobic cocci in pairs or chains with no-haemolysis on SBA; catalase negative.

Staphylococcus spp: Gram positive facultative anaerobic cocci in pairs, tetrads, or more often grouped in irregular clusters, catalase positive.

Pasteurellaceae like: Gram negative, facultative anaerobic pleomorphic coccobacilli or bacilli with characteristic colonial morphology (round, greyish or yellowish, slightly raised and nearly 2mm in diameter after 48hr, often sticky in nature), oxidase positive.

Enterobacteriaceae like: Gram negative, facultative anaerobic bacilli, with small grey colonies, oxidase negative.

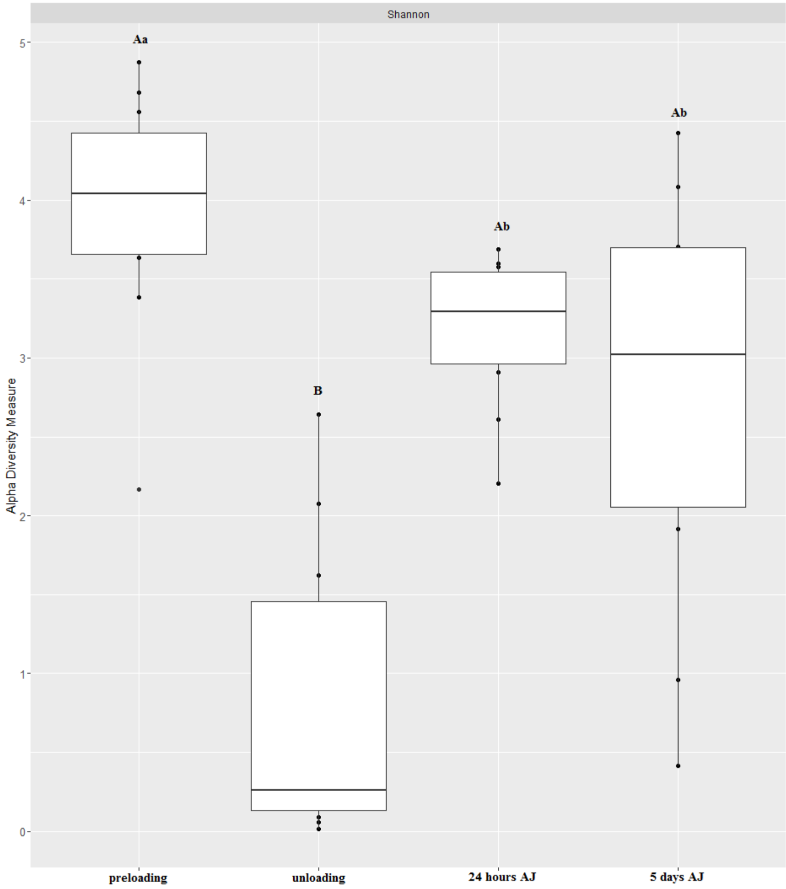
At unloading the predominant bacteria recovered were *Pasteurellaceae* like (Figure 10) in the majority of horses (n=8).

Figure 10. Photo of a plate planted with 10^3 TW collected at unloading after 48h of incubation.

5.3.7.9. Microbial diversity profiling from TW

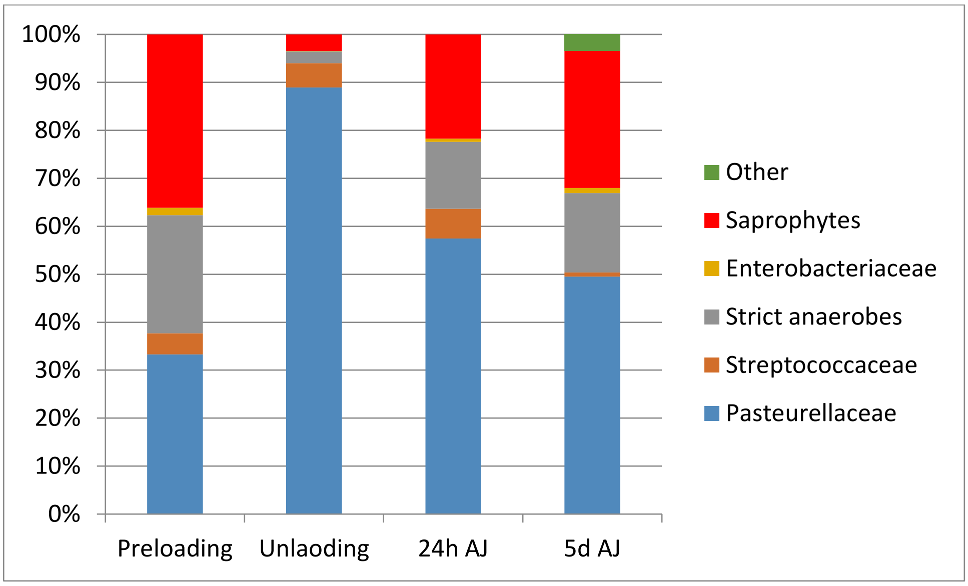
Figure 11 show the results of the Shannon diversity metric, illustrating a significant reduction in bacteria diversity at unloading in comparison with preloading, 24 hours AJ and 5 days AJ(P<0.001), without restoring even after 5 days (P<0.05). The index decreased significantly at unloading, reflecting a dominance of *Pasteurellaceae* bacteria in the majority of cases (n=8) (P<0.001)(Appendix 5, Figure 8). Two horses H7 and H12, which spent more time with their heads down, did not show the predominance of any family in particularly. There was no statistical difference in the Shannon diversity metric calculated between groups (A: 2.64; B: 2.78; t = -0.32, df = 39.986, P = 0.749).

Figure 11. Shannon diversity index calculated on the different type of bacteria present in the TW collected at preloading, unloading, 24h AJ, 5d AJ. Box plot with different letter differ significantly A, B: P<0.01; a, b:P<0.05.



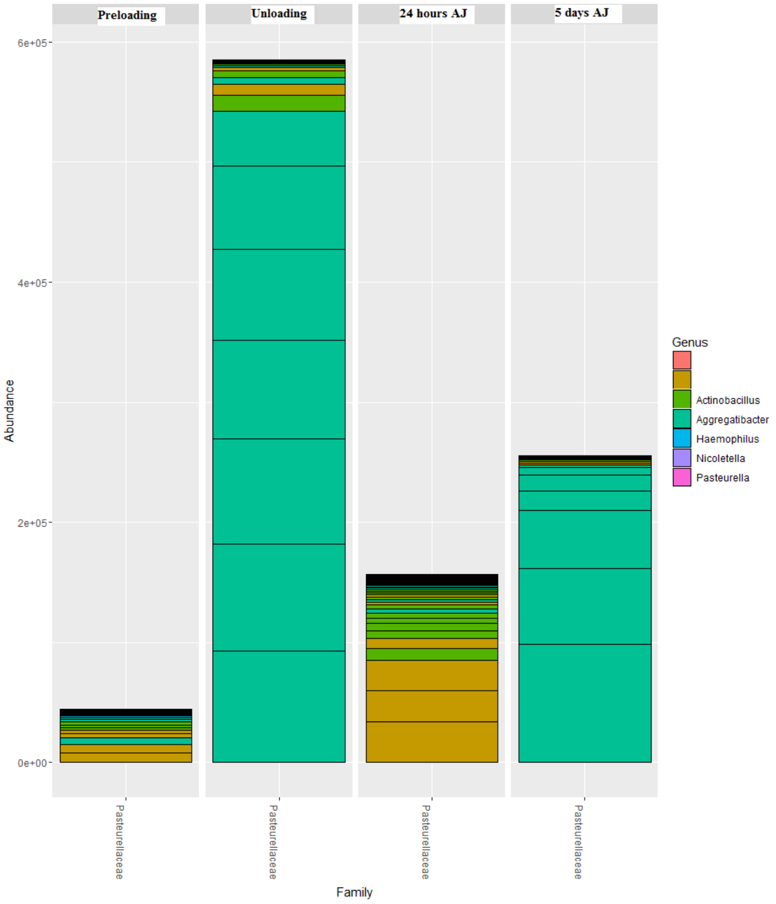
From the mixed linear model results, the time of sampling had a significant effect on the mean percentage of *Pasteurellaceae*, strict anaerobes and saprophytes (P<0.001)(Figure 12) in the total TW microbiome. There was a clear increase of *Pasteurellaceae* and decrease of the other two groups at unloading in comparison with the other times. The effect of group and time*group interaction were not significant on *Pasteurellaceae* (P=0.496; P=0.058). There was no effect of time, group and time*group interaction on *Streptococcaceae* and *Enterobacteriaceae*.

Figure 12. Changes in mean percentage of total bacterial genome of the 5 classes (Saprophytes, *Enterobacteriaceae*, strict anaerobes, *Streptococcaceae*, and *Pasteurellaceae*) at the different time points.



The predominant genera identified within the *Pasteurellaceae* using sequencing was *Aggregatibacter* spp., bacterial species within this genera appeared to increase significantly at unloading and 5d AJ in comparison with preloading (P<0.001)(Figure 13), particularly so in horses who spent more time with their head elevated during transport (Appendix 5, Figure 9). Streptococci increased at unloading in one horses (H5) as the second most predominant family (*Pasteurellaceae* 55% and *Streptococcaceae* 45%)(Appendix 5, Figure 10).

Figure 13. Pilot bar for *Pasteurellaceae* at the different times only obtained in phyloseq (McMurdie and Holmes, 2013). In the legend, if there is no name for a colour, it indicates that for that OTU the identification did not progress to genus, thus family = *Pasteurellaceae*, but no further information for that particular sequence.



5.3.7.10. Thoracic Ultrasound

There was no effect of time and group on the quantity of fluid seen on ultrasonography (P=0.339, P=0.932, respectively). There was no association between the time and group and the loss of aeration (P=0.258, P=0.568, respectively) and pleural changes (P=0.216, P=0.782, respectively) (Appendix 5, Table 2).

5.3.8. Viral parameters

5.3.8.1. EVH-1 and EHV-4 serology

All horses were seronegative for EHV-1, however the seroprevalence for EHV-4 was 92% (11/12). No instances of seroconversion (increase in optical density > 0.2) were observed through comparison of antibody levels prior to and 2 weeks post transportation.

5.3.8.2. Virus detection and shedding

No EHV viruses were recovered from nasal swabs inoculated in cell culture (i.e no evidence of CPE). All samples were negative for EHV-1 and EHV-4 by qPCR however there was a high prevalence of EHV-2 (83%) and EHV-5 (92%) (Table 16). Confirmative melting curves analysis showed single peaks within temperature ranges of 78.95-79.8°C and 79.83-80.83°C for EHV-2 and EHV-5 products respectively. Prevalence of EHV-2 increased over time with 3 horses becoming positive after transport (Table 16).

Table 16. Number of positive horses for gamma herpesviruses by qPCR at various time points.

	Pre	Unloading	24 hours	5 days
EHV-1	0	0	0	0
EHV-4	0	0	0	0
EHV-2	7	8	9	10
EHV-5	9	10	8	8

There was no effect of the time on the viral load (as indicated by cycle threshold (Ct) for EHV-2 or EHV-5 (P=0.1404 and P=0.9237, respectively).

There was no association between the group (A: abnormal; B: normal) and the presence of EHV-2 pre (P=0.558) and after journey (P=1.000) and the presence of EHV-5 pre (P=0.505) and after journey (P=0.221).

<u>5.3.9. Pearson correlation and linear regression analysis: associations between behavioural and clinical parameters</u>

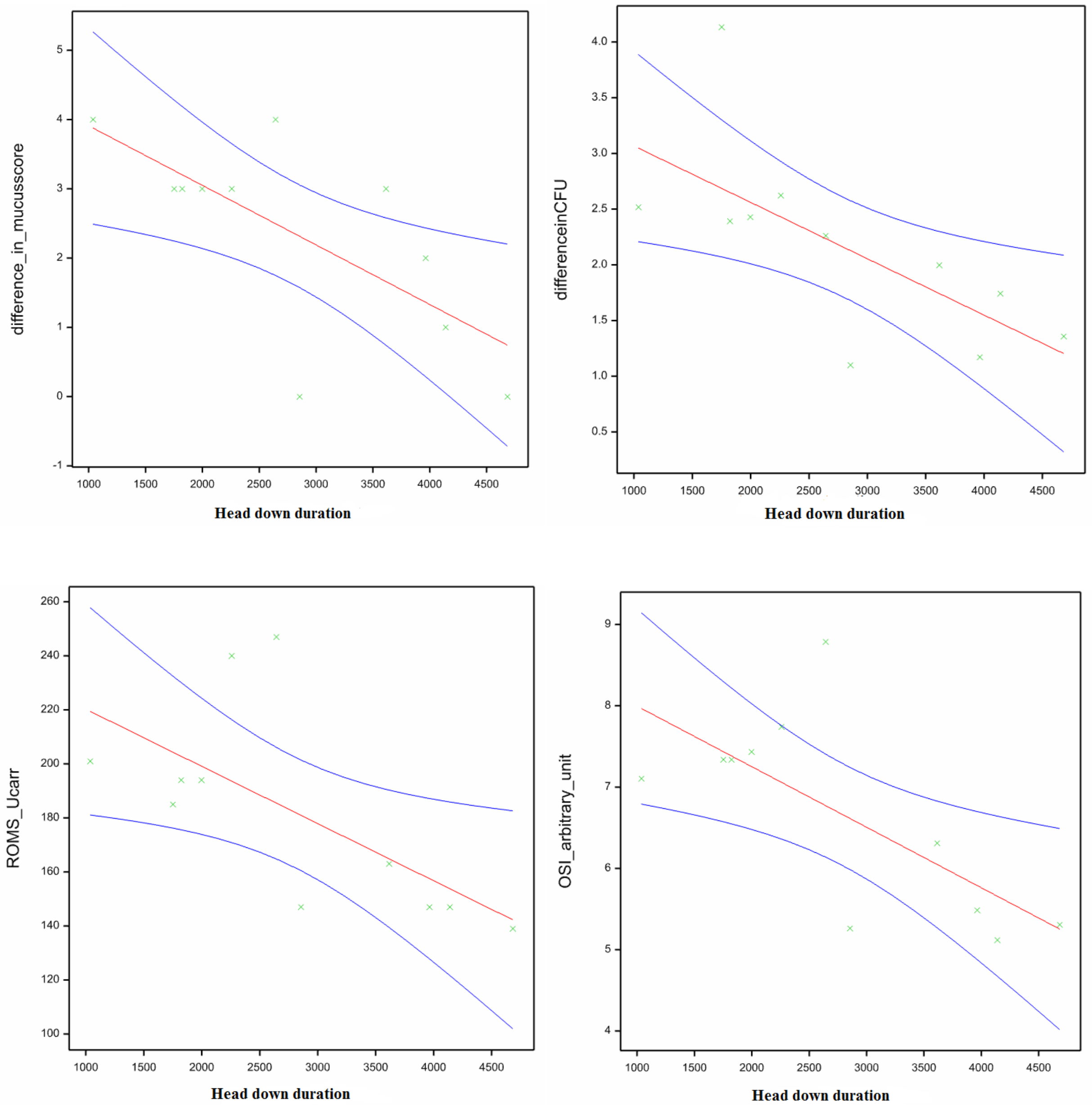
Table 17 shows the results of the Spearman correlation and linear regression analysis.

Table 17. Pearson correlations and linear regression analysis results. Data expressed as r and P^a for the correlation, and as R^2 and P^b for the linear regression.

X	Y	r	P^a	R^2	P^b
Total head down duration	Tracheal mucus score	-0.717	0.012	46.1	0.013
	TW Bacteria log_{10}CFU/ml	-0.686	0.019	41.3	0.020
	OSI	-0.703	0.015	44.0	0.016
	ROMs	-0.654	0.029	36.4	0.29
	Body weight	-0.621	0.041	31.8	0.041
	Tracheal inflammation score	-0.613	0.044	30.7	0.045
	Licking/Chewing	-0.718	0.012	46.2	0.013
	Pawing	-0.637	0.035	34.0	0.035
Total Movements	CK	0.658	0.027	36.9	0.028
	TP	0.619	0.042	31.5	0.042
Touching rubber tie cord	Cortisol	0.724	0.0117	61.5	0.012
	Tracheal inflammation	0.679	0.021	40.2	0.021
	Lower head frequency	-0.725	0.011	47.4	0.011
Total stress related behaviours	Fibrinogen	0.648	0.031	35.5	0.031
HR	Backward movement	0.914	<0.001	81.8	<0.001
	Forward movements	0.729	0.010	48.0	0.011
	Evasive behaviours	0.808	0.002	61.5	0.003
White blood cell	Neutrophils	0.927	<0.001	84.5	<0.001
	Fibrinogen	0.773	0.005	55.4	0.005
	PTAS	0.663	0.025	37.8	0.026
	Globulin	0.684	0.020	40.9	0.020
	Albumin	-0.757	0.007	52.7	0.007
PTAS	Neutrophils	0.831	0.001	65.7	0.002
	Globulin	0.719	0.012	46.5	0.013
ROMs	Mucus Score	0.768	0.005	57.8	0.004
OSI	Mucus Score	0.825	0.001	70.3	<0.001

The head down duration was associated with the tracheal mucus and inflammation scores, the TW bacterial concentration (log_{10} CFU/ml), ROMs concentration and OSI (Figure 14). Thus the more time the horse travelled with its head in a lowered position, the less mucus, inflammation, bacteria, ROMs and OSI observed on arrival. Heavier animals showed more licking/chewing behaviours and spent less time with their heads down. On review of video footage it was apparent that some larger framed horses were physically unable to lower their heads in the available space. The animals that moved more *en route* showed higher total protein and CK on arrival. There was an association between stress indicators, namely cortisol and HR, with behaviours interpreted as indicative of stress such as touching rubber tie cord, evasive behaviours, forward and backward movements. The animals which showed more stress related behaviours *en route* had higher serum fibrinogen concentrations on arrival. The number of white blood cells was positively correlated with PTAS and inflammation markers, namely neutrophils, fibrinogen and globulins. ROMs and OSI were positively correlated with tracheal mucus score. No other significant association/correlations were observed.

Figure 14. Linear regression results between total head down duration (x) and mucus score, TW bacterial load, ROMs and OSI (y).



5.4. Discussion

This multidisciplinary study documents the effects of eight hour transportation devoid of feed and water on behavioural, clinical, haematological, oxidative and respiratory parameters, identifying significant associations and relationships. The data reflects that transportation is a stressful event for the horse and that more stressed horses spent more time with their heads in an elevated position *en route*, accumulating more mucus and bacteria in their lower respiratory tract, triggering inflammation and the production of more systemic oxygen radical metabolites. The shedding of gamma equine herpes virus was seen in many of these horses with 3 negative horses for EHV-2, shedding EHV-2 post transportation suggesting potential reactivation of latent infections after transport or acquisition of the virus from transported or study mates post transport, however no definitive association could be made between EHV and transport. This study is unique in that it has recorded parameters which have enabled a holistic examination of transport stress in horses. The findings may be used to inform future recommendations for improving the management of transported horses and for identifying animals at risk of transport-related respiratory diseases.

During the transport event horses demonstrated stress-related behaviours more than when stabled in the box, and at unloading, illustrated through elevations in their physiological parameters (HR, RR and T) and cortisol levels. While these behavioural, physiological and hormonal parameters are considered as valid animal based stress indicators (Dalla Costa et al., 2014), our data demonstrated that the specific transportation event was stressful. Even though our horses fasted during the journey, blood glucose peaked at unloading as a consequence of the simultaneous cortisol peak (Moberg and Mench, 2000). Cortisol is secreted based on circadian rhythms with higher values in the morning and lower values in the afternoon in horses (Bohák et al., 2013). The tested journey altered the cortisol circadian rhythms only on the day of the journey. As all stress-indicators were restored within 12 hours after the journey, the transport itself should therefore be considered an acute stressor in its own right.

The highest frequency of stress related behaviours was registered during the first hour of transport, suggesting this to be the most stressful part of a journey. Similar findings have been made in other studies using either cortisol or other stress indicators, such as heart rate variability, tiroidal and adrenocortical hormones (Fazio et al., 2008a; Fazio et al., 2013a; Schmidt et al., 2010; Tateo et al., 2012). Based on our study and the findings of the above studies it seems that horses need around 5 hours to adapt to the journey and to the vehicle. However, our behavioural responses might be also interpreted as a sign of fatigue, as evidenced by the increased instability of the horse after the fifth hours of transport, the observed peak in balance related behaviour at the 8th hour, the quiet demeanour and the increases in lactate and CK at unloading.

Our behavioural observations *en route* suggests that there is individual variation in behavioural responses to transport. Individual differences were noticed with the more nervous and agitated horses affecting their neighbours. As social animals, anxiety and arousal can be socially transmitted to herd mates (McGreevy, 2012; Saslow, 2002). This should be taken into account when mixing horses with different temperament and travel experiences (Broom and Johnson, 1993; Fazio et al., 2013b), in particular given our findings that *en route* agitated horses do not lower their heads which is associated with increased tracheal mucus and respiratory bacterial concentrations. Alert horses have a neck angle of 45 degrees relative to the ground compared to a neck angle which is parallel to the ground when they are calm (Fureix et al., 2012). The negative correlation between stress-related behaviours and time spent with their head down was seen also during the rest stop, where horses showed fewer stress-related behaviours and spent more time with their heads in a lower position in comparison to travelling. However the frequency of these stress-related behaviours during the rest stop was higher than when the horses were in their stable boxes. Larger horses were physically limited in their ability to make these postural adaptations. Cross tying was identified as a risk factor in the development of transport-pneumonia and travelling untied or with a long tether has been recommended (Racklyeft and Love, 1990; Raidal et al., 1996; Stull and Rodiek, 2002). However, from our data it seems that horse did not try to lower their head when stressed. Habituation to travel prior to undertaking longer journeys might result in a reduction in transport-stress related responses (Yngvesson et al.)(Chapter 3.3) and therefore limit the time spent with an elevated head *en route*. In our study horses travelled without food, further studies are need to test the hypothesis that offering hay on the floor effects the frequency of head lowering during transport (Allano et al., 2016).While research on the transport density of slaughter horses has been conducted (Collins et al., 2000; Iacono et al., 2007a), the ideal dimension of a bay during the transportation of performance horses is still unclear with differing national regulations (Waran et al., 2007). Further studies are therefore needed to test the effect of bay dimension on head down frequency and duration and consequently respiratory outcomes.

The Canadian, Australian, and the European Animal Transportation Codes allow the transportation of horses without offering water for a maximum of 36, 24 and 8 hours, respectively. While it has been proven that 36 and 24 hours of transport devoid of water causes dehydration (Stull and Rodiek, 2000), this is the first study documenting the effect of 8 hour transportation devoid of food and water on arterial blood gas and haematology. This journey caused mild, but significative effects on fluid and electrolyte balance, with transient increases in albumin, total protein, capillary refill time and weight loss consistent with minor decreases in total body water. Acidosis or alkalosis was not seen in this study, mirroring the transport related blood-gas changes reported in steers (Parker et al., 2003). As there was no evidence of acid-base changes, nor alteration in plasma sodium concentration and haematocrit it is unlikely these changes were of biological significance in the current study. Consequently, the European standard and guidelines on watering (Anon, 2014) seem useful and could be adopted worldwide. It is worth noting, however, that based on the environmental parameters, our horses travelled within the thermo-neutral zone, which is between 5°C and 25°C (Morgan, 1998) and in absence of noxious gases (Pickrell, 1991; Smith et al., 1996). Consequently our findings might be true only in similar conditions. As travelling outside of the thermo-neutral zone and over longer distance could lead to different outcomes (Stull and Rodiek, 2000), in the latter cases travelling with an on board watering system should be considered (Iacono et al., 2007b). Despite their modest magnitude, the observed changes in albumin, protein and body weight were not restored within 24 hours, suggesting that recovery from transportation might require a number of days, as suggested previously (Marlin, 2004). Similarly blood lactate and CK were also elevated at unloading confirming that the continuous movements *en route* affected the musculoskeletal system (Giovagnoli et al., 2002). Our horses were rested in their home stable following the journey but lactate and CK did not restore the day AJ. At least one rest day after eight hour journeys should be recommended and cool down practices, such as walking and stretching (Kohn, 2000)(chapter 3.1), should be considered as possible recovery strategies.

Transportation was associated with an acute phase inflammatory response characterised by neutrophilia, increased fibrinogen, increased reactive oxygen metabolites and mobilisation of antioxidants, as has been demonstrated in previous studies (Leadon, 2000; Niedźwiedź et al., 2013; Stull et al., 2008; Wessely-Szponder et al., 2015)(Chapter 4). However, this is the first study to report the effect of transportation on AOPP, which are biomarkers of protein oxidation (Celi and Gabai, 2015), and ceruloplasmin, which is an acute phase protein (Crisman et al., 2008) also involved in redox-balance (Celi, 2011). Interestingly these parameters peaked at unloading and the day after the journey, while the antioxidants peaked at unloading but reached their minimal value the day after. These data suggest a possible depletion of antioxidants after the journey, which could lead to oxidative stress and predisposition to disease. However this hypothesis warrants future research.

Transport related variations in EBC H_2O_2 were negligible in this study. Thus, 8 hours of transport and the observed responses seen in the respiratory tract did not appear to influence local H_2O_2 measure in EBC. However, our data should be interpreted with caution because they could have been affected by sampling technique (mask instead of a tracheal tube (Schack et al., 2002) or storage. H_2O_2 should be measured soon after the collection (Crowley et al., 2013) or stored at minus 80 for a short period (Rosias, 2012).

The transport related changes in respiratory microflora at unloading are in agreement with the literature (Racklyeft and Love, 1990; Raidal et al., 1997a). The microbial diversity profiling data confirmed the prominence of *Pasteurellaceae* bacteria in the majority of horses immediately after transport (Racklyeft and Love, 1990; Raidal et al., 1997a) and, for the first time, conventional bacteriology and genetic approaches to bacteriology were combined to evaluate the effects of transportation on lower airway contamination. Our study suggests that due to transport-stress related behaviours (i.e. licking/chewing, touching rubber tie cord) and consequently elevated head position, the lower respiratory tract is invaded mainly by oral-pharyngeal commensal and not by environmental bacteria. These findings support the role of *Pasteurellaceae* as early, opportunistic invaders when pulmonary clearance mechanisms are compromised (Raidal et al., 1996). Within the *Pasteurellaceae* family, there was a clear increase in the proportion of *Aggregatibacter* spp. at unloading. In humans, these bacteria are often found as normal oral flora, and have been associated also to gengivitis, endocardititis and pneumonia (Nørskov-Lauritsen, 2014). While it is well known that *Pasteurella*-like bacteria are common commensal of the oral cavity and might be associated with respiratory diseases in horses (Layman et al., 2014), there is currently no information regarding the relevance of *Aggregatibacter* spp. in the horse. Future studies are needed to investigate their role in equine transport related respiratory diseases. Importantly, there was no evidence of concurrent increases in other bacteria, particularly *Enterobacteriaceae* or strict anaerobes, suggesting that these organisms may become part of the disease process at a later stage or under different circumstances. It is also important to note that airborne bacteria identified from the stable and truck air samples were not detected at significant levels in the lower respiratory tract following transportation, confirming earlier suggestions that these organisms make little contribution to the development of respiratory disease associated with transportation (Racklyeft and Love, 1990; Raidal et al., 1997a).

Inflammation and tracheal mucus scores in our horses did not recover in the day after the journey as demonstrated in previous studies (Racklyeft and Love, 1990; Raidal et al., 1997a). This discrepancy could be related to differences in management post journey. In the above mentioned studies horses were kept on pasture while our horses were stabled. The air quality in these stables was relatively poor (more than 3 log_{10} CFU/m^3) and with poor ventilation (i.e. no wind recorded). Given that stabled horses tended to spend less time with the head down (Kiley-Worthington, 1997) and are exposed to irritants such as endotoxins at higher concentrations (Berndt et al., 2010) in comparison with grazing horses, it may not be totally suppressing to have observed a delay in respiratory tract recovery in our study. Further studies are also needed to evaluate if keeping horses on pasture for 24 hours after a journey reduces the risk of transport related respiratory diseases.

At five days AJ, group A horses showed protracted neutrophilia in the air ways. The development of the inflammatory diseases of the respiratory tract in these six horses could have been a combined effect of transportation and being stabled. The pathogenesis of inflammatory airways disease (IAD) remains incompletely defined; while housing is a well-known risk factor in IAD (Couëtil et al., 2016), the role of transportation warrants future research. Group A horses showed abnormal auscultation and coughing, but did not develop pyrexia, and haematological, arterial blood gas and thoracic ultrasound parameters were within normal limits suggesting there was no substantive parenchymal or pleural disease. Hence we cannot generalise our data for transport-associated pneumonia. Since these animals' tracheal inflammation scores prior to transportation were higher, they may have had some degree of minor compromisation of the respiratory tract before the transport event, potentially impacting upon recovery. Thus we may want to suggest that an assessment of fitness for travel should be conducted to avoid transporting animals with subclinical/clinical diseases (http://www.fve.org/news/index.php?id=208#208, 2016). While their tracheal inflammation was diagnosed only by scoping, scoping is recommended especially before long distance journeys (Marlin, 2004).

Shedding of EHV-1 and EHV-4 was not detected before or after transportation in this study, in agreement with literature (Oikawa and Jones, 2000). EHV-2 and EHV-5 are commonly seen in the upper respiratory tract of horses but their relevance in disease is generally thought to be subtle or negligible (Allen and Murray, 2004; Bell et al., 2006). Our data suggest EHV-2 reactivation or possible transmission in three horses associated with transportation. However, as these horses were in close contact before and after the journey, the possibility of circulation within the group and unrelated to transportation cannot be excluded. Consistent with the uncertain relevance of these agents to disease in horses (Allen and Murray, 2004), we were unable to see an association between the shedding of these gamma *herpesviridae* and airway inflammation.

From our data it appears that behaviours *en route* may play a role in influencing respiratory outcomes, with frequency and time the horse spent with an elevated head posture appearing relatively critical (Racklyeft and Love, 1990; Raidal et al., 1997a; Stull and Rodiek, 2002). The elevated head position and licking during transportation appear to favour more abundant contamination of the lower respiratory tract by oropharyngeal bacteria and the increase in tracheal mucus. Each horse reacted differently to the transportation resulting in different respiratory outcomes; therefore monitor horses during the journeys may aid in predicting the horses' disease risk related to transport or may aid in early intervention to prevent more significant disease manifestation. The horses which have greater difficulty coping with transport stress and demonstrate more stress related behaviours, such as: licking/chewing, chewing objects (rubber tie cord), evasive behaviours, spending the majority of the time with their heads in an elevated position, should be monitored very closely after journeys. The associations found between the head position *en route*, airway inflammation scores and ROMs suggest a link between stress, oxidative stress and predisposition to respiratory diseases. The positive correlation between the mucus score and plasmatic ROMs and OSI are in line with the use of plasmatic ROMs and OSI as possible indicators of inflammations in animals (Celi, 2011; Piccione et al., 2013; Tsuzuki et al., 2016). However, our findings should be interpreted with caution due to the low number of observations used for statistical analysis (n=11) and the lack of any evidence of local respiratory oxidative stress. Future studies involving more horses and immediate assessment of H_2O_2 in EBC may help to clarify the role of respiratory oxidative stress in the transported horse.

This study was limited by a number of factors. The horses used for the experiment were research horses and may not have been as well conditioned compared to performance horses. Only 12 horses were used with one being removed due to injury which further decreased the number of observations. The limited number of horses included in the two groups of animals (A: abnormal, B: normal) affected the statistical analysis as the interaction between time and group often did not reach the minimum values to run the statistical model. The horses were transported on two different days, which were not identical for environmental parameters. The physiological parameters, heart rate, respiratory rate and temperature were not recorded *en route*. The monitoring system used did not record sounds, so we could not measure the noise inside the truck nor calculate vocalizations which are recognised a stress indicator in horses (Young et al., 2012). TW samples were not incubated anaerobically and full differential counting of individual colony types and full bacteriological speciation were not performed.

Notwithstanding these limitations, this is the first study to combine a detailed analysis of behaviours throughout a single transport event, and has demonstrated that stress behaviours correlate well with physiological (HR) and endocrine (cortisol) markers of stress and respiratory outcomes. Head position predicted the amount of tracheal mucus and the bacterial load in horses at unloading. This in turn was confirmed by clinical examination parameters, particularly thoracic auscultation, but not by ultrasound imaging of the lungs or by blood gas evaluation, suggesting that bacteria present in lower airway samples had not colonised pulmonary parenchyma significantly in horses in this study. This suggests the pathogenesis of equine transport pneumonia requires other predisposing factors such as the inability of the animals to cope with longer duration journeys, or pre-existing damage or disease of the respiratory tract.

5.5. Conclusions

Overall, this multidisciplinary study showed that the level of stress experienced by the animal *en route* is manifested with increased stress-related behaviour frequency and reduced duration with their head in the downward position. The elevated head position is a known risk factor and in our study correlated with ROMs, OSI and with increases in tracheal mucus and concentrations of respiratory bacterial flora, with prominence of commensals within the *Pasteurellaceae* family. From our findings, accurate clinical examination including auscultation prior to and after journey and monitoring of the horse behaviours *en route* may aid in reduce the incidence of transport-related respiratory disease or at the very least prioritise animals for early disease mitigation. Determinations of oxidative stress indicators and fibrinogen might be useful to identify horses at risk of transport related respiratory diseases. Further studies are needed to test possible strategies, such as habituation training, more comfortable bays and the positioning of food at the floor level to decrease the time horses spend in an elevated head position while travelling. Future studies would benefit from using more horses and new technologies, such as textile electrodes (Lanata et al., 2015), accelerometer, audio and thermo-cameras to evaluate accurately transport related stress response.

CHAPTER 6

General Discussion

6.1. Discussion

This thesis has enhanced the understanding of transportation and its implications for horse health and welfare in Australia. In particular, the studies have reported a large amount of data on road transportation conducted after the publication of the Australian Code of land animal transportation (http://www.animalwelfarestandards.net.au/land-transport/). The thesis provides statistics, practical recommendations for safer travel and suggestions on how to implement the Code. It identifies risk factors and behavioural, physiological and clinical responses to transportation and makes evidence-based recommendations. The findings of Chapters 2 and 3 support the first hypothesis that transportation results in the development of behavioural and health problems during and after travelling, with a different incidence depending on transport management. The findings of Chapters 4 and 5 partially support the second hypothesis, that transportation affects horse behaviour, physiology and redox balance, increasing oxidative stress and thereby predisposing to disease. The effects of medium (8h) and long duration (4 days) transportation on behavioural, clinical, haematological, immunological, oxidative and respiratory responses have been documented, using a holistic approach and addressing potential associations between these factors. Both transportation events activated an acute response with an increase in oxidative products which is in agreement with the literature (Niedźwiedź et al., 2013; Onmaz et al., 2011; Wessely-Szponder et al., 2015). The relation between transport stress and redox balance was seen as a depletion of antioxidants after transportation which has been theorised to be a cause of the development of oxidative stress and a predisposing factor for the development of disease. The depletion of antioxidants after transportation and its implication in the pathophysiology of transport-related respiratory disease and mortality in calves has been reported (Chirase et al., 2004). However, there was no evidence of transport-associated diseases in the horses used in our studies, thus this hypothesis warrants future research.

Transportation by road may result in behavioural and health problems before, during and after the journey (Chapter 1). The majority of Australian equine industry members reported experiencing transport-related problems, with no difference observed between amateurs and professionals (Chapter 3.1). However, comparing the incidence of transport-related diseases between Chapters 2 and 3, it was apparent that the management of transport was a key factor for reducing the incidence of disease and injury. Chapter 2 analysed the records of a company specialising in long distance transportation which complied with the current Australian Code. The incidence of the health problems experienced by this company was approximately 3%. This confirms that transporting horses over long distances can be successful when the management practices comply with the Australian standards and guidelines. In contrast, the survey results suggested 67% respondents reported having experienced a transport-related health problem, and 38% respondents reported behavioural problems. Although this survey data was based on participant recall, and therefore might represent cumulative data (i.e. the sum of all transport events for each respondent), the majority of these journeys were not managed in compliance with the Australian Code (Chapter 3.1). From the survey results it was clear that horse owners and trainers did not comply with the policy because they were uninformed about the recommended practices, and respondents indicated that they would like to have better knowledge of best practices in transportation. Thus the main recommendations to safeguard the health and welfare of the horse during transportation are: i) Australian equine industry members should be educated on risk factors and on the Australian Code guidelines and standards; ii) there should be increased monitoring of compliance with the Code by the relevant authorities.

Transport-related behavioural problems were reported by 38% of the survey respondents, particularly at loading and during travel (Chapter 3.1, 3.3). This incidence is similar to what has been reported in the USA (Lee et al., 2001), but it is higher than recently reported by in a Swedish survey (Yngvesson et al., 2016). However the Swedish study asked only for loading problem behaviours and analysed only 95 surveys. Our survey analysed 797 responses and identified risk factors for preloading, loading, travelling and unloading problem behaviours, a global first. Interestingly, our survey and the other published surveys reached the same conclusion: habituation training is the most useful way to reduce the risk for transport-related problem behaviours and subsequent injuries. Habituation training to all phases of transport has been suggested in the literature (Houpt, 1982; Waran et al., 2002; Waran and Cuddeford, 1995). Unfortunately it was applied only by 20% of our respondents. Australian equine industry members should be educated about the benefits and techniques of habituation training to ensure that they are familiar with, and able to implement, this type of training. In the Swedish survey, 12% of the respondents were injured simultaneously with their horses during loading problems (Yngvesson et al., 2016). Although consideration of human injuries was outside the scope of our survey, other Australian studies have demonstrated an association between transportation and injury to personnel (Noble et al., 2013; Riley et al., 2015; Thompson et al., 2015). Therefore, the application of habituation training for loading and travelling should be recommended not only for horse health and welfare but also for human safety.

In comparison to the reported incidence of behavioural problems, transport-related health problems were reported by 67% of respondents (Chapter 3.1, 3.2). The higher reported incidence of health problems could be interpreted as the impact of transport on horse health, or it could be evidence that health problems arising from transport are more readily identified than are behavioural problems. It must be noted that the findings of the survey need to be interpreted with caution because they are self-reported and biased by what the respondents remembered or recognised as a problem. In the equine industry it is still common to confuse behavioural problems with bad horse temperament or attitude, which accounts for high rates of wastage due to behavioural causes (Hayek, 2004; McGreevy, 2012). In the future it would be useful to organise a prospective study, to evaluate horse behaviours associated with transportation and also the effects of different training methods.

Laminitis, heat stroke and particularly muscular problems are reported as common consequences of transportation (Mansmann and Woodie, 1995; Marlin, 2004; Weeks et al., 2012). Surprisingly, there were no records of these problems in our epidemiological study (Chapter 2) and a very low incidence in the survey (Chapter 3.1, 3.4). This could be due to a lack of recognition of the clinical signs of these conditions, or a lack of association of these pathologies with prior transport by both the transport company clients and survey respondents. However, even if laminitis was under-reported, risk factors were recognised for the condition, and the use of recovery strategies after journey was suggested to decrease its incidence (Chapter 3.2). Heat stroke was associated with the respondents' status (amateurs/professionals) and with access to food and water before travelling (Chapter 3.2). To reduce the risk of heat stroke, journeys should be planned in relation to the weather forecast and in relation to the environmental parameters inside the vehicle. Transport during the cooler hours of the day (http://www.animalwelfarestandards.net.au/land-transport/), and monitoring of the vehicle environment are recommended to reduce the likelihood of heat stroke (Chapter 1). In Chapter 5, the HI increased progressively from 9.00 to 17.00, even when ventilation of the truck was appropriate. Consequently travel during these times should be minimised particularly during spring and summer. As dehydration is a major contributor to the development of heat stroke, horses should have access to both water and feed *ad libitum* before journeys, and they should be watered at least every four hours *en route* (Anon, 2014). Horses within the thermal comfort zone can also develop heat stroke due to agitation (Weeks et al., 2012); it is therefore vitally important that they are not stressed during the journey. In Chapter 5, it was shown that the first hour of the journey was the most stressful; therefore it is possible that the horses might suffer from heat stroke also during short journeys or at the beginning of a long journey. In Chapter 3.4, heat stroke was not associated with any specific journey duration nor with breed, and so could be interpreted to be a risk for all types of journey and horses. Muscular problems have been associated with the lack of assessment of the fitness for travel (Chapter 3.2), with journeys of medium duration and non-commercial transportation (Chapter 3.4). In Chapter 4, we found an increase in AST and a decrease in potassium; in Chapter 5, we found an increase in CK and lactate and a decrease in potassium and calcium. These findings confirm that transportation affects the muscular system. The difference in the responses between CK and AST might be related to the different journey duration (4 days vs 8 hours) and normal enzyme dynamics, as CK levels rise and fall more quickly than AST after muscle insult (Eades and Bounous, 1997). It has been shown that the type of road and driving skills of drivers (Cockram et al., 2004; Cockram and Spence, 2012; Giovagnoli et al., 2002) are key risk factors contributing to muscle related problems during transport. Consequently, a specific driving licence for animal transportation could reduce the risk of transport-related muscular problems. Overall, it appears that high levels of competence in driving and all aspects of transport management are fundamental to reduce the incidence of laminitis, heat stroke and muscular problems.

The most common transport-related health problems reported in the survey were traumatic injuries (Chapter 3.1). The incidence reported was higher than expected (Noble et al., 2013), and could be due to the fact that in the survey we targeted respondents who moved their horses on a regular and frequent basis. Additionally, many respondents reported moving large numbers of horses (>30), which was also identified as a risk factor (Chapter 3.2). Injuries were associated with horse handling and driving skills, the use of protective measures, such as boots and sedation (Chapter 3.2). The importance of experience was confirmed by the lower incidence of injuries reported from the transport company (Chapter 2). In the latter case injuries were associated with the beginning of the journey, mainly relating to loading and travelling problem behaviours, identified as risk factors in Chapter 3.3. During the experimental 8h journey, one horse got injured due to problem behaviours during travel (Chapter 5). It has been shown that aggressive behaviours from some horses can be the cause of injuries to others (Iacono et al., 2007a) and it is reported that these behaviours are related to the dominance and the temperament of the horse more than to travel management (Chapter 1). Thus the temperament of the horse should be evaluated before mixing horses in the same truck, and the interior of vehicles should always be covered by rubber to reduce traumatic injuries due to kicking, pawing and other travelling problem behaviours (Weeks et al., 2012). The association between short journeys and traumatic injuries was found also in Chapter 3.4. The behavioural observations conducted in Chapter 5 documented that in the first hour of the journey horses showed more stress-related behaviour and lost their balance more frequently, probably because they were less attentive to maintaining their balance. This also highlights that the initial part of the journey might be a higher risk for injuries. These data also confirm that short journeys (1h) are likewise stressful (Fazio et al., 2008a; Tateo et al., 2012). While short journeys (< 2 hours) were the most common in Australia (Chapter 3.1), our data highlight the importance of planning short journeys appropriately and in compliance with the Code.

The respiratory and gastrointestinal systems are more affected by protracted transport stress (Leadon and Hodgson, 2014)(Chapter 1) and our data supports this (Chapter 2, 3.4). In Chapter 2 the frequency of gastrointestinal and respiratory problems, amongst problems reported during the long distance transportation was identical (27%). In Chapter 3.4, the reported incidences of respiratory and gastrointestinal problems amongst transport related problems were 37.7% and 23.8%, respectively. From our findings, it appears that in Australia enterocolitis is a common transport-related condition which is frequently fatal (Chapter 2, 3.1, 3.4). This pathology has been linked to transportation (McClintock and Begg, 1990) and it appears that transport stress may affect the gastrointestinal microbiota such that *Salmonella* spp and *Clostridium* spp might become predominant (Feary and Hassel, 2006; Staempfli et al., 1991) leading to enterocolitis. Future studies are needed to document the effects of transport on the gastrointestinal microbiota using pyrosequencing as per the respiratory microbiota investigation in Chapter 5. While the respondent status (amateur/professional) was the only factor associated with diarrhoea, highlighting the importance of professional management to reduce this risk, an association between colic and transport management was not identified (Chapter 3.2). Colic is a multi-factorial syndrome (Cohen and Peloso, 1996; Tinker et al., 1997) and transport-related colic requires future research. While sex, age and breed were not associated with gastrointestinal problems in Chapter 2, breed (i.e. Arabians, Warmboods) was identified as a risk factor when a larger dataset was analysed (Chapter

3.4). However, this finding needs to be interpreted with caution and should be confirmed by future research. A prospective longitudinal study would be useful to clarify the nexus between horse characteristics, transport management and gastrointestinal problems.

Transport pneumonia, also named shipping fever or pleuropneumonia, is commonly associated with long distance transportation and is an area that has been studied in some detail previously (Austin et al., 1995; Copas, 2011; Leadon et al., 1989; Oikawa et al., 2005; Oikawa et al., 1994; Oikawa et al., 2004; Raidal, 1995; Raidal et al., 1997a; Raidal et al., 1996). We reported an incidence of transport pneumonia of 9.2% (Chapter 3.2) which was similar to a Japanese study which included journeys of 1000-1300 km (Oikawa and Jones, 2000). Race horses, in particular Thoroughbreds, were confirmed to be at greater risk (Austin et al., 1995). Journey duration and the head position were confirmed as risk factors (Chapter 2, Chapter 3.4, Chapter 5), as has been previously reported (Austin et al., 1995; Raidal et al., 1996; Stull and Rodiek, 2002). In Chapter 5 a positive correlation between stress-related behaviours, elevated head position and increased mucus and bacteria load in the airways was demonstrated. From our findings, monitoring the behaviour *en route* (licking/chewing frequency, evasive behaviours frequency, head down duration) may be useful in identifying of horses at risk of developing respiratory diseases after the journey. However, based on the thesis findings, it seems that in order to adopt the beneficial lower head position horses need to experience low levels of stress prior to and during transport. This could be achieved by using larger bays, tying long or not tying at all, travelling in a quiet environment, motivating them to lower their head by offering food and habituating them to travelling by appropriate training methods. In Chapter 5, the increase in bacteria load was due to an increase of oropharyngeal commensally bacteria (mainly *Pasteurellaceae*) in agreement with the literature (Racklyeft and Love, 1990; Raidal et al., 1995). For the first time a genetic approach to bacteriology was used to document the changes in respiratory flora induced by transportation (Chapter 5). The study clearly illustrates that transport enables opportunistic proliferation of specific commensally bacteria in so reducing the respiratory flora diversity and hence potentially enabling these key species (mostly those in the *Pasteurellaceae* family at least in this study) to invade the lower respiratory tract. From our results, *Streptococcus* spp were identified relatively infrequently, however, animals where streptococci were evident in the airways before the journey tended to have more *Streptococcus* spp at unloading. Like *Pasteurellaceae*, *Streptococcus equi* subsp. *zooepidemicus* has been identified in cases of transport pneumonia (Mair and Lane, 1989;

Oikawa et al., 1994), and our data identified that species in this genus can also proliferate and potentially become an opportunistic invader when the respiratory system is compromised by stress and postural adjustments during transportation (Mair and Lane, 1989; Oikawa et al., 1994)(Chapter 1). From our data, the tracheal inflammation score was greatly increased in horses which had a higher score at preloading, confirming the popular motto that "if we let a sick horse travel, we will have a sicker one" (Leadon and Hodgson, 2014). The assessment of the fitness for travel is mandatory in Australia, but it does not include a specific clinical examination of the respiratory system. Based on the thesis findings, we suggest that lung auscultation could be conducted pre transport and in doubtful cases endoscopy performed to evaluate respiratory health prior to transport. The assessment of fitness after travel including auscultation is as important as the fitness assessment prior to travel. Post travel management is also important to reduce the incidence of respiratory disease. However, the Australian Code does not include any guidelines for the post transport period. In Chapter 4, following transport, horses were at pasture during daytime and within 24 hours all signs of respiratory insult had disappeared, and the respiratory system was completely restored by 7 days. Contrariwise, in Chapter 5 the horses were stabled after the journey and when scoped 5 days after the journey, 6 out of 11 showed protracted inflammation of the airways. In the literature, it has been suggested that horses confined by cross ties in stables and during travel accumulate mucus and bacteria in the airways, but they are able to clear these within 24 hours when kept on pasture (Racklyeft and Love, 1990; Raidal et al., 1996). Keeping the animal on pasture for 24 hours after transportation to facilitate self-clearance of the respiratory system during grazing is therefore recommended. However, since this thesis was mainly composed of surveys and observational studies, there is a need for applied research comparing the effects of these factors on horse behaviour *en route*, after the journey and the consequent inflammation of the respiratory tract. Moreover, none of the animals used in our experiments developed transport-pneumonia, thus our findings cannot be generalised for this pathology. A prospective study should be carried out collecting and comparing behavioural, haematological, immunological, oxidative stress and respiratory (including bacteria and virus) data between horses who do and don't develop transport pneumonia.

Transportation can be fatal for animals (Chapter 1). The mortality rate reported in Chapter 2 was higher than that reported in other countries (Roy et al., 2015; Stefancic and Martin, 2005). This could be related to the particularly long duration of the journey (4000 km), the Australian climate and the type of horses (i.e. performance and not slaughter horses). However, the mortality rate calculated in Chapter 3.1 is more consistent with what expected, suggesting that the mortality rate reported in Chapter 2 may not be valid for all Australian equine transportation. The major cause of death/euthanasia *en route* was trauma (bone fractures, Chapter 2, 3.1, 3.4). However, some horses were found dead *en route* or after arrival, and it was not possible to determine a diagnosis. These findings highlight the importance of surveillance cameras in animal transport vehicles and of assessment of fitness after travel. Many horses may be saved if they were monitored to enable identification and management of their distress before they had the fatal accident. Surveillance cameras are mandatory in commercial animal vehicles for journeys longer than 8 hours in Europe (http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=uriserv:f83007), but the use of surveillance cameras is only a guideline in the Australian code, and only 20% of Australian survey respondents used them (Chapter 3.1). As transport-related health problems usually manifest within days after the journey (Chapter 1), monitoring the animals using the clinical examination sheet reported in Chapter 5 could be recommended at unloading and at least once daily for 5 days after journeys longer than 20-24 hours (Chapter 2, Chapter 3.4), which have been identified as a greater risk. Our findings suggest this requirement could be a valuable addition to the Australian Code.

This thesis found new risk factors for transport-related health problems, such as the season (i.e. spring), some breeds, management practices, but critically journey duration is the major risk factor (Chapter 2, Chapter 3.4). It is clear that journeys longer than 24 hours pose a greater risk for horse health and welfare. In Australia, the maximum time without water is 24 hours, however horses may be transported for periods up to 36 hours if water is offered during the journey (http://www.animalwelfarestandards.net.au/land-transport/). In Europe the maximum time without water is 8 hours and when water is offered the maximum journey duration is 24 hours (http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=uriserv:f83007). In Chapter 5, the haematological responses were not of biological significance and none of the horses developed pneumonia. Accordingly the current Australian maximum duration of food and water curfew and journey duration may need to be revised to mirror European watering guidelines (Anon, 2014). While distances in Australia are clearly greater than in Europe, and increased intra-journey rest stops with unloading could increase the total journey duration and the risk of injuries, the installation of on board watering system in vehicles (Iacono et al., 2007b), or more frequent use of transportation by plane for interstate journeys may be worth considering. It has been suggested that transportation by plane is less stressful than by road (Munsters et al., 2013) but few studies have been conducted to compare the two (Chapter 1). Further studies are needed to investigate the effects of airplane transportation on behavioural, clinical, haematological, oxidative, respiratory and immunological parameters, in a similar manner as done for road transport in this thesis (Chapters 4 and 5).

The association between journey duration and transport-related severe health problems (Chapter 2, Chapter 3.4) could be explained by the decreased immunological ability to react to a stimulus (i.e. pathogen) after long distance transportation (Chapter 4). In Chapter 4 a decrease in lymphocyte activity was documented. Chapter 4 was the first multidisciplinary study ever conducted documenting the effect of a 4000 km journey on immunology, haematology and oxidative parameters. Unfortunately we were not able to assess viral or bacteriological load in this study. However, in Chapter 5 we observed possible reactivation/shedding of gamma herpes virus associated with 8h transportation, and their possible role of immunosuppression following long distance transportation warrants future research.

Transportation induces dehydration and weight loss (Marlin, 2004). We found different grades of dehydration dependent on journey duration (Chapter 4 and 5). Interestingly in both studies increased haematocrit after the journey was not observed, however increases in other indicators of dehydration (capillary refill time (CRT), body weight (BW) lost, total protein (TP), albumin (Alb)) were observed. CRT has been identified as a reliable on-farm equine welfare indicator (Dalla Costa et al., 2014), but it was implemented only by two of our survey respondents (Chapter 3.1). Since CRT is a non-invasive and simple method of assessing the absence of prolonged thirst (Dalla Costa et al., 2014), and we found good association between CRT and transport-induced dehydration in our experiments, we suggest that CRT should be monitored before, during and after journey. When CRT is >3s, water should be offered and, if necessary, other rehydration techniques implemented. In Chapter 4 we monitored the BW using a tape. This is a cheaper way than using a scale, thus monitoring the BW lost by tape could also be recommended. In Chapter 5 we noticed that BW, TP and Alb did not recover within 24 hours of arrival, and we suggest at least one day of rest after an 8h journey. Horses should not be exercised until they recover; assessment of CRT and BW might be useful to plan when start working the horses after transport.

Transportation induces an acute phase response (Chapter 4, 5), with neutrophilia, hyperglobulinemia, increases in oxidative products and acute phase proteins (APPs). Among the APPs we monitored the concentration of fibrinogen, serum amyloid A and ceruloplasmin (CP). Fibrinogen was suggested as a good biomarker of respiratory diseases after travel (Leadon, 2000) and and our data also demonstrated increased fibrinogen following transportation. Serum amyloid A increases rapidly after tissue injury, infection or inflammation (Crisman et al., 2008), but we did not find any effect of transportation on this. CP is an α_2 globulin, which usually increases in case of acute inflammatory disorders, but it is not commonly determined in horses (Crisman et al., 2008; Murata et al., 2004). CP has several functions including the transport of copper, ferroxidase activity, modulation of coagulation, angiogenesis, inactivation of biogenic amines and defense against oxidative stress (Chauhan et al., 2004). However, CP appears to have multiple and contrasting functions in the redox balance because pro-oxidant activity has also been reported. It has been identified as a biomarker for cardiovascular disease in humans (Shukla et al., 2006). Higher CP levels were found in horses with *Rhodococcus equi* pneumonia (Barton and Embury, 1987) and laminitis (Fagliari et al., 1998). In Chapter 5, CP increased at unloading and the day after travel. A slight increase of CP was found at unloading in camel transported for one hour (Baghshani et al., 2010), but CP has not previously been evaluated in transported horses. This thesis provides reference values and future studies are needed to investigate whether CP might be a possible new biomarker in transport-related diseases in horses.

When free radicals are not balanced by antioxidants, the host experiences oxidative stress (Celi, 2011). Oxidative stress has been linked to many diseases in horses: colic (Kirschvink et al., 2008), laminitis (Treiber et al., 2009; Yin et al., 2009) and respiratory disease (Crowley et al., 2013; Deaton et al., 2006; Kirschvink et al., 2002; Po et al., 2013). In view of the similarity between oxidative and transport stress-induced diseases, it was hypothesed that the development of oxidative stress after transportation could be involved in the development of disease after transport. In both experimental studies (Chapters 4, 5) transportation caused a mobilisation of antioxidants, as shown by PTAS, which firstly was increased at unloading, and then significantly decreased. Antioxidants are stored in different tissues, particularly in fat (Celi, 2011), and horses are able to *de novo* synthesis of antioxidants, namely ascorbic acid (vitamin C) in their liver (Grollman and Lehninger, 1957; Jaeschke, 1984). Under stress, horses mobilise antioxidants to balance the stress-induced increase in oxidative products (Ralston and Stives, 2012). The different values of PTAS

(Chapter 4: ~5000 and Chapter 5: ~3000 U.Carr), registered at unloading in the two studies, might be related to the difference in journey duration, but also different in horse management, diet, breed and age (Kusano et al., 2016). The horses used in Chapter 4 were show jumping horses of different breed, aged from 5 to 15 years (10.3 ± 3.2), with a BCS of 3 out of 5 (Carroll and Huntington, 1988), kept on pasture and fed with a commercial food balanced for vitamins and electrolytes. The horses used in Chapter 5 were research horses (6 Standarbreds and 6 Thoroughbred), aged from 3 to 8 years (4.9 ± 1.9) with a lower BCS, stabled and fed with oats. Differences in antioxidant storage might explain therefore the discrepancy in the values. Even if the values were different the trend was similar, with a significant decrease in antioxidants after the journey. Stress of any type, including transport stress is capable of depleting the body's antioxidant resources (Sconberg et al., 1993). Thus, our findings suggest that a depletion of antioxidant due to transport stress might lead to oxidative stress, facilitating the development of health problems. Supplementation with antioxidants before and after the journey might be useful to decrease the risk of oxidative stress and transport-related disease, but further testing is required. Low levels of vitamin C in TW and BAL were related to RAO in horses (Deaton et al., 2004). In Chapter 5 a positive correlation between the stress behaviours and the mucus score and OSI was found. While further research is required on the nexus between transport stress, oxidative stress and transport-related respiratory diseases, our findings suggest that oxidative stress parameters may be useful as an indicator of the welfare of transported horses as is currently reported in ewes (Piccione et al., 2013) and camels (Nazifi et al., 2009).

It is important to note that transportation is also a biosecurity hazard (Herholz et al., 2008). Our behavioural data documented that licking trailers' wall and partitions, sniffing around and interacting with the neighbour animals are common behaviours (Chapter 5). Thus it is important that horses are tested for infectious diseases prior to transport and that each part of the interior of the vehicles is disinfected after each journey. The Federation Equestre Internationale (FEI) has recently issued new biosecurity rules for high performance horses which travel internationally (OIE, 2016). These new rules, which include mandatory health check, should be considered also for national movements of other performance horses.

This thesis clearly demonstrated that transportation is a health and welfare concern in horses. A trans-disciplinarily approach was used, in agreement with the current recommendations on best practice in the conduct of animal welfare research (Veissier and Miele, 2014, 2015). Our holistic approach is a validated model for studying the implications of transportation in other species in the future.

6.2. Conclusions

This thesis achieved its aim of increasing the understanding of transportation stress and its implications for horse health, wellbeing and welfare. It has also identified a number of potential areas for further research. The findings of this thesis will assist horse owners, breeders, trainers, nutritionists, veterinarians, and researchers to improve transport management to decrease the incidence of transport-related behavioural and health problems.

Transportation may be a human-related risk to horses and it might also be a horse-related risk for humans due to economical (Gordon, 2001), psychological (Foster and Maples, 2014) and physical (Riley et al., 2015; Thompson et al., 2015) involvement. It should always be carried out with high levels of professional competence, applying evidence-based best practices to reduce its negative impacts on horse and human health and welfare.

A full list of recommendations from the studies presented in this thesis to promote horse and human health, wellbeing and welfare are as follows:

1. Equine industry members need to be educated on equine transportation risk factors, best practices and policies.
2. Policing of compliance of the equine movements with the Australian Code should be implemented.
3. Horse movements should be planned and managed carefully to minimise transport stress, particularly where journeys are longer than 20 h, carried out during the Australian spring or involve the transport of racing horses, particularly Thoroughbreds.
4. Protective equipment such as boots and rugs should be used only where horses have been habituated, checked during the travel and used only for short journeys.
5. The use of sedation and other medication before transportation should be minimised and administrated only after veterinary consultation.
6. Horses should be trained for loading and travelling using habituation and self-loading training approaches.

7. Horses should have access to hay and water *ad libitum* before travelling.
8. Fitness for travel must be always correctly assessed; before long journeys, respiratory endoscopy should be conducted to avoid transporting animals with subclinical respiratory diseases.
9. Transportation should be always conducted with the highest levels of professional competence including horse handling and driving skills.
10. Horses should be allowed to lower their head to floor level during transportation.
11. Arousing stimuli during transportation should be kept to a minimum.
12. Environmental parameters should be monitored during transport.
13. Horse behaviour *en route* should be monitored using surveillance cameras.
14. The maximum journey time and the maximum time without water should be decreased in Australia, as already done in Europe.
15. Horse health should be assessed after transportation. Visual inspection, monitoring temperature and gut and lung sounds auscultation should be carried out at least once daily for 5 days after arrival, to promptly identify animals with possible diseases.
16. Horses that spend most of their journey in an elevated head position, performing often stress-related behaviours (i.e. licking/chewing, touching rubber tie, pawing), should be monitored closely after a journey.
17. Horses should be allowed to rest for at least 24 hours following 8 hours of transportation.
18. Horses should be allowed to lower their heads as long as possible after transportation therefore keeping horses on pasture post travel is recommended.
19. Monitoring fibrinogen levels before and after a journey is beneficial to identify horses at risk of transport/related health problems.
20. Monitoring oxidative balance (by ROMs and PTAS) might be a useful tool to assess horse welfare during and after travelling.
21. Monitoring the hydration status by CRT and weight loss (using a weight tape or scales) is recommended following transport to enable appropriate rehydration strategies to be implemented where required.

Based on the research questions arising from this thesis, a list of future research projects are as follows:

1. Effects of an administration of antioxidants before and after a journey on clinical, behavioural, immunological, oxidative and respiratory parameters in horses.
2. Effects of transportation on gastric pH and gastrointestinal microbiota.
3. Effects of an administration of an immune-modulator before a journey on clinical, behavioural, immunological, oxidative and respiratory parameters in horses.
4. Effects of different management practices after a journey (i.e. housing vs pasture) on clinical, behavioural, immunological, oxidative and respiratory parameters in horses.
5. Effects of transportation by air on clinical, behavioural, immunological, oxidative and respiratory parameters in horses.
6. Effects of different loading and travelling training methods on behavioural and physiological parameters.
7. Effects of space allocation of the individual stall in the transport vehicle on horse health, behaviour and welfare.
8. Effects of different position *en route* on horse health, behaviour and welfare.
9. Effects of different two horse trailer design on horse health, behaviour and welfare.
10. A survey on horse transportation issues and practice worldwide.
11. A longitudinal prospective study on transport related behavioural and health problems.
12. A novel scale of behavioural indicators of stress in travelling horse.
13. The development of reliable animal base welfare indicators during transportation.

References

Adenkola, A., Ayo, J., Sackey, A., Adelaiye, A., 2011. Modulatory role of ascorbic acid on behavioural responses of pigs transported by road during the Harmattan Season. Nigerian Journal of Physiological Sciences 26.

Alexander, S., Irvine, C., 1998. Stress in the racing horse: coping vs not coping. Journal of equine science 9, 77-81.

Allano, M., Labrecque, O., Batista, E.R., Beauchamp, G., Bédard, C., Lavoie, J.-P., Leclere, M., 2016. Influence of short distance transportation on tracheal bacterial content and lower airway cytology in horses. The Veterinary Journal 214, 47-49.

Allen, G., Murray, M., 2004. Equid herpesvirus 2 and equid herpesvirus 5 infections, In: Infectious diseases of livestock. Oxford University Press, Newmarket (United Kingdom), p. 860.

Anderson, N., DeBowes, R., Nyrop, K., Dayton, A., 1985. Mononuclear phagocytes of transport-stressed horses with viral respiratory tract infection. American Journal Veterinary Research 46, 2272-2277.

Anon, 2014. Practical guidelines on the watering of equine animals transported by road. World Horse Welfare, London,Europe, p. 36.

Anzai, T., Fukunaga, Y., Matsumura, T., Imagawa, H., Oikawa, M.-a., 2001. Serological examination for viral infection among young racehorses transported by vehicle over a long distance. Journal of Equine Science 12, 135-137.

Artursson, K., Wallgren, P., Alm, G.V., 1989. Appearance of interferon-α in serum and signs of reduced immune function in pigs after transport and installation in a fattening farm. Veterinary Immunology and Immunopathology 23, 345-353.

Atiyah, M., Bott, R., Patodi, V.K., 1973. On the heat equation and the index theorem. Inventiones Mathematicae 19, 279-330.

Austin, S.M., Foreman, J.H., Hungerford, L.L., 1995. Case-control study of risk factors for development of pleuropneumonia in horses. Journal of the American Veterinary Medical Association 207, 325-328.

371Averós, X., Knowles, T.G., Brown, S.N., Warriss, P.D., Gosálvez, L.F., 2010. Factors affecting the mortality of weaned piglets during commercial transport between farms. Veterinary Record 167, 815-819.

References

Baghshani, H., Nazifi, S., Saeb, M., Saeb, S., 2010. Influence of road transportation on plasma concentrations of acute phase proteins, including fibrinogen, haptoglobin, serum amyloid A, and ceruloplasmin, in dromedary camels (Camelus dromedarius). Comparative Clinical Pathology 19, 193-198.

Baker, M.A., Cerniglia, G.J., Zaman, A., 1990. Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples. Analytical Biochemistry 190, 360-365.

Baragli, P., Padalino, B., Telatin, A., 2015. The role of associative and non-associative learning in the training of horses and implications for the welfare (a review). Annali Istituto Superiore di Sanita’ 51, 40-51.

Baron, A., 1991. Avoidance and punishment, In: Experimental analysis of behavior. Elsevier, New York, USA, pp. 173-217.

Barton, M., Embury, D., 1987. Studies of the pathogenesis of Rhodococcus equi infection in foals. Australian Veterinary Journal 64, 332-339.

Baucus, K.L., Ralston, S.L., Nockels, C.F., McKinnon, A.O., Squires, E.L., 1990a. Effects of transportation on early embryonic death in mares. Journal of Animal Science 68, 345-351.

Baucus, K.L., Squires, E.L., Ralston, S.L., McKinnon, A.O., Nett, T.M., 1990b. Effect of transportation on the estrous cycle and concentrations of hormones in mares. Journal of Animal Science 68, 419-426.

Beaunoyer, D., Chapman, J., 1987. Trailering stress on subsequent submaximal exercise performance, In: Proceedings of 11th Equine Nutrition and Physiology Symposium, pp. 379-384.

Bell, S.A., Balasuriya, U.B., Gardner, I.A., Barry, P.A., Wilson, W.D., Ferraro, G.L., MacLachlan, N.J., 2006. Temporal detection of equine herpesvirus infections of a cohort of mares and their foals. Veterinary Microbiology 116, 249-257.

Benzie, I.F., Strain, J., 1996. The ferric reducing ability of plasma (FRAP) as a measure of “antioxidant power”: the FRAP assay. Analytical Biochemistry 239, 70-76.

Berndt, A., Derksen, F.J., Robinson, N.E., 2010. Endotoxin concentrations within the breathing zone of horses are higher in stables than on pasture. The Veterinary Journal 183, 54-57.

Blecha, F., Boyles, S.L., Riley, J.G., 1984. Shipping suppresses lymphocyte blastogenic responses in Angus and Brahman× Angus feeder calves. Journal of Animal Science 59, 576-583.

Boden, L.A., Parkin, T.D.H., Yates, J., Mellor, D., Kao, R.R., 2013. An online survey of horse-owners in Great Britain. BMC Veterinary Research 9, 188-199.

References

Bohák, Z., Szabo, F., Beckers, J.-F., de Sousa, N.M., Kutasi, O., Nagy, K., Szenci, O., 2013. Monitoring the circadian rhythm of serum and salivary cortisol concentrations in the horse. Domestic Animal Endocrinology 45, 38-42.

Böhm, R., 1998. Disinfection and hygiene in the veterinary field and disinfection of animal houses and transport vehicles. International Biodeterioration & Biodegradation 41, 217-224.

Bordignon, M., Da Dalt, L., Marinelli, L., Gabai, G., 2014. Advanced oxidation protein products are generated by bovine neutrophils and inhibit free radical production in vitro. Veterinary Journal 199, 162-168.

Broom, D., 2005. The effects of land transport on animal welfare. Revue Scientifique et Technique (International Office of Epizootics) 24, 683-691.

Broom, D., 2008. The welfare of livestock during road transport. Long distance transport and the welfare of farm animals. CABI, Wallingford, UK, 157-181.

Broom, D.M., Johnson, K.G., 1993. Stress and animal welfare. Springer Science & Business Media.

Bruce, G., 2009. How to click with your horse : clicker training for groundwork, riding & problem solving. Click With Horses, Kuranda, Qld.

Burrell, M.H., 1985. Endoscopic and virological observations on respiratory disease in a group of young Thoroughbred horses in training. Equine Veterinary Journal 17, 99-103.

Calabrese, R., Friend, T.H., 2009. Effects of density and rest stops on movement rates of unrestrained horses during transport. Journal of Equine Veterinary Science 29, 782-785.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K., Gordon, J.I., 2010. QIIME allows analysis of high-throughput community sequencing data. Nature Methods 7, 335-336.

Carroll, C., Huntington, P., 1988. Body condition scoring and weight estimation of horses. Equine Veterinary Journal 20, 41-45.

Casamassima, D., Palazzo, M., Presutti, T., Cinone, M., 2008. Effects of two tame systems on physiological parameters of Arab horses subjected to load in the trailer. Ippologia 19, 13-19.

Casella, S., Fazio, F., Giannetto, C., Giudice, E., Piccione, G., 2012. Influence of transportation on serum concentrations of acute phase proteins in horse. Research in Veterinary Science 93, 914-917.

References

Cavallone, E., Di Giancamillo, M., Secchiero, B., Belloli, A., Pravettoni, D., Rimoldi, E., 2002. Variations of serum cortisol in Argentine horses subjected to ship transport and adaptation stress. Journal of Equine Veterinary Science 22, 541-545.

Cave, J.G., Callinan, A.P.L., Woonton, W.K., 2005. Mortalities in bobby calves associated with long distance transport. Australian Veterinary Journal 83, 82-84.

Celi, P., 2011. Biomarkers of oxidative stress in ruminant medicine. Immunopharmacology and Immunotoxicology 33, 233-240.

Celi, P., Gabai, G., 2015. Oxidant/antioxidant balance in animal nutrition and health: the role of protein oxidation. Frontiers in Veterinary Science 2, 48.

Chauhan, A., Chauhan, V., Brown, W.T., Cohen, I., 2004. Oxidative stress in autism: increased lipid peroxidation and reduced serum levels of ceruloplasmin and transferrin-the antioxidant proteins. Life Sciences 75, 2539-2549.

Chirase, N.K., Greene, L.W., Purdy, C.W., Loan, R.W., Auvermann, B.W., Parker, D.B., Walborg Jr, E.F., Stevenson, D.E., Xu, Y., Klaunig, J.E., 2004. Effect of transport stress on respiratory disease, serum antioxidant status, and serum concentrations of lipid peroxidation biomarkers in beef cattle. America Journal Veterinary Research 65, 860-864.

Christley, R., 2016. Questionnaire survey response rates in equine research. Equine Veterinary Journal 48, 138-139.

Clark, D.K., Friend, T.H., Dellmeier, G., 1993. The effect of orientation during trailer transport on heart rate, cortisol and balance in horses. Applied Animal Behaviour Science 38, 179-189.

Cockram, M., Baxter, E., Smith, L., Bell, S., Howard, C., Prescott, R., Mitchell, M., 2004. Effect of driver behaviour, driving events and road type on the stability and resting behaviour of sheep in transit. Animal Science 79, 165.

Cockram, M., Spence, J., 2012. The effects of driving events on the stability and resting behaviour of cattle, young calves and pigs. Animal Welfare 21, 403-417.

Codazza, D., Maffeo, G., Redaelli, G., 1974. Serum enzyme changes and hemato-chemical levels in Thoroughbreds after transport and exercise. Journal of the South African Veterinary Association 45, 331-335.

Cohen, J.M., Boniface, S., Watkins, S., 2014. Health implications of transport planning, development and operations. Journal of Transport & Health 1, 63-72.

References

Cohen, N.D., O'Conor, M.S., Chaffin, M.K., Martens, R.J., 2005. Farm characteristics and management practices associated with development of Rhodococcus equi pneumonia in foals. Journal of the American Veterinary Medical Association 226, 404-413.

Cohen, N.D., Peloso, J.G., 1996. Risk factors for history of previous colic and for chronic, intermittent colic in a population of horses. Journal of the American Veterinary Medical Association 208, 697-703.

Collins, M.N., Friend, T.H., Jousan, F.D., Chen, S.C., 2000. Effects of density on displacement, falls, injuries, and orientation during horse transportation1. Applied Animal Behaviour Science 67, 169-179.

Copas, V., 2011. Diagnosis and treatment of equine pleuropneumonia. Practice 33, 155-162.

Couëtil, L., Cardwell, J., Gerber, V., Lavoie, J.P., Léguillette, R., Richard, E., 2016. Inflammatory airway disease of horses — revised consensus statement. Journal of Veterinary Internal Medicine, 30, 503-515.

Council, A.H., 2005. The economic impact of the horse industry on the United States National report. Barents Group LLC, USA.

Covalesky, M., Russoniello, C., Malinowski, K., 1992. Effects of show-jumping performance stress on plasma cortisol and lactate concentrations and heart rate and behavior in horses. Journal of Equine Veterinary Science 12, 244-251.

Crabb, B., MacPherson, C., Reubel, G., Browning, G., Studdert, M., Drummer, H., 1995. A type-specific serological test to distinguish antibodies to equine herpesviruses 4 and 1. Archives of Virology 140, 245-258.

Cray, C., Zaias, J., Altman, N.H., 2009. Acute phase response in animals: a review. Comparative Medicine 59, 517-526.

Cregier, S.E., 1982. Reducing equine hauling stress: a review. Journal of Equine Veterinary Science 2, 186-198.

Cregier, S.E., 1984. Reducing equine hauling stress. Proceedings of the International Congress on Applied Ethology in Farm Animals, Kiel 1984, 175-178.

Cregier, S.E., 2010. Best practices: surface transport of the horse. Proceedings of Animal Transportation Association, AATA Education Committee, Canada, 1e29.

Cregier, S.E., Gimenez, R., 2015. Non-commercial horse transport: new standard for trailer in Canada. Cregier SE, Montague, Canada.

Crisman, M.V., Scarratt, W.K., Zimmerman, K.L., 2008. Blood proteins and inflammation in the horse. Veterinary Clinics of North America: Equine Practice 24, 285-297.

Cross, N., van Doorn, F., Versnel, C., Cawdell-Smith, J., Phillips, C., 2008. Effects of lighting conditions on the welfare of horses being loaded for transportation. Journal of Veterinary Behavior: Clinical Applications and Research 3, 20-24.

Crotty, S., Aubert, R.D., Glidewell, J., Ahmed, R., 2004. Tracking human antigen-specific memory B cells: a sensitive and generalized ELISPOT system. Journal of Immunological Methods 286, 111-122.

Crowley, J., Po, E., Celi, P., Muscatello, G., 2013. Systemic and respiratory oxidative stress in the pathogenesis and diagnosis of Rhodococcus equi pneumonia. Equine Veterinary Journal 45, 20-25.

Cullinane, A., 2014. Equine influenza and air transport. Equine Veterinary Education 26, 456-457.

Curcio-Wolfe, J., 1996. Tellington-touch, In: Proceedings of the 1996 American Holistic Veterinary Medical Association Annual Conference, pp. 12-13.

Dalla Costa, E., Murray, L., Dai, F., Canali, E., Minero, M., 2014. Equine on-farm welfare assessment: a review of animal-based indicators. Animal Welfare 23, 323-341.

Darth, A.C. 2014. Identifying causes and preventing injuries to horses. Thesis in Biology, Swedish University of Agricultural Sciences.

de Silva, K., Begg, D., Carter, N., Taylor, D., Di Fiore, L., Whittington, R., 2010. The early lymphocyte proliferation response in sheep exposed to Mycobacterium avium subsp. paratuberculosis compared to infection status. Immunobiology 215, 12-25.

de Witte, K., 2009. Development of the Australian animal welfare standards and guidelines for the land transport of livestock: process and philosophical considerations. Journal of Veterinary Behavior: Clinical Applications and Research 4, 148-156.

Dean, R., 2015. The use and abuse of questionnaires in veterinary medicine. Equine Veterinary Journal 47, 379-380.

De Araugo, J., McLaren, S., McManus, P., McGreevy, P., 2016. Improving the understanding of psychological factors contributing to horse-related accident and injury: context, loss of focus, cognitive errors and rigidity. Animals 6, 12.

Deaton, C., Marlin, D., Deaton, L., Smith, N., Harris, P., Schroter, R., Kelly, F., 2006. Comparison of the antioxidant status in tracheal and bronchoalveolar epithelial lining fluids in recurrent airway obstruction. Equine Veterinary Journal 38, 417-422.

Deaton, C.M., Marlin, D.J., Smith, N.C., Smith, K.C., Newton, R.J., Gower, S.M., Cade, S.M., Roberts, C.A., Harris, P.A., Schroter, R.C., 2004. Breath condensate hydrogen peroxide correlates with both airway cytology and epithelial lining fluid ascorbic acid concentration in the horse. Free Radical Research 38, 201-208.

De Santis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., Huber, T., Dalevi, D., Hu, P., Andersen, G.L., 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Applied and Environmental Microbiology 72, 5069-5072.

Dunlap, K.L., Reynolds, A.J., Duffy, L.K., 2006. Total antioxidant power in sled dogs supplemented with blueberries and the comparison of blood parameters associated with exercise. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 143, 429-434.

Dwyer, J., Johnson, C., 1981. The use of concanavalin A to study the immunoregulation of human T cells. Clinical and Experimental Immunology 46, 237.

Eades, S.C., Bounous, D.I., 1997. Laboratory profiles of equine diseases. Mosby, St. Louis, Mo.

Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26, 2460-2461.

Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R., 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27, 2194-2200.

Fagliari, J., McClenahan, D., Evanson, O., Weiss, D., 1998. Changes in plasma protein concentrations in ponies with experimentally induced alimentary laminitis. American Journal Veterinary Research 59, 1234-1237.

Fallon, K., Fallon, S., Boston, T., 2001. The acute phase response and exercise: court and field sports. British Journal of Sports Medicine 35, 170-173.

Fazio, E., Ferlazzo, A., 2003. Evaluation of stress during transport. Veterinary Research Communications 27, 519-524.

Fazio, E., Medica, P., Aronica, V., Grasso, L., Ferlazzo, A., 2008a. Circulating β-endorphin, adrenocorticotrophic hormone and cortisol levels of stallions before and after short road transport: stress effect of different distances. Acta Veterinaria Scandinavica 50, 1-7.

Fazio, E., Medica, P., Cravana, C., Ferlazzo, A., 2008b. Effects of competition experience and transportation on the adrenocortical and thyroid responses of horses. Veterinary Record: Journal of the British Veterinary Association 163, 713-716.

References

Fazio, E., Medica, P., Cravana, C., Aveni, F., Ferlazzo, A., 2013a. Comparative endocrinological responses to short transportation of Equidae (Equus asinus and Equus caballus). Animal Science Journal 84, 258-263.

Fazio, E., Medica, P., Cravana, C., Ferlazzo, A., 2013b. Cortisol response to road transport stress in calm and nervous stallions. Journal of Veterinary Behavior: Clinical Applications and Research 8, 231-237.

Fazio, E., Medica, P., Cravana, C., Ferlazzo, A., 2015. Comparative effects of simulated and conventional transportations on the thyroid response of stallions (Equus caballus). Journal of Equine Veterinary Science, 35, 894-900.

Feary, D.J., Hassel, D.M., 2006. Enteritis and colitis in horses. Veterinary Clinics of North America - Equine Practice 22, 437-479.

Felippe, M.J.B., 2016. The Immune System, In: Equine Clinical Immunology. John Wiley & Sons, Inc., pp. 1-10.

Ferguson, D.L., Rosales-Ruiz, J., 2001. Loading the problem loader: the effect of target training and shaping on trailer-loading behavior of horses. Journal of Applied Behavior Analysis 34, 409-423.

Firestone, S.M., Schemann, K.A., Toribio, J.A.L.M.L., Ward, M.P., Dhand, N.K., 2011. A case-control study of risk factors for equine influenza spread onto horse premises during the 2007 epidemic in Australia. Preventive Veterinary Medicine 100, 53-63.

Fong, D., Gaulin, C., Lê, M.-L., Shum, M., 2011. Effectiveness of Alternative Antimicrobial Agents for Disinfection of Hard Surfaces. National Collaborating Centre for Environmental Health, 1-19.

Foster, S.M., Maples, E.H., 2014. Occupational stress in veterinary support staff. Journal of Veterinary Medical Education 41, 102-110.

Friend, T., 2001. A review of recent research on the transportation of horses. Journal of Animal Science 79, 9.

Friend, T.H., 2000. Dehydration, stress, and water consumption of horses during long-distance commercial transport. Journal of Animal Science 78, 2568-2580.

Fureix, C., Jego, P., Henry, S., Lansade, L., Hausberger, M., 2012. Towards an ethological animal model of depression? A study on horses. PLoS One 7, e39280.

Gajewski, T.F., Fitch, F.W., 1988. Anti-proliferative effect of IFN-gamma in immune regulation. I. IFN-gamma inhibits the proliferation of Th2 but not Th1 murine helper T lymphocyte clones. The Journal of Immunology 140, 4245-4252.

Garey, S.M., Friend, T.H., Sigler, D.H., Berghman, L.R., 2010. The effects of loose group versus individual stall transport on glucocorticosteriods and dehydroepiandrosterone in yearling horses. Journal of Equine Veterinary Science 30, 696-700.

Gerber, V., Straub, R., Marti, E., Hauptman, J., Herholz, C., King, M., Imhof, A., Tahon, L., Robinson, N., 2004. Endoscopic scoring of mucus quantity and quality: observer and horse variance and relationship to inflammation, mucus viscoelasticity and volume. Equine Veterinary Journal 36, 576-582.

Gibbs, A., Friend, T., 1999. Horse preference for orientation during transport and the effect of orientation on balancing ability. Applied Animal Behaviour Science 63, 1-9.

Gibbs, P., Benefield, M., Potter, G., McNeill, J., Johnson, B., Moyer, W., 1997. Profile of horse ownership and use in 8 Texas counties–phase 2 of the Texas horse industry quality audit. Proceedings of the 15th Equine Nutrition and Physiology Society, Texas, USA, 321-325.

Gibbs, P., Potter, G., Jones, L., Benefield, M., McNeill, J., Johnson, B., Moyer, B., 1998. The Texas horse industry. Retrieved April 15, 2004.

Gilkerson, J., Whalley, J., Drummer, H., Studdert, M., Love, D., 1999. Epidemiology of EHV-1 and EHV-4 in the mare and foal populations on a Hunter Valley stud farm: are mares the source of EHV-1 for unweaned foals. Veterinary Microbiology 68, 27-34.

Giovagnoli, G., 2008. Manuale teorico-pratico sul trasporto del cavallo. Mursia Editore, Milano, Italy, 1.

Giovagnoli, G., Trabalza Marinucci, M., Bolla, A., Borghese, A., 2002. Transport stress in horses: an electromyographic study on balance preservation. Livestock Production Science 73, 247-254.

González, L.A., Schwartzkopf-Genswein, K.S., Bryan, M., Silasi, R., Brown, F., 2012. Relationships between transport conditions and welfare outcomes during commercial long haul transport of cattle in North America. Journal of Animal Science 90, 3640-3651.

Gordon, J., 2001. The horse industry contributing to the Australian economy, Canberra, Australia, p. 58.

Grandin, T., McGee, K., Lanier, J.L., 1999. Prevalence of severe welfare problems in horses that arrive at slaughter plants. Journal of the American Veterinary Medical Association 214, 1531-1533.

References

Grandin, T., Shivley, C., 2015. How farm animals react and perceive stressful situations such as handling, restraint, and transport. Animals 5, 04-09.

Grollman, A.P., Lehninger, A.L., 1957. Enzymic synthesis of L-ascorbic acid in different animal species. Archives of Biochemistry and Biophysics 69, 458-467.

Hargreaves, B., Kronfeld, D., Waldron, J., Lopes, M., Gay, L., Saker, K., Cooper, W., Sklan, D., Harris, P., 2002. Antioxidant status and muscle cell leakage during endurance exercise. Equine Veterinary Journal 34, 116-121.

Hayakawa, Y., Komae, H., Ide, H., Nakagawa, H., Yoshida, Y., Kamada, M., Kataoka, Y., Nakazawa, M., 1993. An occurrence of equine transport pneumonia caused by mixed infection with Pasteurella caballi, Streptococcus suis and Streptococcus zooepidemicus. The Journal of Veterinary Medical Science/the Japanese Society of Veterinary Science 55, 455-456.

Hayek, A. 2004. Epidemiology of horses leaving the racing and breeding industries, University of Sydney.

Heidenreich, B., 2007. An introduction to positive reinforcement training and its benefits. Journal of Exotic Pet Medicine 16, 19-23.

Hendriksen, P., Elmgreen, K., Ladewig, J., 2011. Trailer-loading of horses: is there a difference between positive and negative reinforcement concerning effectiveness and stress-related signs? Journal of Veterinary Behavior: Clinical Applications and Research 6, 261-266.

Henry, S., Richard-Yris, M.A., Hausberger, M., 2006. Influence of various early human-foal interferences on subsequent human-foal relationship. Developmental Psychobiology 48, 712-718.

Herholz, C., Füssel, A.E., Timoney, P., Schwermer, H., Bruckner, L., Leadon, D., 2008. Equine travellers to the Olympic Games in Hong Kong 2008: a review of worldwide challenges to equine health, with particular reference to vector-borne diseases. Equine Veterinary Journal 40, 87-95.

Hill, C., 2000. Trailering Your Horse: A Visual Guide to Safe Training and Traveling. Storey Publishing, LLC, North Adams, MA, USA.

Hillström, A., Tvedten, H., Lilliehöök, I., 2010. Evaluation of an in-clinic Serum Amyloid A (SAA) assay and assessment of the effects of storage on SAA samples. Acta Veterinaria Scandinavica 52, 1-6.

Hillyer, M.H., Taylor, F.G.R., Proudman, C.J., Edwards, G.B., Smith, J.E., French, N.P., 2002. Case control study to identify risk factors for simple colonic obstruction and distension colic in horses. Equine Veterinary Journal 34, 455-463.

Hines, T., 2000. Effects of transit on the immune system of the horse, In: Guidelines for horse transport by road &air. AHSA/MSPCA, New York, USA, pp. 93-102.

Hobo, S., Oikawa, M., Kuwano, A., Yoshida, K., Yoshihara, T., 1997. Effect of transportation on the composition of bronchoalveolar lavage fluid obtained from horses. American Journal Veterinary Research 58, 531-534.

Hockenhull, J., Creighton, E., 2014. Management practices associated with owner-reported stable-related and handling behaviour problems in UK leisure horses. Applied Animal Behaviour Science 155, 49-55.

Hodgson, J.L., Hodgson, D.R., 2007. Collection and analysis of respiratory tract samples. Equine Respiratory Medicine and Surgery, 119-149.

Hotchkiss, J., Reid, S., Christley, R., 2007. A survey of horse owners in Great Britain regarding horses in their care. Part 2: risk factors for recurrent airway obstruction. Equine Veterinary Journal 39, 301-308.

Houpt, K., 1982. Misbehavior of horses: trailer problems. Equine Practice 4, 12-16.

Houpt, K., Lieb, S., 1993. Horse handling and transport. Livestock handling and transport, 233-252.

Houpt, K.A., 1986. Stable vices and trailer problems. Veterinary Clinics of North America: Equine Practice 2, 623-633.

Houpt, K.A., Wickens, C.L., 2014. Handling and transport of horses, In: Livestock handling and transport: fourth edition, pp. 315-341.

http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=uriserv:f83007 (accessed 01/10/2015).

http://faostat.fao.org (accessed 25/02/2015 2015).

http://statulator.com/descriptive.html (accessed 20/09/2015).

http://www.animalwelfarestandards.net.au/land-transport/ (accessed 01/10/2015).

http://www.fve.org/news/index.php?id=208#208, 2016. Practical Guidelines to Assess Fitness for Transport of Equidae. http://www.fve.org/news/index.php?id=208#208 (accessed 14/02/2016 2016).

https://github.com/lh3/seqtk. https://github.com/lh3/seqtk (accessed 17/07/2016 2016).

Huber, W., Carey, V.J., Gentleman, R., Anders, S., Carlson, M., Carvalho, B.S., Bravo, H.C., Davis, S., Gatto, L., Girke, T., 2015. Orchestrating high-throughput genomic analysis with Bioconductor. Nature Methods 12, 115-121.

Iacono, C., Friend, T., Keen, H., Martin, T., Krawczel, P., 2007a. Effects of density and water availability on the behavior, physiology, and weight loss of slaughter horses during transport. Journal of Equine Veterinary Science 27, 355-361.

Iacono, C.M., Friend, T.H., Johnson, R.D., Krawczel, P.D., Archer, G.S., 2007b. A preliminary study on the utilization of an onboard watering system by horses during commercial transport. Applied Animal Behaviour Science 105, 227-231.

Internationale, F.E., 2009. Rules for Eventing. Switzerland.

ISES, 2016. Principles of learning theory in equitation. http://www.equitationscience.com/learning-theory-in-equitation (accessed 30/04/2016 2016).

Ishida, N., Hobo, S., Takahashi, T., Nanbo, Y., Sato, F., Hasegawa, T., Mukoyama, H., 1999. Chronological changes in superoxide-scavenging ability and lipid peroxide concentration of equine serum due to stress from exercise and transport. Equine Veterinary Journal Supplement 30, 430-433.

Jaeschke, G., 1984. Influence of ascorbic acid on the physical development and performance of racehorses. A possible role in the equine sport medicine, In: Proceedings of Workshop on Ascorbic acid in Domestic Animals, pp. 153-161.

Jarratt, I.S., Tobin, J.G., Saal, R.L., 1982. Railed cattle mortalities in Queensland, In: Animal production in Australia. Pergamon Press, Sydney, Australia, pp. 253-256.

Johns, I., Marr, C., Durham, A., Mair, T., McParland, T., 2016. Causes of pleural effusions in horses resident in the UK. Equine Veterinary Education, in press.

Jones, W.E., 2003. Transporting horses: minimizing the stress. Journal of Equine Veterinary Science 23, 543-545.

Judge, N., 1969. Transport of horses. Australian Veterinary Journal 45, 465-469.

Julia, M., Felippe, B., 2016. Immunologic testing, In: Equine clinical immunology. John Wiley & Sons, Inc., pp. 205-212.

Katayama Y, O.M., Yoshihara T, 1995. Clinico-pathological effects of atmosferic ammonia exposure on horses. Journal of Equine Science 6, 99-104.

Kay, R., Hall, C., 2009. The use of a mirror reduces isolation stress in horses being transported by trailer. Applied Animal Behaviour Science 116, 237-243.

Kiley-Worthington, M., 1997. The behaviour of horses: in relation to management and training. JA Allen, London, UK.

Kirschvink, N., Art, T., Moffarts, B., Smith, N., Marlin, D., Roberts, C., Lekeux, P., 2002. Relationship between markers of blood oxidant status and physiological variables in healthy and heaves-affected horses after exercise. Equine Veterinary Journal 34, 159-164.

Kirschvink, N., de Moffarts, B., Lekeux, P., 2008. The oxidant/antioxidant equilibrium in horses. The Veterinary Journal 177, 178-191.

Knowles, T.G., Warriss, P.D., 2000. Stress physiology of animals during transport. Livestock handling and transport 2, 385-407.

Kohn, C.W., 2000. Guidelines for horse transport by road and air. AHSA/MSPCA, New York, USA.

Kruisbeek, A.M., Shevach, E., Thornton, A.M., 2004. Proliferative assays for T cell function. Current Protocols in Immunology, 3.12. 11-13.12. 20.

Kusano, K., Yamazaki, M., Kiuchi, M., Kaneko, K., Koyama, K., 2016. Reference range of blood biomarkers for oxidative stress in Thoroughbred racehorses (2–5 years old). Journal of Equine Science 27, 125-129.

Kushner, I., 1982. The phenomenon of the acute phase response. Annals of the New York Academy of Sciences 389, 39-48.

Laegreid, W., Huston, L., Basaraba, R., Crisman, M., 1988. The effects of stress on alveolar macrophage function in the horse: an overview. Equine Practice (USA).

Lanata, A., Guidi, A., Baragli, P., Paradiso, R., Valenza, G., Scilingo, E.P., 2015. Removing movement artifacts from equine ECG recordings acquired with textile electrodes, In: 2015 37th Annual International Conference of the IEEE Engineering in Medicine and Biology Society (EMBC), pp. 1955-1958.

Layman, Q.D., Rezabek, G.B., Ramachandran, A., Love, B.C., Confer, A.W., 2014. A retrospective study of equine actinobacillosis cases 1999–2011. Journal of Veterinary Diagnostic Investigation 26, 365-375.

Leadon, D., 2000. Environmental, hematological and blood biochemical changes in equine transit stress, In: Guidelines for horse transit by road &air. AHSA/MSPCA, New York, USA, pp. 63-70.

Leadon, D., 2015. A review of long distance elite horse transport and its effects and consequences. Pferdeheilkunde 31, 500-501.

Leadon, D., Frank, C., Backhouse, W., 1989. A preliminary report on studies on equine transit stress. Journal of Equine Veterinary Science 9, 200-202.

Leadon, D., Waran, N.K., Herholz, C., Klay, M., 2008. Veterinary management of horse transport. Veterinaria Italiana 44, 149-163.

Leadon, D.P., 1994. Studies of the effects of transporting horses: better to arrive than to travel. Equine Veterinary Journal 26, 346-347.

Leadon, D.P., Daykin, J., Backhouse, W., Frank, C., Atock, M.A., 1991. Environmental, hematological and blood biochemical changes in equine transit stress. Proceedings of the Annual Convention of the American Association of Equine Practitioners, 485-490.

Leadon, D.P., Hodgson, D.R., 2014. Transport of horses, In: The athletic horse: principles and practice of equine sports medicine: second edition. Elsevier-Saunders, St. Louis, USA, pp. 155-161.

LeDoux, J.E., 1994. Emotion, memory and the brain. Scientific American 270, 50-57.

Lee, J., Houpt, K., Doherty, O., 2001. A survey of trailering problems in horses. Journal of Equine Veterinary Science 21, 237-241.

Linden, A., Art, T., Amory, H., Desmecht, D., Lekeux, P., 1991. Effect of 5 different types of exercise, transportation and ACTH administration on plasma cortisol concentration in sport horses. Equine Exercise Physiology 3, 391-396.

Lykkesfeldt, J., Svendsen, O., 2007. Oxidants and antioxidants in disease: oxidative stress in farm animals. The Veterinary Journal 173, 502-511.

Mair, T., Lane, J., 1989. Pneumonia, lung abscesses and pleuritis in adult horses: a review of 51 cases. Equine Veterinary Journal 21, 175-180.

Mal, M., Friend, T., Lay, D., Vogelsang, S., Jenkins, O., 1991. Physiological responses of mares to short term confinement and social isolation. Journal of Equine Veterinary Science 11, 96-102.

Mansmann, R.A., Woodie, B., 1995. Equine transportation problems and some preventives: a review. Journal of Equine Veterinary Science 15, 141-144.

Marahrens, M., Kleinschmidt, N., Di Nardo, A., Velarde, A., Fuentes, C., Truar, A., Otero, J.L., Di Fede, E., Villa, P.D., 2011. Risk assessment in animal welfare - Especially referring to animal transport. Preventive Veterinary Medicine 102, 157-163.

Markey, B., Leonard, F., Archambault, M., Cullinane, A., Maguire, D., 2013. Clinical veterinary microbiology. Elsevier Health Sciences, St.Louis, USA.

Marlin, D., Kettlewell, P., Parkin, T., Kennedy, M., Broom, D., Wood, J., 2011. Welfare and health of horses transported for slaughter within the European Union Part 1: Methodology and descriptive data. Equine Veterinary Journal 43, 78-87.

Marlin, D., Meldrum, K., White, J., Westen, H., Parkin, T., Wood, J., Broom, D., Kennedy, M., Johnson, B., Coombs, S., Heard, C., 2009. Welfare of horses transported long distances in Europe. Veterinary Record 165, 270.

Marlin, D.J., 2004. Transport of horses, In: Equine Sports Medicine and Surgery. Elsevier-Saunders, St.Louis, USA, pp. 1239-1250.

Mars, L.A., Kiesling, H., Ross, T., Armstrong, J., Murray, L., 1992. Water acceptance and intake in horses under shipping stress. Journal of Equine Veterinary Science 12, 17-20.

McClintock, S.A., Begg, A.P., 1990. Suspected salmonellosis in seven broodmares after transportation. Australian Veterinary Journal 67, 265-267.

McClure, S.R., Carithers, D.S., Gross, S.J., Murray, M.J., 2005. Gastric ulcer development in horses in a simulated show or training environment. Journal of the American Veterinary Medical Association 227, 775-777.

McCord, J.M., 2000. The evolution of free radicals and oxidative stress. The American Journal of Medicine 108, 652-659.

McDonnell, S., Haviland, J., 1995. Agonistic ethogram of the equid bachelor band. Applied Animal Behaviour Science 43, 147-188.

McGlone, J., Salak, J., Lumpkin, E., Nicholson, R., Gibson, M., Norman, R., 1993. Shipping stress and social status effects on pig performance, plasma cortisol, natural killer cell activity, and leukocyte numbers. Journal of Animal Science 71, 888-896.

McGorum, B.C., 2007. Equine respiratory medicine and surgery. Saunders Elsevier, Edinburgh.

McGreevy, P., 2012. Equine behavior: a guide for veterinarians and equine scientists. Saunders/Elsevier, New York/Edinburgh.

McGreevy, P.D., 2007. The advent of equitation science. The Veterinary Journal 174, 492-500.

McGreevy, P.D., Cripps, P.J., French, N.P., Green, L.E., Nicol, C.J., 1995. Management factors associated with stereotypic and redirected behaviour in the thoroughbred horse. Equine Veterinary Journal 27, 86-91.

McGreevy, P.D., McLean, A., 2011. Equitation Science. Wiley, Hoboken, USA.

McGreevy, P.D., McLean, A.N., 2007. Roles of learning theory and ethology in equitation. Journal of Veterinary Behavior: Clinical Applications and Research 2, 108-118.

McLean, A., 2003. The truth about horses. Penguin, Camberwell, Victoria, AU.

McLean, A.N., MCLean, M., 2008. Academic horse training. Australian equine behaviour centre, Clonbinane, Victoria, AU.

McMurdie, P.J., Holmes, S., 2013. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PloS one 8, e61217.

Messori, S., Visser, E.K., Buonanno, M., Ferrari, P., Barnard, S., Borciani, M., Ferri, N., 2016. A tool for the evaluation of slaughter horse welfare during unloading. Animal Welfare 25, 101-113.

Millar, H., Simpson, J., Stalker, A., 1971. An evaluation of the heat precipitation method for plasma fibrinogen estimation. Journal of Clinical Pathology 24, 827-830.

Mills, D., Clarke, A., 2007. Housing, management and welfare, In: The welfare of horses. Springer, pp. 77-97.

Minka, N., Ayo, J., 2009. Physiological responses of food animals to road transportation stress. African Journal of Biotechnology 9, 6601-6613.

Minka, N.S., Ayo, J.O., 2013. Physiological and behavioral responses of goats to 12-hour road transportation, lairage and grazing periods, and the modulatory role of ascorbic acid. Journal of Veterinary Behavior: Clinical Applications and Research 8, 349-356.

Miranda-de la Lama, G.C., 2013. Transport and pre-slaughter logistics: definitions and current tendencies in animal welfare and meat quality. Veterinaria Mexico 44, 31-56.

Mitchell, M.A., Kettlewell, P.J., 2008. Engineering and design of vehicles for long distance road transport of livestock (ruminants, pigs and poultry). Veterinaria Italiana 44, 201-213.

Moberg, G.P., Mench, J.A., 2000. The biology of animal stress: basic principles and implications for animal welfare. CABI Publishing, Oxfordshire, UK.

Mongillo, P., Bertotto, D., Pitteri, E., Stefani, A., Marinelli, L., Gabai, G., 2015. Peripheral leukocyte populations and oxidative stress biomarkers in aged dogs showing impaired cognitive abilities. Age 37, 39.

Morgan, K., 1998. Thermoneutral zone and critical temperatures of horses. Journal of Thermal Biology 23, 59-61.

Morresey, P.R., 2014. Ultrasonography of the pleural cavity, lung, and diaphragm. Atlas of equine ultrasonography, Wiley-Blackwell, USA, 365-378.

Munsters, C., de Gooijer, J.W., van den Broek, J., van Oldruitenborgh-Oosterbaan, M.S., 2013. Heart rate, heart rate variability and behaviour of horses during air transport. Veterinary Record 172, 15.

Murata, H., 2007. Stress and acute phase protein response: an inconspicuous but essential linkage. The Veterinary Journal 173, 473-474.

Murata, H., Shimada, N., Yoshioka, M., 2004. Current research on acute phase proteins in veterinary diagnosis: an overview. The Veterinary Journal 168, 28-40.

Murata, H., Takahashi, H., Matsumoto, H., 1987. The effects of road transportation on peripheral blood lymphocyte subpopulations, lymphocyte blastogenesis and neutrophil function in calves. British Veterinary Journal 143, 166-174.

Nazifi, S., Saeb, M., Baghshani, H., Saeb, S., 2009. Influence of road transportation during hot summer conditions on oxidative status biomarkers in Iranian dromedary camels (Camelus dromedarius). African Journal of Biochemistry Research 3, 282-287.

Nestved, A., 1996. Evaluation of an immunostimulant in preventing shipping stress related respiratory disease. Journal of Equine Veterinary Science 16, 78-82.

Niedźwiedź, A., Kubiak, K., Nicpoń, J., 2013. Plasma total antioxidant status in horses after 8-hours of road transportation. Acta Veterinaria Scandinavica 55, 1.

Nielsen, B.L., Dybkjær, L., Herskin, M.S., 2011. Road transport of farm animals: effects of journey duration on animal welfare. Animal 5, 415-427.

Noble, B., Riley, C.B., Thompson, K., 2013. Incidence and risk factors associated with horse injuries sustained during noncommercial transport, In: International large animal rescue ILAR, Adelaide, p. 10.

Nørskov-Lauritsen, N., 2014. Classification, identification, and clinical significance of Haemophilus and Aggregatibacter species with host specificity for humans. Clinical Microbiology Reviews 27, 214-240.

Ohmura, H., Hobo, S., Hiraga, A., Jones, J.H., 2012. Changes in heart rate and heart rate variability during transportation of horses by road and air. American Journal Veterinary Research 73, 515-521.

OHS, 2011. OHS Act NSW. http://www.safework.nsw.gov.au/law-and-policy/legislation-and-codes/work-health-and-safety-legislation (accessed 30/08/2016).

OIE, 2016. Handbook for the management of high health, high performance horses. http://www.oie.int/fileadmin/Home/eng/Our_scientific_expertise/docs/pdf/Chevaux/HHP_Handbook_May_2016_V2.pdf (accessed 15/09/16 2016).

Oikawa, M., Hobo, S., Oyamada, T., Yoshikawa, H., 2005. Effects of orientation, intermittent rest and vehicle cleaning during transport on development of transport-related respiratory disease in horses. Journal of Comparative Pathology 132, 153-168.

Oikawa, M., Jones, J.H., 2000. Studies of the causes and effects of road transport stress in athletic horses, In: Guidelines for horse transport by road and air. AHSA/MSPCA, New York, USA, pp. 35-62.

Oikawa, M., Kamada, M., Yoshikawa, Y., Yoshikawa, T., 1994. Pathology of equine pneumonia associated with transport and isolation of Streptococcus equi subsp. zooepidemicus. Journal of Comparative Pathology 111, 205-212.

Oikawa, M., Takagi, S., Anzai, R., Yoshikawa, H., Yoshikawa, T., 1995. Pathology of equine respiratory disease occurring in association with transport. Journal of Comparative Pathology 113, 29-43.

Oikawa, M.A., Takagi, S., Yashiki, K., 2004. Some aspects of the stress responses to road transport in thoroughbred horses with special reference to shipping fever. Journal of Equine Science 15, 99-102.

Olsson, K., 2010. A review of methods used to measure temperamental characteristics in horses. Student report 338, Swedish University of Agricultural Sciences.

Onmaz, A., Van Den Hoven, R., Gunes, V., Cinar, M., Kucuk, O., 2011. Oxidative stress in horses after a 12-hours transport period. Revue de Médicine Véterinaire 162, 213-217.

Padalino, B., 2015. Effects of the different transport phases on equine health status, behavior, and welfare: a review. Journal of Veterinary Behavior: Clinical Applications and Research 10, 272-282.

Padalino, B., Maggiolino, A., Boccaccio, M., Tateo, A., 2012. Effects of different positions during transport on physiological and behavioral changes of horses. Journal of Veterinary Behavior: Clinical Applications and Research 7, 135-141.

Pagan, J.D., 2004. Problems in equine nutrition. Ippologia 15, 5-22.

Pagan, J.D., Geor, R.J., 2005. Advances in equine nutrition III. Nottingham University Press, England.

Parelli, P., Kadash, K., Parelli, K., 1993. Natural horse-man-ship. Western Horseman, Colorado Springs, Colorado, USA.

Parish, C.R., 1999. Fluorescent dyes for lymphocyte migration and proliferation studies. Immunology and Cell Biology 77, 499-508.

Parker, A., Hamlin, G., Coleman, C., Fitzpatrick, L., 2003. Quantitative analysis of acid-base balance in steers subjected to transportation of long duration. Journal of Animal Science 81, 1434-1439.

Piccione, G., Casella, S., Giannetto, C., Bazzano, M., Giudice, E., Fazio, F., 2013. Oxidative stress associated with road transportation in ewes. Small Ruminant Research 112, 235-238.

Pickrell, J., 1991. Hazards in confinement housing - gases and dusts in confined animal houses for swine, poultry, horses and humans. Veterinary and Human Toxicology 33, 32-39.

Pineiro, M., Pineiro, C., Carpintero, R., Morales, J., Campbell, F.M., Eckersall, P.D., Toussaint, M.J., Lampreave, F., 2007. Characterisation of the pig acute phase protein response to road transport. The Veterinary Journal 173, 669-674.

Po, E., Williams, C., Muscatello, G., Celi, P., 2013. Assessment of oxidative stress biomarkers in exhaled breath condensate and blood of Thoroughbred foals. The Veterinary Journal 196, 269-271.

Pregel, P., Bollo, E., Cannizzo, F., Biolatti, B., Contato, E., Biolatti, P., 2005. Antioxidant capacity as a reliable marker of stress in dairy calves transported by road. Veterinary Record 156, 53-54.

Pryor, J.H., 1982. Transportation of horses by sea during the era of the crusades, eight century to 1285 AD: part I. The Mariner's Mirror 68, 9-27.

Purswell, J.L., Gates, R.S., Lawrence, L.M., Davis, J.D., 2010. Thermal environment in a four-horse slant-load trailer. Transactions of the ASABE 53, 1885-1894.

Purswell, J.L., Gates, R.S., Lawrence, L.M., Jacob, J.D., Stombaugh, T.S., Coleman, R.J., 2006. Air exchange rate in a horse trailer during road transport. Transactions of the ASABE 49, 193-201.

R Core Team, R: A Language and Environment for Statistical Computing. https://www.r-project.org. (accessed 22/08/2016 2016).

Racklyeft, D., Love, D., 1990. Influence of head posture on the respiratory tract of healthy horses. Australian Veterinary Journal 67, 402-405.

Racklyeft, D.J., Raidal, S., Love, D.N., 2000. Towards an understanding of equine pleuropneumonia: factors relevant for control. Australian Veterinary Journal 78, 334-338.

Raidal, S.L., 1995. Equine pleuropneumonia. British Veterinary Journal 151, 233-262.

Raidal, S.L., Bailey, G.D., Love, D.N., 1997a. Effect of transportation on lower respiratory tract contamination and peripheral blood neutrophil function. Australian Veterinary Journal 75, 433-438.

Raidal, S.L., Love, D.N., Bailey, G.D., 1995. Inflammation and increased numbers of bacteria in the lower respiratory tract of horses within 6 to 12 hours of confinement with the head elevated. Australian Veterinary Journal 72, 45-50.

Raidal, S.L., Love, D.N., Bailey, G.D., 1996. Effects of posture and accumulated airway secretions on tracheal mucociliary transport in the horse. Australian Veterinary Journal 73, 45-49.

Raidal, S.L., Taplin, R.H., Bailey, G.D., Love, D.N., 1997b. Antibiotic prophylaxis of lower respiratory tract contamination in horses confined with head elevation for 24 or 48 hours. Australian Veterinary Journal 75, 126-131.

Ralston, S., Stives, M., 2012. Supplementation of ascorbic acid in weanling horses following prolonged transportation. Animals 2, 184-194.

Reece, V.P., Friend, T.H., Stull, C.H., Grandin, T., Cordes, T., 2000. Equine slaughter transport-update on research and regulations. Journal of American Veterinary Medical Association 216, 1253-1257.

Reed, S.M., Bayly, W.M., Sellon, D.C., 2003. Equine Internal Medicine: Second Edition, Elsevier, Canada.

Riley, C.B., Liddiard, J.R., Thompson, K., 2015. A cross-sectional study of horse-related injuries in veterinary and animal science students at an Australian university. Animals 5, 951-964.

Ritter, M., Ellis, M., Bowman, R., Brinkmann, J., Curtis, S., DeDecker, J., Mendoza, O., Murphy, C., Orellana, D., Peterson, B., 2008. Effects of season and distance moved during loading on transport losses of market-weight pigs in two commercially available types of trailer. Journal of Animal Science 86, 3137-3145.

Robbin, M.G., Wagner, B., Noronha, L.E., Antczak, D.F., de Mestre, A.M., 2011. Subpopulations of equine blood lymphocytes expressing regulatory T cell markers. Veterinary Immunology and Immunopathology 140, 90-101.

Roberts, T., 1990. Staying upright in a moving trailer. The equine athlete 3, 1-8.

Robin, C.A., Wylie, C.E., Wood, J.L.N., Newton, J.R., 2011. Making use of equine population demography for disease control purposes: preliminary observations on the difficulties of counting and locating horses in Great Britain. Equine Veterinary Journal 43, 372-375.

Robinson, M.D., McCarthy, D.J., Smyth, G.K., 2010. EdgeR: a bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26, 139-140.

Rosanowski, S.M., Cogger, N., Rogers, C.W., Bolwell, C.F., Benschop, J., Stevenson, M.A., 2013. Analysis of horse movements from non-commercial horse properties in New Zealand. New Zealand Veterinary Journal 61, 245-253.

Rosanowski, S.M., Rogers, C.W., Bolwell, C.F., Cogger, N., 2015. The movement pattern of horses around race meetings in New Zealand. Animal Production Science 55, 1075-1080.

Rosias, P., 2012. Methodological aspects of exhaled breath condensate collection and analysis. Journal of Breath Research 6, 027102.

Roy, R.C., Cockram, M.S., Dohoo, I.R., 2015. Welfare of horses transported to slaughter in Canada: assessment of welfare and journey risk factors affecting welfare. Canadian Journal of Animal Science 95, 509-522.

Sanada, Y., Noda, H., Nagahata, H., 1992. Development of lymphocyte blastogenic response in the neonatal period of foals. Journal of Veterinary Medicine Series A 39, 69-75.

Saslow, C.A., 2002. Understanding the perceptual world of horses. Applied Animal Behaviour Science 78, 209-224.

Schack, S., Fey, K., Sasse, H.H.L., 2002. Collection of breath condensate in horses. Pferdeheilkunde 18, 612-616.

Schaefer, A., Cook, N., Bench, C., Chabot, J., Colyn, J., Liu, T., Okine, E., Stewart, M., Webster, J., 2012. The non-invasive and automated detection of bovine respiratory disease onset in receiver calves using infrared thermography. Research in Veterinary Science 93, 928-935.

Schemann, K., Taylor, M.R., Toribio, J.A.L.M.L., Dhand, N.K., 2011. Horse owners' biosecurity practices following the first equine influenza outbreak in Australia. Preventive Veterinary Medicine 102, 304-314.

Schmidt, A., Möstl, E., Wehnert, C., Aurich, J., Müller, J., Aurich, C., 2010. Cortisol release and heart rate variability in horses during road transport. Hormones and Behavior 57, 209-215.

Schwartzkopf-Genswein, K.S., Faucitano, L., Dadgar, S., Shand, P., González, L.A., Crowe, T.G., 2012. Road transport of cattle, swine and poultry in North America and its impact on animal welfare, carcass and meat quality: a review. Meat Science 92, 227-243.

Scoggins, R., 1996. Horses and claustrophobia. AVSAB Newsletter 18, 2-3.

Sconberg, S., Nockels, C., Bennett, B., Bruyninckx, W., Blancquaert, A., Craig, A., 1993. Effects of shipping, handling, adrenocorticotropic hormone, and epinephrine on alpha-tocopherol content of bovine blood. American Journal of Veterinary Research 54, 1287-1293.

Shanahan, S., 2003. Trailer loading stress in horses: behavioral and physiological effects of nonaversive training (TTEAM). Journal of Applied Animal Welfare Science 6, 263-274.

Sheng, H., Wang, Y., Jin, Y., Zhang, Q., Zhang, Y., Wang, L., Shen, B., Yin, S., Liu, W., Cui, L., 2008. A critical role of IFNγ in priming MSC-mediated suppression of T cell proliferation through up-regulation of B7-H1. Cell Research 18, 846-857.

Shimabukuro, Y., Murakami, S., Okada, H., 1992. Interferon-gamma-dependent immunosuppressive effects of human gingival fibroblasts. Immunology 76, 344.

Shukla, N., Maher, J., Masters, J., Angelini, G.D., Jeremy, J.Y., 2006. Does oxidative stress change ceruloplasmin from a protective to a vasculopathic factor? Atherosclerosis 187, 238-250.

Siniscalchi, M., Padalino, B., Lusito, R., Quaranta, A., 2014. Is the left forelimb preference indicative of a stressful situation in horses? Behavioural Processes 107, 61-67.

Slade, L.M.J., 1987. Trailer transportation and racing performance, In: 10th Equine Nutrition and Physiology Symposium, Fort Collins, CO, Texas, USA, pp. 511-514.

Slater, C., Dymond, S., 2011. Using differential reinforcement to improve equine welfare: shaping appropriate truck loading and feet handling. Behavioural Processes 86, 329-339.

Smith, B.L., Jones, J.H., Carlson, G.P., Pascoe, J.R., 1994. Effect of body direction on heart rate in trailered horses. American Journal of Veterinary Research 55, 1007-1011.

Smith, B.L., Jones, J.H., Hornof, W., Miles, J., Longworth, K.E., Willits, N., 1996. Effects of road transport on indices of stress in horses. Equine Veterinary Journal 28, 446-454.

Smyth, G.B., Dagley, K., 2015. Demographics of Australian horse owners: results from an internet-based survey. Australian Veterinary Journal 93, 433-438.

Söder, J., Bröjer, J.T., Nostell, K.E., 2012. Interday variation and effect of transportation on indirect blood pressure measurements, plasma endothelin-1 and serum cortisol in Standardbred and Icelandic horses. Acta Veterinaria Scandinavica 54, 37.

Soffler, C., 2007. Oxidative stress. Veterinary Clinics of North America: Equine Practice 23, 135-157.

Staempfli, H.R., Townsend, G.G., Prescot, J.F., 1991. Prognostic features and clinical presentation of acute idiopathic enterocolitis in horses. Canadian Veterinary Journal 32, 232-237.

Starling, M., McLean, A., McGreevy, P., 2016. The contribution of equitation science to minimising horse-related risks to humans. Animals 6, 15.

Stefancic, I., Martin, D., 2005. Influence of transport conditions on animal welfare, In: ISAH, Warsaw, Poland, pp. 148-152.

Stewart, M., Foster, T.M., Waas, J.R., 2003. The effects of air transport on the behaviour and heart rate of horses. Applied Animal Behaviour Science 80, 143-160.

Studdert, M., Blackney, M., 1979. Equine herpesviruses: on the differentiation of respiratory from foetal strains of type 1. Australian Veterinary Journal 55, 488-492.

Studdert, M., Turner, A., Peterson, J., 1970. 1. Isolation and characterisation of equine rhinopneumontis virus and other equine herpesviruses from horses. Australian Veterinary Journal 46, 83-89.

Stull, C., 2001. Evolution of the proposed federal slaughter horse transport regulations. Journal of Animal Science 79, E12-E15.

Stull, C., Rodiek, A., 2000. Physiological responses of horses to 24 hours of transportation using a commercial van during summer conditions. Journal of Animal Science 78, 1458-1466.

Stull, C.L., 1999. Responses of horses to trailer design, duration, and floor area during commercial transportation to slaughter. Journal of Animal Science 77, 2925-2933.

Stull, C.L., Morrow, J., Aldridge, B.A., Stott, J.L., McGlone, J.J., 2008. Immunophysiological responses of horses to a 12-hour rest during 24 hours of road transport. Veterinary Record 162, 609-614.

Stull, C.L., Rodiek, A.V., 2002. Effects of cross-tying horses during 24 h of road transport. Equine Veterinary Journal 34, 550-555.

Stull, C.L., Spier, S.J., Aldridge, B.M., Blanchard, M., Stott, J.L., 2004. Immunological response to long-term transport stress in mature horses and effects of adaptogenic dietary supplementation as an immunomodulator. Equine Veterinary Journal 36, 583-589.

Styrt, B., Sugarman, B., 1991. Estrogens and infection. Reviews of Infectious Diseases 13, 1139-1150.

Sunderman, F.W., Nomoto, S., 1970. Measurement of human serum ceruloplasmin by its p-phenylenediamine oxidase activity. Clinical Chemistry 16, 903-910.

Sundra, T.M., Harrison, J.L., Lester, G.D., Raidal, S.L., Phillips, J.K., 2012. The influence of spasmolytic agents on heart rate variability and gastrointestinal motility in normal horses. Research in Veterinary Science 93, 1426-1433.

Tadich, T., Leal, F., Gallo, C., 2015. Preliminary study on the effects of long distance road transport on some blood constituents in recently weaned Thoroughbred foals. Journal of Equine Veterinary Science 35, 697-699.

Tarantola, M., Valle, E., De Marco, M., Bergagna, S., Dezzutto, D., Gennero, M.S., Bergero, D., Schiavone, A., Prola, L., 2016. Effects of abrupt housing changes on the welfare of Piedmontese cows. Italian Journal of Animal Science 15, 103-109.

Tarrant, P., 1990. Transportation of cattle by road. Applied Animal Behaviour Science 28, 153-170.

References

Tateo, A., Padalino, B., Boccaccio, M., Maggiolino, A., Centoducati, P., 2012. Transport stress in horses: effects of two different distances. Journal of Veterinary Behavior: Clinical Applications and Research 7, 33-42.

Tee, S., Dart, A., MacDonald, M., Perkins, N., Horadagoda, N., Jeffcott, L., 2012. Effects of collecting serial tracheal aspirate and bronchoalveolar lavage samples on the cytological findings of subsequent fluid samples in healthy Standardbred horses. Australian Veterinary Journal 90, 247-251.

Thompson, K., McGreevy, P., McManus, P., 2015. A critical review of horse-related risk: a research agenda for safer mounts, riders and equestrian cultures. Animals 5, 561-575.

Thompson, R.F., Spencer, W.A., 1966. Habituation: a model phenomenon for the study of neuronal substrates of behavior. Psychological Review 73, 16-43.

Tinker, M.K., White, N.A., Lessard, P., Thatcher, C.D., Pelzer, K.D., Davis, B., Carme, D.K., 1997. Prospective study of equine colic risk factors. Equine Veterinary Journal 29, 454-458.

Toscano, M., Friend, T., 2001. A note on the effects of forward and rear-facing orientations on movement of horses during transport. Applied Animal Behaviour Science 73, 281-287.

Traub-Dargatz, J., McKinnon, A., Bruyninckx, W., Thrall, M., Jones, R., Blancquaert, A., 1988. Effect of transportation stress on bronchoalveolar lavage fluid analysis in female horses. American Journal of Veterinary Research 49, 1026-1029.

Treiber, K., Carter, R., Gay, L., Williams, C., Geor, R., 2009. Inflammatory and redox status of ponies with a history of pasture-associated laminitis. Veterinary Immunology and Immunopathology 129, 216-220.

Tsuzuki, N., Sasaki, N., Kusano, K., Endo, Y., Torisu, S., 2016. Oxidative stress markers in Thoroughbred horses after castration surgery under inhalation anesthesia. Journal of Equine Science 27, 77-79.

Turner, A., Studdert, M., Peterson, J., 1970. 2. Persistence of equine herpesviruses in experimentally infected horses and the experimental induction of abortion. Australian Veterinary Journal 46, 90-98.

Veissier, I., Miele, M., 2014. Animal welfare: towards transdisciplinarity - the European experience. Animal Production Science 54, 1119-1129.

Veissier, I., Miele, M., 2015. Short historical overview of animal welfare sciences: how a societal concern has become a transdisciplinary subject. Inra Productions Animales 28, 399-409.

Vieira, F.M.C., Silva, I.J.O., Barbosa Filho, J.A.D., Vieira, A.M.C., Broom, D.M., 2011. Preslaughter mortality of broilers in relation to lairage and season in a subtropical climate. Poultry Science 90, 2127-2133.

Waldron, L.A., 2012. Essential equine nutrition. Nottingham University Press, Nottingham, UK.

Waran, N., Leadon, D., Friend, T., 2002. The effects of transportation on the welfare of horses. Kluwer Academic Publishers, Dordrecht, Netherlands.

Waran, N., Leadon, D., Friend, T., 2007. The effects of transportation on the welfare of horses, In: The welfare of horses. Springer Netherlands, pp. 125-150.

Waran, N.K., 1993. The behaviour of horses during and after transport by road. Equine Veterinary Education 5, 129-132.

Waran, N.K., Cuddeford, D., 1995. Effects of loading and transport on the heart rate and behaviour of horses. Applied Animal Behaviour Science 43, 71-81.

Waran, N.K., Robertson, V., Cuddeford, D., Kokoszko, A., Marlin, D.J., 1996. Effects of transporting horses facing either forwards or backwards on their behaviour and heart rate. Veterinary Record 139, 7-11.

Waring, G.H., 2003. Horse behavior. Noyes Publications/William Andrew Pub, Norwich, New York, USA.

Warriss, P., Knowles, T., Brown, S., Edwards, J., Kettlewell, P., Mitchell, M., Baxter, C., 1999. Effects of lairage time on body temperature and glycogen reserves of broiler chickens held in transport modules. The Veterinary Record 145, 218-222.

Weeks, C.A., McGreevy, P., Waran, N.K., 2012. Welfare issues related to transport and handling of both trained and unhandled horses and ponies. Equine Veterinary Education 24, 423-430.

Werner, M., Gallo, C., 2008. Effects of transport, lairage and stunning on the concentrations of some blood constituents in horses destined for slaughter. Livestock Science 115, 94-98.

Wessely-Szponder, J., Bełkot, Z., Bobowiec, R., Kosior-Korzecka, U., Wójcik, M., 2015. Transport induced inflammatory responses in horses. Polish Journal of Veterinary Sciences 18, 407-413.

Wessely-Szponder, J., Bełkot, Z., Bobowiec, R., Wójcik, M., Kosior-Korzecka, U., 2014. Crosstalk between adiponectin and cytokines (IL-6 and IL-8) during transportation stress in horses. Medycyna Weterynaryjna 70, 546-549.

West, R., French, D., Kemp, R., Elander, J., 1993. Direct observation of driving, self reports of driver behaviour, and accident involvement. Ergonomics 36, 557-567.

White, A., Reyes, A., Godoy, A., Martínez, R., 1991. Effects of transport and racing on ionic changes in thoroughbred race horses. Comparative Biochemistry and Physiology Part A: Physiology 99, 343-346.

Whiting, T., 1999. Maximum loading density of loose horses. Canadian Journal of Animal Science 79, 115-118.

Wilkins, P.A., 2003. Lower airway diseases of the adult horse. Veterinary Clinics of North America: Equine Practice 19, 101-121.

Witko-Sarsat, V., Friedlander, M., Khoa, T.N., Capeillère-Blandin, C., Nguyen, A.T., Canteloup, S., Dayer, J.-M., Jungers, P., Drüeke, T., Descamps-Latscha, B., 1998. Advanced oxidation protein products as novel mediators of inflammation and monocyte activation in chronic renal failure1, 2. The Journal of Immunology 161, 2524-2532.

Yin, C., Pettigrew, A., Loftus, J., Black, S., Belknap, J., 2009. Tissue concentrations of 4-HNE in the black walnut extract model of laminitis: indication of oxidant stress in affected laminae. Veterinary Immunology and Immunopathology 129, 211-215.

Yngvesson, J., de Boussard, E., Larsson, M., Lundberg, A., Loading horses (Equus caballus) onto trailers - Behaviour of horses and horse owners during loading and habituating. Applied Animal Behaviour Science, 184, 59-65.

Young, T., Creighton, E., Smith, T., Hosie, C., 2012. A novel scale of behavioural indicators of stress for use with domestic horses. Applied Animal Behaviour Science 140, 33-43.

Zhang, J., Kobert, K., Flouri, T., Stamatakis, A., 2014. PEAR: a fast and accurate Illumina paired-end reAd mergeR. Bioinformatics 30, 614-620.

APPENDIX 1

Appendix 1

THE UNIVERSITY OF SYDNEY

Dear horse friend,

If you or your organization has transported horses in the last two years, we'd greatly appreciation your participation in this important survey of horse transport practices and transport-related illnesses.

We want to get an insight into how you manage and prepare your horses for transportation, the act of transportation of horses and how you manage horses after a transport event. If any diseases or injuries have occurred to your horses related to a transport event in the last two years we are interested in documenting and investigating the underlying potential reasons behind these events.

The aim of this study is to gather data to identify transportation risk factors, and therefore potentially making horse transportation safer and less stressful. The survey results will potentially help reduce the incidence of transport-related diseases and injuries and thereby be used by the industry as it strives to implement best practices in horse transportation. However, we need your 'real world' input to do this.

By filling in this survey you will be making a valuable contribution to improving horse health and welfare.

If you are interested in the results of this survey please leave your email address. You will be invited to a presentation of results where we will discuss these results and have a forum on horse transportation issues. Your email details will be kept confidential.

Thanks very much for your help.

Best wishes,

For the horses

Barbara Padalino

Appendix 1

Survey of Horse transportation: Issues and practices

Please, fill this data about your detail, circle the appropriate option.

Sex: M/F   Age: 20-30; 30-40; 40-50; 50-60: 60-70

Your postcode:………………………………………………..

State:...

How did you get the questionnaire?

on line, by postal mail, by a researcher an information session, by a researcher at a horse event

Which section of the horse industry are you primarily involved in?

Thoroughbred Racing, Standardbred racing, Equestrian sport (dressage, eventing, jumping, reining, driving, vaulting), Endurance, Horse breeding, Recreational non-competitive riding

Please, tick the boxes: in many questions several answers are possible.

Q1. What is your relationship with the horse?

1. Professional (e.g. horse trainer, stud manager, riding teacher)
2. Amateur (e.g. horse owner, rider)

Q2. In your busiest week of the year, approximately how many horses are on your property?

1. <5 horses
2. 5- 10 horses
3. 11-30 horses
4. 31-50 horses
5. 50-100 horses
6. More than 100 horses

And how many now? ………….

Q3. How often do you or your organisation transport horses?

1. Daily
2. Twice a week
3. Weekly

4. Fortnightly

5. Monthly

And what is the average number of horses transported in a given trip? ....

Q4. What is the most common duration of your horse transport?

1. Less than 2 hours
2. 2- 4 hours
3. 4- 8 hours
4. More than 8 hours

Q5. Please tick any (all) of the following strategies you commonly use to prepare your horses for transportation (tick all that apply)

1. Administration of antibiotics
2. Administration of tranquilizers
3. Administration of oral supplement (e.g electrolytes, vitamins)
4. Ad libitum access to hay and water
4. Removing of shoes
5. Application of bandages
7. Wearing of rug
8. Other: (Please specify)……………………
9. No treatment

Q6. Have you used any training to aid in transporting your horses? If so, describe the training tool (i.e. training in loading and unloading the vehicle).

……………………………………………………………………………………………

……………………………………………………………………………………………

Q7. Do your horses show behavioural problems (i.e. anxiety, flight response, fight response, kicking, rearing, refusal to load) associated with loading, unloading or during travel

1. Yes

2. No

If Yes, in which phase of the travel have you noted behavioural problems?

1. Preloading (e.g. anxiety, pawing)
2. Loading (e.g. refuse to load)
3. Travelling (e.g. scrambling, kicking, biting)
4. Unloading (e.g. refuse to load, jumping)

Q8. Who assesses the health (fitness for travel) of the horse before transportation?

1. A veterinarian
2. Non-veterinary staff (myself / my assistant(s))
3. Routine checks are not conducted

If you or your staff members conduct the health check, please tick the specific parameters that you assess

1. Temperature
2. Heart rate
3. Feeding behaviour
4. Drinking behaviour
5. Weight
6. General health
7. Other:………………….

Q9: What is the positioning or arrangement commonly used when your horses are transported?

1. Forward
2. Backward
3. At an angle (eg. 45°)
4. Unrestrained

Q10: When your horses travel for <u>competition purposes</u>, how much rest is usually given after transportation before starting the sporting activities (e.g. training, racing, competing or riding), and how does this vary with trip duration? Please fill this quick table, tick how much rest you give (row) according with the different trip duration (column)

	Trips<2hours	Trip of 2-4 hours	Trip 4-8 hours	Trip >8 hours
No rest				
Less than 2 hours rest				
Between 2 and 4 hours of rest				
Between 4 and 8 hours of rest				
Between 8 and 12 hours of rest				
One day of rest				
Other:……………..				

Q11 . Who assesses the health (fitness after travel) of the horse after transportation?

1. A veterinarian
2. Non-veterinary staff (myself / my assistant(s))
3. Routine checks are not conducted

If you or your staff members conduct the health check, please tick the specific parameters that you assess

1. Temperature
2. Heart rate
3. Feeding behaviour
4. Drinking behaviour
5. Weight
6. General health
7. Other:………………….

Q12: Which health problems related to transport have you observed in transported horses over the last two years? Did they occur during (between loading and unloading) or after the journey? Please tick the box and circle when. (Tick all that apply)

Eg. 1. Injuries |V| **during after**

1. Injuries such as cuts, bruises |_| during after
2. Respiratory problems |_| during after
3. Colic |_| during after
4. Laminitis |_| during after
5. Muscular problems |_| during after
6. Diarrhoea |_| during after
7. Overheating/sunstroke |_| during after
8. Other:………………………… |_| during after
9. No health problems observed |_|

Q13.Please describe the last time your horses experienced a transported related illness (e.g pathology, type of horse, type of trip)

Horse details (i.e. age, breed, sex):____

Trip details (i.e. duration, transport mean):__________________________________

Illness:____

Your comments about the possible cause:

____

____

Q14: Have any of your horses suffered from travel sickness (i.e. equine transport-related respiratory illness or transport pneumonia) in the last two years?

1. Yes
2. No

If Yes, please describe the last time your horse(s) experienced travel sickness (i.e. equine transport-related respiratory illness or transport pneumonia) (e.g type of horse, type of trip, nature of illness)

Horse details (i.e. age, breed, sex):____

Trip details (i.e. duration, transport mean):__________________________________

Position of the horse during the trip:______________________________________

Your comments about the possible cause: ____________________________________

____

Did the horse recover? If so, after how much time?

____

____

Q15. Have any of your horses died/been euthanized due to a transport-related problem in the last two years?

1. Yes
2. No

If **Yes**, how many horses and what was/were the health problems related to the transport event that resulted in death or required euthanasia.

____

____

Q16. In your opinion, what product may be useful in preventing horse transport-related illness and improve the safety of travelling horses ?

1. A non-doping non prescriptive supplement to administer before the trip
2. A monitoring system to alert you if the horse is in trouble during the trip
3. A system for monitoring the health of the horse before and after the trip
4. Other :…………………………………………

Q17 .Who transports your horses?

1. You (please answer the question from 18 to 22)
2. A horse Transport Company (please go to question 23)

PLEASE ANSWER THESE QUESTIONS, IF <u>YOU</u> TRANSPORT YOUR HORSES

18. What mode of road transport do you use?? [tick all that apply]

1. A one horse trailer
2. A two horse straight load trailer
3. A horse angle load trailer
4. A 3-4 horse angle load trailer

5. A gooseneck-trailer

6. A truck

Q19: Do you tie up your horses during the trip?

1. Yes
2. No

If **YES**, is the horse able to lower its head to wither height or below when he is travelling?

1. Yes
2. No

Q20: Do you monitor the horse or the horses' environment during the trip?

1. By video camera
2. By weather station
3. Other:………………………………………………………
4. No monitor

Q21. Do your horses have access to food and/or water whist travelling?

1. Yes
2. No

If Yes, please tick all that apply?

1. Water
2. Hay in net
3. Hard feed in a manger or bucket

Q22. In your mind, how important are the following factors to the quality of equine transportation?

Driver quality / experience / horsemanship **(1=not important; 5= very important)**

1 2 3 4 5

Size of space allocated per horse

1 2 3 4 5

Speed of trip (shorter travel times)

1 2 3 4 5

Cleanliness of trailer/horse trailer

1 2 3 4 5

Appendix 1

Trailer/trailer age and quality

1 2 3 4 5

Ventilation system of the trailer/trailer

1 2 3 4 5

Monitoring of horses before and after transportation

1 2 3 4 5

Monitoring of the horse during the travel

1 2 3 4 5

Stopping for an unloading rest during a long trip (>8 hours)

1 2 3 4 5

Please, complete this section if you usually use a Transport Company

Q23. In column 1 please mark HOW IMPORTANT these factors are IN YOUR DECISION on which transport company to use. In column 2 please SCORE YOUR CURRENT TRANPORT COMPANY in that area.

Please mark from (from 1= not important, 5 = very important)

FACTOR	IMPORTANCE TO YOU					RATING OF CURRENT TRANPORT COMPANY				
Reliable, on time service	1	2	3	4	5	1	2	3	4	5
Reputation / Brand / Longevity	1	2	3	4	5	1	2	3	4	5
Respect and care for horse health	1	2	3	4	5	1	2	3	4	5
Sponsorship of racing / breeding organisations	1	2	3	4	5	1	2	3	4	5
Transporting gear / additional items/ Handling of special requests	1	2	3	4	5	1	2	3	4	5
Size of space allocated per horse	1	2	3	4	5	1	2	3	4	5
Cleanliness of vehicles	1	2	3	4	5	1	2	3	4	5
Internet systems / ease of interaction	1	2	3	4	5	1	2	3	4	5
Ventilation of vehicles	1	2	3	4	5	1	2	3	4	5
Stopping for an unloading rest during a long trip (>8 hours)	1	2	3	4	5	1	2	3	4	5
Monitoring of the horse during the travel	1	2	3	4	5	1	2	3	4	5
Certainty of completion (will be done as requested)	1	2	3	4	5	1	2	3	4	5
Marketing / advertising	1	2	3	4	5	1	2	3	4	5
Familiarity / relationships with key staff	1	2	3	4	5	1	2	3	4	5
Speed of trip (shorter travel times)	1	2	3	4	5	1	2	3	4	5
Fleet age and maintenance	1	2	3	4	5	1	2	3	4	5
Occupational health & safety (driver fatigue management, etc.)	1	2	3	4	5	1	2	3	4	5
Price	1	2	3	4	5	1	2	3	4	5
Communication / notification	1	2	3	4	5	1	2	3	4	5
Driver quality / experience / horsemanship	1	2	3	4	5	1	2	3	4	5
Stable quality and 24 hour monitoring at stabling	1	2	3	4	5	1	2	3	4	5
Staff training	1	2	3	4	5	1	2	3	4	5

If you are interested in the results of this survey please leave your email or postal mail address and we will contact you when the results are available. Your details will be kept in confidence and separately from the information you have provided for this survey.

Name:…………………………………………………………………………
Email and/or postal mail address:………………………………………………………….

Thank you for participating

Yours sincerely

Dr Gary Muscatello, Lecturer in Applied Animal Microbiology, Equine Research, Faculty of Veterinary Science, The University of Sydney and **Dr Barbara Padalino**, PhD candidate, Faculty of Veterinary Science, The University of Sydney.

APPENDIX 2

Appendix 2

Table 1. Wald test's P values generated from univariate regression analysis. Wald test's P values calculated in the univariate regression analyses for all predictive variables on the following outcomes: traumatic injuries, colic, diarrhoea, heat stroke, laminitis, transport pneumonia (BJ: before journey; AJ: after journey).

Predictive Variables	Traumatic Injuries	Diarrhoea	Muscular Problem	Heat stroke	Laminitis	Transport Pneumonia	Colic
Age	<0.001	0.224	0.932	0.378	0.387	0.190	0.331
Address	0.159	0.176	0.090	0.591	0.562	0.669	0.808
Sector	0.120	0.246	0.534	0.879	0.164	<0.001	0.588
Background	0.144	0.046	0.430	0.063	0.630	<0.001	0.760
Number of horses	<0.001	0.756	0.690	0.167	0.412	<0.001	0.171
Journey frequency	0.392	0.796	0.381	0.769	0.207	<0.001	0.317
Journey duration	0.593	0.846	0.423	0.696	0.882	<0.001	0.625
Antibiotics	0.099	0.994	0.409	0.429	0.655	0.155	0.693
Tranquilizers	0.009	0.317	0.198	0.358	0.737	0.838	0.701
Oral supplements	0.535	0.906	0.601	0.864	0.278	<0.001	0.780
***Ad libitum* hay/water**	0.237	0.152	0.074	0.003	0.135	0.421	0.316
Protections	<0.001	0.356	0.847	0.930	0.454	0.325	0.773
Wearing of rug	0.166	0.886	0.800	0.892	0.781	0.004	0.968
Health assessment BJ	0.482	0.753	0.085	0.745	0.786	0.030	0.274
Temperature BJ	0.039	0.753	0.630	0.316	0.331	<0.001	0.937
Heart rate BJ	0.404	0.530	0.799	0.413	0.912	0.449	0.459
Feeding behaviour BJ	0.079	0.633	0.344	0.458	0.725	0.003	0.373
Drinking behaviour BJ	0.596	0.583	0.842	0.990	0.522	0.002	0.345
Weight BJ	0.055	0.178	0.134	0.354	0.080	0.550	0.401
General health BJ	0.007	0.744	0.781	0.646	0.339	0.720	0.825
Vehicle	0.064	0.929	0.829	0.697	0.773	<0.001	0.462
Tying	0.452	0.677	0.983	0.097	0.516	0.644	0.395
Monitoring	0.128	0.167	0.201	0.418	0.586	0.95	0.151
Feeding	0.752	0.813	0.052	0.868	0.093	0.025	0.788
Health assessment AJ	0.112	0.365	0.243	0.645	0.846	0.095	0.713
Temperature AJ	0.016	0.540	0.308	0.065	0.346	<0.001	0.353
Heart rate AJ	0.613	0.315	0.680	0.892	0.915	0.493	0.656

Feeding behaviour AJ	0.004	0.185	0.401	0.559	0.781	0.004	0.968
Drinking behaviour AJ	0.004	0.104	0.194	0.967	0.941	0.003	0.977
Weight AJ	0.006	0.874	0.395	0.602	0.233	0.777	0.167
General health AJ	<0.001	0.224	0.461	0.187	0.347	0.427	0.713
Recovery Strategies	0.006	0.248	0.821	0.707	0.034	0.022	0.759

Table 2. Results of the univariate regression analysis with injuries as the outcome. Respondents' details and transport management risk factors for transport-related injuries with a Wald test P value less than 0.250 identified using univariate logistic regression. In the third and fourth column the frequency of the respondent (not reporting and reporting injuries) are reported as total number (n) and percentage in each category.

Variable	**Category**	**No Injuries n(%)**	**Injuries n(%)**	**OR**	**95%CI**	**P [a]**
Age	>61	47(71.2)	19(28.8)	Ref	1	<.001
	51-60	115(68.9)	52(31.1)	1.11	0.59-2.08	
	41-50	122(61.6)	76(38.4)	1.54	0.84-2.81	
	31-40	79(49.4)	81(50.6)	2.53	1.37-4.69	
	20-30	67(35.0)	124(65)	4.57	2.49-8.40	
Address	ACT	17 (62.9)	10 (37.1)	Ref	1	0.159
	NSW	180 (56.6)	138(43.4)	1.30	0.57-2.93	
	NT	18 (72)	7(28)	0.66	0.20-2.12	
	QLD	43(45.3)	52(54.7)	2.05	0.85-4.94	
	SA	26(48.1)	28(51.9)	1.83	0.71-4.71	
	TAS	13(72.2)	5(27.8)	0.65	0.17-2.37	
	VIC	101(54.3)	85(45.7)	1.43	0.62-3.28	
	WA	40(21.5)	34(78.5)	1.44	0.58-3.56	
Sector	Recreational	104 (54.1)	88(45.9)	Ref	1	0.120
	Endurance	34(66.7)	17(33.3)	0.59	0.30-1.12	
	Equestrian Sport	218(56.0)	171(44.0)	0.92	0.65-1.31	
	Horse Breeding	34(46.6)	39(53.4)	1.35	0.78-2.32	
	SB racing	22(64.7)	12(35.3)	0.64	0.30-1.37	
	TB racing	26(44.8)	32(55.2)	1.45	0.80-2.62	
Backgrounds	Professionals	123(51.0)	118(49.0)	Ref	1	0.144
	Amateurs	315(56.6)	241(43.4)	0.79	0.58-1.08	
Horse Number	<5	207(60.7)	134(39.3)	Ref	1	<.001
	5-10	102(53.1)	90(46.9)	1.36	0.95-1.94	

	11-30	85(57.8)	62(42.2)	1.12	0.76-1.66	
	31-50	20(36.4)	35(63.6)	2.70	1.50-4.87	
	>51	24(38.7)	38(61.3)	2.44	1.40-4.25	
Antibiotics	No	433(53.4)	349(44.6)	Ref	1	0.099
	Yes	5(33.3)	10(66.7)	2.48	0.84-7.30	
Tranquilizers	No	431(54.0)	341(42.7)			0.009
	Yes	7(0.8)	18(2.2)	3.25	1.34-7.85	
Protections	No	278(34.8)	181(22.7)	Ref	1	<.001
	Yes	160(20.0)	178(22.3)	1.70	1.28-2.62	
Rugs	No	288(56.8)	219(43.2)	Ref	1	
	Yes	150(51.7)	140(48.3)	1.22	0.91-1.64	
Temperature BJ	No	337(42.2)	253(31.7)			0.039
	Yes	101(12.6)	106(13.2)	1.39	1.01-1.92	
Feeding Behaviour BJ	No	223(55.9)	176(44.1)	Ref	1	0.079
	Yes	215(54.0)	183(46.0)	1.28	0.97-1.69	
Weight BJ	No	375(85.6)	63(14.4)	Ref	1	0.055
	Yes	289(80.5)	70(19.5)	1.44	0.99-2.09	
General health BJ	No	113(14.1)	64(8.0)			0.007
	Yes	325(40.7)	295(37.0)	1.60	1.13-2.26	
Vehicle	Truck	96(60.0)	64(40.0)	Ref	1	0.064
	Two horses straight trailer	207(50.9)	199(49.1)	1.44	0.99-2.09	
	Two horses angle trailer	48(64.0)	27(36.0)	0.84	0.47-1.48	
	3-4 horses angle trailer	56(51.8)	52(48.2)	1.39	0.85-2.27	
	3-4 gooseneck trailer	31(64.5)	17(35.5)	0.82	0.42-1.60	
Monitoring	No monitor	176(60.7)	114(39.3)	Ref	1	0.128
	By camera	81(51.9)	75(48.1)	1.42	0.96-2.11	
	At fuel stop	151(53.9)	129(46.1)	1.31	0.94-1.84	
Health assessment AJ	A veterinarian	24(53.3)	21(46.7)	Ref	1	0.112
	Non veterinary staff	354(53.6)	306(46.4)	0.98	0.53-1.81	
	No assessment	60(65.2)	32(34.8)	0.60	0.29-1.25	
Temperature AJ	No	338(42.4)	250(31.6)			0.016

	Yes	100(12.5)	109(13.6)	1.47	1.07-2.02	
Feeding Behaviour AJ	No	179(22.4)	111(13.9)			0.004
	yes	259(32.4)	248(31.1)	1.54	1.15-2.07	
Drinking Behaviour AJ	No	175(21.9)	108(13.5)			0.004
	yes	263(32.9)	251(31.4)	1.54	1.15-2.07	
Weight AJ	No	364(58.2)	270(33.8)			0.006
	Yes	74(9.2)	89(11.1)	1.62	1.14-2.29	
General Health AJ	No	120(67.4)	58(32.6)	Ref	1	<.001
	Yes	318(51.4)	301(48.6)	1.95	1.38-2.77	
Recovery Strategies	No	226(28.3)	150(18.8)			0.006
	Yes	212(26.5)	209(26.2)	1.48	1.21-1.96	

Odds ratio (OR); 95% confidence interval (95%CI); [a] P value calculated using Wald's test (P). ACT: Australian Capital Territory; NSW: New South Wales; NT: Northern Territory; QLD: Queensland; SA: South Australia; TAS: Tasmania, VIC: Victoria; WA: Western Australia; SB: Standardbred, TB: Thoroughbred; BJ: before journey; AJ: after journey.

Table 3. Results of the univariate regression analysis with diarrhoea as the outcome. Respondents' details and transport management risk factors for transport-related diarrhea with a Wald test P value less than 0.250 identified using univariate logistic regression. In the third and fourth column the frequency of the respondent (not reporting and reporting diarrhea) are reported as total number (n) and percentage in each category.

Variable	**Category**	**No Diarrhea n(%)**	**Diarrhoea n(%)**	**OR**	**95%CI**	**P [a]**
Age	>61	51(77.2)	15(22.8)	Ref	1	0.224
	51-60	138(82.6)	29(17.4)	0.71	0.35-1.43	
	41-50	164(82.8)	34(17.2)	0.70	0.35-1.39	
	31-40	128(80.0)	32(20.0)	0.85	0.42-1.69	
	20-30	142(74.3)	49(25.7)	1.17	0.60-2.26	
Address	ACT	25(92.6)	2(7.4)	Ref	1	0.176
	NSW	254(79.9)	64(20.1)	3.15	0.72-13.64	
	NT	19(76.0)	6(24)	3.94	0.71-21.74	
	QLD	73(76.8)	22(23.2)	3.76	0.82-17.17	
	SA	37(68.5)	17(31.5)	5.74	1.21-27.05	
	TAS	16(88.9)	2(11.1)	1.56	0.19-12.23	
	VIC	149(80.1)	37(19.9)	3.10	0.70-13.69	
	WA	64(86.5)	10(13.5)	1.95	0.39-9.54	
Sector	Recreational	154(80.2)	38(19.8)	Ref	1	0.246

	Endurance	44(86.3)	7(13.7)	0.64	0.26-1.54	
	Equestrian Sport	303(77.9)	86(22.1)	1.15	0.75-1.76	
	Horse Breeding	60(82.2)	13(17.8)	0.87	0.43-1.76	
	SB racing	32(94.1)	2(5.9)	0.25	0.05-1.10	
	TB racing	44(75.9)	14(24.1)	1.29	0.64-2.58	
Backgrounds	Professionals	203(25.4)	38(4.7)	Ref	1	0.046
	Amateur	434(54.4)	122(15.3)	1.50	1.0-2.24	
Ad libitum hay/water	Yes	242(82.6)	51(17.4)	Ref	1	0.152
	No	395(78.4)	109(21.6)	1.30	0.90-1.89	
Weight BJ	No	525(79.1)	139(20.9)	Ref	1	0.178
	Yes	112(84.2)	21(15.8)	0.70	0.42-1.17	
Monitoring	No monitor	235(81.0)	55(19.0)	Ref	1	0.167
	By camera	116(74.3)	40(25.7)	1.47	0.92-2.34	
	At fuel stop	228(81.4)	52(18.6)	0.97	0.64-1.48	
Feeding Behaviour AJ	No	239(82.4)	51(17.6)	Ref	1	0.185
	Yes	398(78.5)	109(21.5)	1.28	0.88-1.85	
Drinking Behaviour AJ	No	235(83.0)	48(17.0)	Ref	1	0.104
	Yes	402(78.2)	112(21.8)	1.36	0.93-1.98	
General Health AJ	No	148(83.1)	30(16.9)	Ref	1	0.224
	Yes	489(78.9)	130(21.1)	1.31	0.84-2.03	
Recovery Strategies	No	169(78.2)	47(21.8)	Ref	1	
	Yes	468(80.5)	113(19.5)	1.22	0.86-1.73	

Odds ratio (OR); 95% confidence interval (95%CI); [a] P value calculated using Wald's test (P). ACT: Australian Capital Territory; NSW: New South Wales; NT: Northern Territory; QLD Queensland; SA: South Australia; TAS: Tasmania, VIC: Victoria; WA: Western Australia; SB: Standardbred, TB: Thoroughbred; BJ: before journey; AJ: after journey.

Appendix 2

Table 4. Results of the univariate regression analysis with muscular problem as the outcome. Respondents' details and transport management risk factors for transport-related muscular problems with a Wald test P value less than 0.250 identified using univariate logistic regression. In the third and fourth column the frequency of the respondent (not reporting and reporting muscular problems) are reported as total number (n) and percentage in each category.

Variable	Category	No Muscular problems n(%)	Muscular Problems n(%)	OR	95%CI	[a] P
Address	ACT	25(92.6)	2(7.3)	Ref	1	0.090
	NSW	282(88.7)	36(11.3)	1.59	0.36-7.01	
	NT	21(84.0)	4(16.0)	2.38	0.39-14.31	
	QLD	75(78.9)	20(21.1)	3.33	0.72-15.27	
	SA	48(88.9)	6(11.1)	1.56	0.29-8.31	
	TAS	14(77.7)	4(22.3)	3.57	0.58-21.98	
	VIC	168(90.3)	18(9.7)	1.33	0.29-6.12	
	WA	60(81.1)	14(18.9)	2.91	0.61-13.78	
Ad libitum hay/water	Yes	263(89.8)	30(10.2)	Ref	1	0.074
	No	430(85.3)	74(14.7)	1.50	0.96-2.36	
Health assessment BJ	A veterinarian	36(94.7)	2(5.3)	Ref	1	0.085
	Non veterinary staff	570(87.4)	82(12.6)	2.58	0.61-10.92	
	No assessment	87(81.3)	20(18.7)	4.13	0.92-18.56	
Weight BJ	No	572(86.1)	92(13.9)	Ref	1	0.134
	Yes	121(90.9)	12(9.1)	0.61	0.32-1.16	
Monitoring	No monitor	244(84.1)	46(15.9)	Ref	1	0.201
	By camera	140(89.7)	16(10.3)	0.60	0.33-1.11	
	At fuel stop	246(87.9)	34(12.1)	0.73	0.45-1.18	
Feeding	No	258(84.0)	49(16.0)	Ref	1	0.052
	Yes	386(88.9)	48(11.1)	0.65	0.42-1.00	
Health assessment AJ	A veterinarian	43(95.5)	2(4.5)	Ref	1	0.243
	Non veterinary staff	570(86.4)	90(13.6)	3.39	0.81-14.12	
	No assessment	80(86.9)	12(13.1)	3.22	0.69-14.94	
Drinking behaviour AJ	No	252(89.0)	31(11.0)	Ref	1	0.194

	Yes	441(85.8)	73(14.2)	1.34	0.86-2.10	

Odds ratio (OR); 95% confidence interval (95%CI); [a] P value calculated using Wald's test (P). ACT: Australian Capital Territory; NSW: New South Wales; NT: Northern Territory; QLD Queensland; SA: South Australia; TAS: Tasmania, VIC: Victoria; WA: Western Australia; BJ: before journey; AJ: after journey.

Table 5. Results of the univariate regression analysis with heat stroke as the outcome. Respondents' details and transport management risk factors for transport-related heat stroke with a Wald test P value less than 0.250 identified using univariate logistic regression. In the third and fourth column the frequency of the respondent (not reporting and reporting heat stroke) are reported as total number (n) and percentage in each category.

Variable	**Category**	**No Heat Stroke n(%)**	**Heat Stroke n(%)**	**OR**	**95%CI**	**[a] P**
Backgrounds	Professionals	223(92.5)	18(7.5)	Ref	1	0.063
	Amateur	490(88.1)	66(11.9)	1.66	0.97-2.86	
Horse Number	<5	304(89.1)	37(10.9)	Ref	1	0.167
	5-10	165(85.9)	27(14.1)	1.34	0.76-2.28	
	11-30	136(95.5)	11(7.5)	0.66	0.32-1.34	
	31-50	53(96.4)	2(3.6)	0.31	0.07-1.29	
	>51	55(88.7)	7(11.3)	1.04	0.44-2.46	
Ad libitum hay/water	Yes	275(34.5)	18(2.2)	Ref	1	0.003
	No	438(54.9)	66(8.2)	2.30	1.33-3.95	
Tying	No	65(95.6)	3(4.4)	Ref	1	0.097
	Yes	602(88.9)	75(11.1)	2.69	0.83-8.73	
Temperature BJ	No	519(88.3)	69(11.7)	Ref	1	0.065
	Yes	194(92.8)	15(7.2)	0.58	0.32-1.03	
General Health AJ	No	164(92.1)	14(7.9)	Ref	1	0.187
	Yes	549(88.7)	70(11.3)	1.49	0.82-2.71	

Odds ratio (OR); 95% confidence interval (95%CI); [a] P value calculated using Wald's test (P); BJ: before journey; AJ: after journey.

Table 6. Results of the univariate regression analysis with laminitis as the outcome. Respondents' details and transport management risk factors for transport-related laminitis with a Wald test P value less than 0.250 identified using univariate logistic regression. In the third and fourth column the frequency of the respondent (not reporting and reporting laminitis) are reported as total number (n) and percentage in each category.

Variable	**Category**	**No Laminitis**	**Laminitis**	**OR**	**95%CI**	**[a] P**
Sector	Recreational	184(95.8)	8(4.2)	Ref	1	0.164
	Endurance	50(98.0)	1(2.0)	0.46	0.05-3.71	
	Equestrian Sport	382(98.2)	7(1.8)	0.42	0.15-1.17	
	Horse Breeding	72(98.6)	1(1.4)	0.31	0.04-2.49	
	SB racing	31(91.2)	3(8.8)	2.22	0.55-8.85	
	TB racing	55(94.8)	3(5.2)	1.25	0.32-4.89	
Journey frequency	Monthly	248(96.5)	9(3.5)	Ref	1	0.207
	Fortnightly	160(99.4)	1(0.6)	0.17	0.22-1.33	
	Weekly	194(97.0)	6(3.0)	0.85	0.29-2.43	
	Twice a week	121(97.6)	3(2.4)	0.68	0.18-2.56	
	Daily	51(92.7)	4(7.3)	2.16	0.64-7.28	
Ad libitum hay/water	Yes	288(98.3)	5(1.7)	Ref	1	0.135
	No	486(96.4)	18(3.6)	2.13	0.79-5.75	
Weight BJ	No	618(97.5)	16(3.5)	Ref	1	0.080
	Yes	156(95.7)	7(4.3)	2.25	0.90-5.57	
Feeding	No	295(96.1)	12(3.9)	Ref	1	0.093
	Yes	426(98.2)	8(1.8)	0.46	0.18-1.13	
Weight AJ	No	618(97.5)	16(2.5)	Ref	1	0.233
	Yes	156(95.7)	7(4.3)	1.73	0.70-4.28	
Recovery strategies	Yes	414(98.3)	7(1.7)	Ref	1	0.003
	No	360(95.7)	16(4.3)	2.62	1.07-6.4	

Odds ratio (OR); 95% confidence interval (95%CI); [a] P value calculated using Wald's test (P); SB: Standardbred, TB: Thoroughbred; BJ: before journey; AJ: after journey.

Appendix 2

Table 7. Results of the univariate regression analysis with transport pneumonia as the outcome. Respondents' details and transport management risk factors for transport pneumonia with a Wald test P value less than 0.250 identified using univariate logistic regression. In the third and fourth column the frequency of the respondent (not reporting and reporting pneumonia) are reported as total number (n) and percentage in each category.

Variable	Category	No Transport Pneumonia	Transport Pneumonia	OR	95%CI	[a] P
Age	>61	59(89.4)	7(10.6)	Ref	1	0.190
	51-60	150(89.2)	17(10.8)	0.95	0.37-2.42	
	41-50	175(88.4)	23(11.6)	1.10	0.45-2.71	
	31-40	145(90.6)	15(9.4)	0.87	0.33-2.24	
	20-30	182(95.3)	9(4.7)	0.41	0.14-1.16	
Sector	Recreational	186(96.9)	6(3.1)	Ref	1	<.001
	Endurance	45(88.2)	6(1.8)	4.12	1.29-13.20	
	Equestrian Sport	369(94.8)	20(5.2)	1.67	0.67-4.16	
	Horse Breeding	61(83.6)	12(6.4)	6.08	2.23-16.62	
	SB racing	27(79.4)	7(20.6)	8.02	2.55-25.26	
	TB racing	36(62.1)	22(37.9)	18.92	7.29-49.03	
Backgrounds	Amateur	553(96.0)	23(4.0)	Ref	1	<.001
	Professionals	191(79.2)	50(20.8)	6.06	3.61-10.17	
Horse Number	<5	330(96.7)	11(3.3)	Ref	1	<.001
	5-10	177(92.2)	15(7.8)	2.53	1.15-5.58	
	11-30	132(89.8)	15(10.2)	3.40	1.54-7.52	
	31-50	45(81.8)	10(18.2)	6.65	2.70-16.40	
	>51	40(64.5)	22(35.5)	16.48	7.53-36.07	
Journey frequency	Monthly	238(92.6)	19(7.4)	Ref	1	<.001
	Fortnightly	155(96.3)	6(3.7)	0.48	0.19-1.22	
	Twice a week	108(87.1)	16(12.9)	1.85	0.91-3.74	
	Weekly	186(93)	14(7)	0.94	0.46-1.9	
	Daily	37(67.3)	18(32.7)	6.09	2.9-12.65	
Journey duration	< 2hours	434(93.7)	29(6.3)	Ref	1	<.001
	2-4 hours	205(90.3)	22(9.7)	1.60	0.90-2.86	
	5-8 hours	65(82.3)	14(17.7)	3.22	1.61-6.41	
	> 8 hours	20(71.4)	8(28.6)	5.98	2.43-14.72	
Antibiotics	No	712(91.0)	70(9.0)	Ref	1	0.155
	Yes	12(80.0)	3(20.0)	2.54	0.70-9.20	
Oral supplements	No	613(92.9)	47(7.1)	Ref	1	<.001

	Yes	111(81.0)	26(19.0)	3.05	1.18-5.13	
Wearing rugs	No	449(88.6)	58(11.4)	Ref		0.004
	Yes	275(94.8)	15(5.2)	2.36	1.31-4.25	
Health assessment BJ	No assessment	104(97.2)	3(2.8)	Ref		0.030
	Non veterinary staff	588(90.2)	64(9.8)	6.48	1.5-26.50	
	A veterinarian	32(84.2)	6(15.8)	3.76	1.20-11.71	
Temperature BJ	No	562(95.2)	28(4.8)	Ref	1	<.001
	Yes	162(78.3)	45(21.7)	5.57	3.37-9.20	
Feeding Behaviour BJ	No	364(94.0)	23(6.0)	Ref	1	0.003
	yes	360(87.8)	50(12.2)	2.19	1.31-3.67	
Drinking Behaviour BJ	No	375(93.9)	24(6.1)	Ref	1	0.002
	yes	349(87.7)	49(12.3)	2.19	1.31-3.65	
Vehicle	Two horses angle trailer	71(94.7)	4(5.3)	Ref	1	<.001
	Truck	127(79.4)	33(20.6)	4.61	1.57-13.51	
	Two horses straight trailer	388(95.6)	18(4.4)	0.82	0.27-2.49	
	3-4 horses angle trailer	95(87.9)	13(12.1)	2.42	0.76-7.74	
	3-4 gooseneck trailer	43(89.6)	5(10.4)	2.06	0.52-8.09	
Do you offer food	Yes	404(93.1)	30(6.9)	Ref	1	0.025
	No	271(88.3)	36(11.7)	1.78	1.07-2.97	
Health assessment AJ	A veterinarian	39(86.7)	6(13.3)	Ref	1	0.095
	Non veterinary staff	596(90.3)	64(9.7)	0.69	0.28-1.71	
	No assessment	89(96.7)	3(3.3)	0.21	0.05-0.90	
Temperature AJ	No	559(95.1)	29(4.9)	Ref	1	<.001
	Yes	165(78.9)	44(21.1)	5.14	3.12-8.4	

Feeding Behaviour AJ	No	275(94.8)	15(5.2)	Ref	1	0.004
	yes	449(88.6)	58(11.4)	2.36	1.31-4.25	
Drinking Behaviour AJ	No	269(95.1)	14(4.9)	Ref	1	0.003
	yes	455(88.5)	59(11.5)	2.49	1.36-4.54	
Recovery strategies	No	373(88.6)	48(11.4)	Ref		0.022
	Yes	351(93.3)	25(6.7)	1.80	1.09-2.99	

Odds ratio (OR); 95% confidence interval (95%CI); [a] P value calculated using Wald's test (P); SB: Standardbred, TB: Thoroughbred; BJ: before journey; AJ: after journey.

APPENDIX 3

Appendix 3

Table 1. Univariate and multivariate logistic analysis with **preloading** problem behaviour (PLPB) as outcome. Data are presented as odds ratio (OR), confidence interval (95% CI) and P value (P).

Variable	**Category**	**No PLPB (%)**	**PLPB (%)**	**Univariate**			**Multivariate**		
				OR	**95%CI**	**P**	**OR**	**95%CI**	**P**
Type of Training	Habituation	155 (96.9)	5 (3.1)	Ref			Ref		
	Self- loading	78(90.7)	8(9.3)	3.17	1.02-9.84	0.016	2.76	0.89-8.58	0.079
	No training	313 (90.7)	32 (9.3)	3.16	1.23-8.10	0.045	3.43	1.34-8.79	0.010
	Operant Conditioning	161 (79.7)	41 (20.3)	7.88	3.10-20.02	<.001	7.64	3.02-19.37	<.001
Equipment	No equipment	82 (91.1)	4 (8.9)	Ref		Ref			
	Halter and leadrope	525 (90.4)	56 (9.6)	1.09	0.50-2.37	0.822			ns
	Hindquarter rope	28 (75.7)	9 (24.3)	3.29	1.16-9.34	0.025			ns
	Whip	51 (82.3)	11 (17.7)	2.21	0.83-5.86	0.111			ns
Vehicle	3-4 horses angle trailer	103 (95.4)	5 (4.6)	Ref			Ref		
	3-4 gooseneck trailer	44 (91.7)	4 (8.3)	1.87	0.48-7.28	0.365	1.90	0.48-7.47	0.356
	Two horses angle trailer	64 (85.3)	11 (14.7)	3.54	1.18-10.61	0.024	3.30	1.10-9.98	0.034
	Truck	149 (93.1)	11 (6.9)	1.52	0.51-4.48	0.448	1.51	0.51-4.49	0.450
	Two horses straight trailer	351 (93.1)	55 (6.9)	3.22	1.26-8.23	0.014	3.13	1.21-8.06	0.018

Appendix 3

Table 2. Univariate and multivariate logistic analysis with **loading** problem behaviour as outcome. Data are presented as odds ratio (OR), confidence interval (95% CI) and P value (P).

Variable	Category	No LPB (%)	LPB (%)	Univariate			Multivariate		
				OR	95%CI	P	OR	95%CI	P
Type of Training	Habituation	152 (95.0)	8 (5.0)	Ref			Ref		
	Self-loading	82 (95.3)	4 (4.7)	0.92	0.27-3.15	0.903	0.86	0.25-2.93	0.810
	No training	293 (84.9)	52 (15.1)	3.37	1.56-7.26	0.002	3.51	1.62-7.58	0.001
	Operant Conditioning	109 (54.0)	93 (46.0)	16.21	7.57-34.68	<.001	16.60	7.74-35.61	<.001
Equipment	No equipment	81 (94.4)	5 (5.8)	Ref			Ref		
	Halter and leadrope	479 (82.4)	102 (17.6)	3.62	1.43-9.13	0.006			ns
	Hindquarter rope	20 (54.1)	17 (45.9)	14.45	4.77-43.78	<.001			ns
	Whip	37 (59.7)	25 (40.3)	11.49	4.08-32.28	<.001			ns
Vehicle	3-4 horses angle trailer	97 (89.8)	11 (10.2)	Ref			Ref		
	3-4 gooseneck trailer	43 (89.6)	5 (10.4)	1.025	0.33-3.13	0.965	0.99	0.30-3.19	0.991
	Two horses angle trailer	123 (89.8)	14 (10.2)	2.02	0.86-4.74	0.105	1.81	0.73-4.46	0.199
	Truck	123 (76.9)	37 (23.1)	2.65	1.28-5.46	0.008	2.97	1.38-6.38	0.005
	Two horses straight trailer	316 (77.8)	90 (22.2)	2.51	1.29-4.88	0.007	2.61	1.29-5.26	0.007

Appendix 3

Table 3. Univariate and multivariate logistic analysis with **travelling** problem behaviour as outcome. Data are presented as odds ratio (OR), confidence interval (95% CI) and P value (P).

Variable	Category	No TPB(%)	TPB (%)	Univariate			Multivariate		
				OR	95%CI	P	OR	95%CI	P
Type of Training	Habituation	148 (92.5)	12 (7.5)	Ref			Ref		
	Self-loading	74 (86.0)	12(14.0)	2.00	0.85-4.66	**0.109**	2.04	0.87-4.82	0.101
	No training	286 (82.9)	59 (17.1)	2.54	1.32-4.88	**0.005**	2.13	1.10-4.12	0.025
	Operant Conditioning	154 (76.2)	48 (23.8)	3.84	1.96-7.52	**<.001**	3.52	1.78-6.95	<.001
Sector	Endurance	50 (98.0)	1 (2.0)	Ref			Ref		
	Equestrian Sport	324 (83.3)	65 (16.7)	9.88	1.57-61.96	**0.014**	10.05	1.64-61.48	0.013
	Breeding	67 (91.8)	6 (8.2)	4.41	0.59-32.57	**0.146**	5.05	0.69-36.58	0.109
	Recreational	162 (84.4)	30 (15.6)	9.12	1.42-58.46	**0.020**	9.40	1.50-58.81	0.017
	ST racing	25 (73.5)	9 (26.5)	17.74	2.47-127.0	**0.004**	18.60	2.63-131.5	0.003
	TH racing	38 (65.5)	20 (34.5)	25.93	3.89-172.5	**<.001**	24.92	3.81-162.8	<.001

Appendix 3

Table 4. Univariate logistic analysis with **unloading** problem behaviour as outcome. Data are presented as odds ratio (OR), confidence interval (95% CI) and P value (P).

Variable	Category	No UPB(%)	UPB(%)	OR	95%CI	P
Type of Training	Habituation	155 (96.9)	5 (3.1)	Ref		
	Self-loading	82 (95.3)	4 (4.7)	1.51	0.40-5.66	**0.54**
	No training	328 (95.1)	17 (4.9)	1.60	0.59-4.33	**0.35**
	Operant Conditioning	180(89.1)	22 (10.9)	3.78	1.42-10.01	**0.007**

APPENDIX 4

Item 1. Survey of Horse transportation: Issues and practices

Part 1: respondent details

Q1. What is the section of the Australian equine industry in which you are primarily involved in? For what do you use horses?

1. Thoroughbred Racing
2. Standardbred racing
3. Equestrian sport (dressage, eventing, jumping, reining, driving, vaulting)
4. Endurance
5. Horse breeding
6. Recreational non-competitive riding

Q2. What is your relationship with the horse?

1. Professional (e.g. horse trainer, stud manager, riding teacher)
2. Amateur (e.g. horse owner, rider)

Part 4: Details of the most recent case of transport-related health problem

Q1.Please describe the last time your horses experienced a transported related illness (e.g pathology, type of horse, type of trip)

Horse details (i.e. age, breed, sex):____

Trip details (i.e. duration, transport mean):__________________________________

Illness:____

Your comments about the possible cause:

____

Appendix 4

Table 2. Distribution of the data

Variable	Category	Count	%
	Horse-level predictive variables		
Sex	Gelding	103	52.3
	Mare	74	37.6
	Stallion	20	10.1
	Total	197	100
	Missing	17	7.9
Breed	Arabian	22	10.6
	Quarter horse	17	8.2
	Thoroughbred	86	41.3
	Warmblood	67	32.2
	Standardbred	16	7.8
	Total	208	100
	Missing	6	2.8
Age	8-24 months	23	11.8
	2-5 years	66	32.8
	6-10 years	70	34.8
	>10 years	42	20.9
	Total	201	100
	Missing Values	13	6.0
Use	Endurance	12	5.6
	Equestrian sport	94	43.9
	Horse breeding	27	12.6
	Standardbred racing	13	6.1
	Thoroughbred Racing	41	19.2
	Recreational non-competitive riding	27	12.6
	Total	214	100
Status	Amateur	104	48.6
	Professionals	110	51.4
	Total	214	100
	Journey-level predictive variables		
Vehicle	Horse trailer	101	50.5
	Truck	99	49.5
	Total	200	100
	Missing Values	14	7
Journey duration	<8 hours	97	45.3
	8-24 hours	50	23.4
	> 24 hours	67	31.3
	Total	214	100
	no	113	55.9

Commercial transport	yes	89	44.1
	Total	202	100
	Missing Values	12	5.6
	Outcome		
Health Problem Category	Injuries	35	16.3
	Muscular Problems	12	5.6
	Heat stroke	13	6.1
	Gastrointestinal Problems	51	23.8
	Respiratory Problems	72	33.7
	Death	31	14.5
	Total	214	100

Table 3. Health outcomes in transported horses. The frequency is expressed in raw numbers and percentage within the same category.

	Injuries n(%)	**Muscular n(%)**	**Heat Stroke n(%)**	**Gastro. n(%)**	**Resp. n(%)**	**Death n(%)**
Use						
Endurance	1(8.3)	1(8.3)	0(0.0)	5(41.7)	4(33.3)	1(8.3)
Equestrian	16(17.0)	7(7.4)	7(7.4)	30(31.9)	26(27.6)	8(8.5)
Breeding	2(7.4)	1(3.7)	1(3.7)	3(11.1)	12(44.4)	8(29.6)
Standardbred racing	6(46.1)	0(0.0)	0(0.0)	0(0.0)	4(30.8)	3(23.1)
Thoroughbred Racing	4(9.7)	0(0.0)	3(7.3)	5(12.2)	19(46.3)	10(24.4)
Recreational	6(22.2)	3(3.0)	2(7.4)	8(29.6)	7(25.9)	1(3.7)
Sex						
Gelding	13(12.6)	4(3.9)	4(3.9)	29(28.1)	37(35.9)	16(15.5)
Mare	16(21.6)	5(6.7)	7(9.4)	15(20.3)	21(28.4)	10(13.5)
Stallion	1(5)	2(10)	1(10)	5(25)	9(45)	2(10)
Breed						
Arabian	1(4.5)	3(13.6)	2(9.1)	10(45.4)	6(27.3)	0(0)
Quarter horse	6(35.2)	1(5.8)	1(5.9)	4(23.5)	4(23.5)	1(5.8)
Thoroughbred	13(15.1)	3(6.9)	6(6.9)	13(15.1)	33(38.4)	18(20.9)
Warmblood	5(7.4)	4(5.9)	4(5.9)	22(32.8)	24(35.8)	8(11.9)
Standardbred	7(43.7)	1(6.2)	0(0)	1(6.2)	4(25)	3(18.75)
Age						
8 – 24 months	5(21.7)	0(0)	1(4.3)	3(13.4)	12(52.1)	2(8.6)
2-5 years	11(16.6)	2(3.0)	6(9.1)	11(16.6)	23(34.8)	13(19.7)
6-10 years	9(12.8)	6(8.6)	4(5.7)	17(24.3)	23(32.8)	11(15.7)
>10 years	6(14.3)	3(7.1)	1(2.4)	17(40.4)	12(28.6)	3(7.1)
Use						
Endurance	1(8.3)	1(8.3)	0(0.0)	5(41.7)	4(33.3)	1(8.3)

Equestrian	16(17.0)	7(7.4)	7(7.4)	30(31.9)	26(27.6)	8(8.5)
Breeding	2(7.4)	1(3.7)	1(3.7)	3(11.1)	12(44.4)	8(29.6)
ST racing	6(46.1)	0(0.0)	0(0.0)	0(0.0)	4(30.8)	3(23.1)
TH Racing	4(9.7)	0(0.0)	3(7.3)	5(12.2)	19(46.3)	10(24.4)
Recreational	6(22.2)	3(3.0)	2(7.4)	8(29.6)	7(25.9)	1(3.7)
Status						
Amateur	8(8.8)	7(7.7)	8(8.8)	34(37.4)	24(26.4)	10(10.9)
Professional	5(4.9)	5(4.9)	5(4.9)	17(16.8)	48(47.6)	21(20.9)
Vehicle						
Horse trailer	30(29.7)	6(5.9)	12(11.9)	32(31.7)	17(16.8)	4(4.0)
Truck	4(4.0)	6(6.1)	1(1.0)	16(16.2)	48(48.5)	24(24.2)
Duration						
<8	29(29.9)	7(7.2)	11(11.3)	32(33.0)	11(11.3)	7(7.2)
8-24	5(10.0)	5(10.0)	1(2.0)	7(14.0)	27(54.0)	5(10)
>24	1(1.5)	0(0.0)	1(1.5)	12(18.0)	34(50.7)	19(28.3)
Operator						
non-commercial	27(36.3)	12(23.9)	9(8.0)	41(6.2)	17(15.0)	7
commercial	8(9.0)	0(0.0)	3(3.4)	8(24.7)	48(53.9)	22

Table 4. Associations between journey duration, vehicle type and operator used for horse transport. Results reported in percentage with observed frequency and expected frequency in brackets.

	Short Journey (<8h)	**Intermediate Journey (8_24h)**	**Long journey (>24h)**
Truck	17.3%(47.5%)	34.3%(23.7%)	48.4%(28.8%)
Horse Trailer	76.2%(47.5%)	13.9 %(24.2%)	9.9%(28.3%)
Non-Commercial	71.7%(45.6%)	18.6 %(23.2%)	9.7 %(31.2%)
Commercial	12.3%(45.5%)	29.3 %(23.3%)	58.4%(31.2%)

Appendix 4

Table 5. Multinomial regression model of associations between Health outcomes and horse and journey variables

		Health Outcome Category							
		Death		Gastrointestinal		Respiratory		Muscular	
		OR (CI)	P	OR (CI)	P	OR (CI)	P	OR (CI)	P
Journey Duration (hours)	< 8	referent	-	-	-	-	-	-	-
	8-24	3.4 (0.7-16.5)	0.12	1.7 (0.4-6.9)	.403	15.7 (4.3-56.7)	.000	5.8 (1.1-29.5)	0.03
	>24	101.6 (10.2- 1010.5)	<0.001	14.2 (1.5-133.8)	.020	113.9 (12.2-1060.7)	.000	0 (0-0)	>0.9
Breed	Standardbred	referent	-	-	-	-	-	-	-
	Arabian	0 (0-0)	>0.9	95.8 (4.6-1990.3)	.003	20.8(1.2-345.2)	.034	25.6(1.0-610.9)	0.04
	Quarter horse	0.5(0-10.2)	0.7	5.4(0.4-71.2)	.195	1.5(0.1-15.1)	.691	1.2(0.1-27.1)	0.9
	Thoroughbred	7.5 (1.0-56)	0.05	9.6(0.9-100.0)	.057	7.4(1.2-45.7)	.031	1.3(0.1-16.8)	0.8
	Warmblood	7.6 (0.8-69.9)	.070	43.0(3.8-485.9)	.002	18.5 (2.5-136.9)	.004	7.7(0.6-100.9)	0.1

APPENDIX 5

Appendix 5

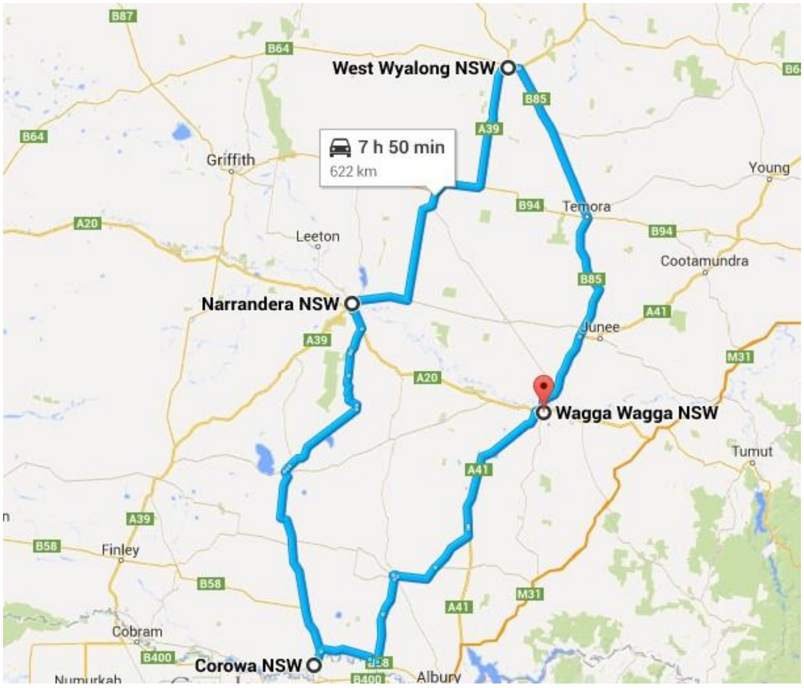
Figure 1.Horse stable

Figure 2. Outside of the truck used for the experiment

Appendix 5

Figure 3. Inside of the truck used for the experiment

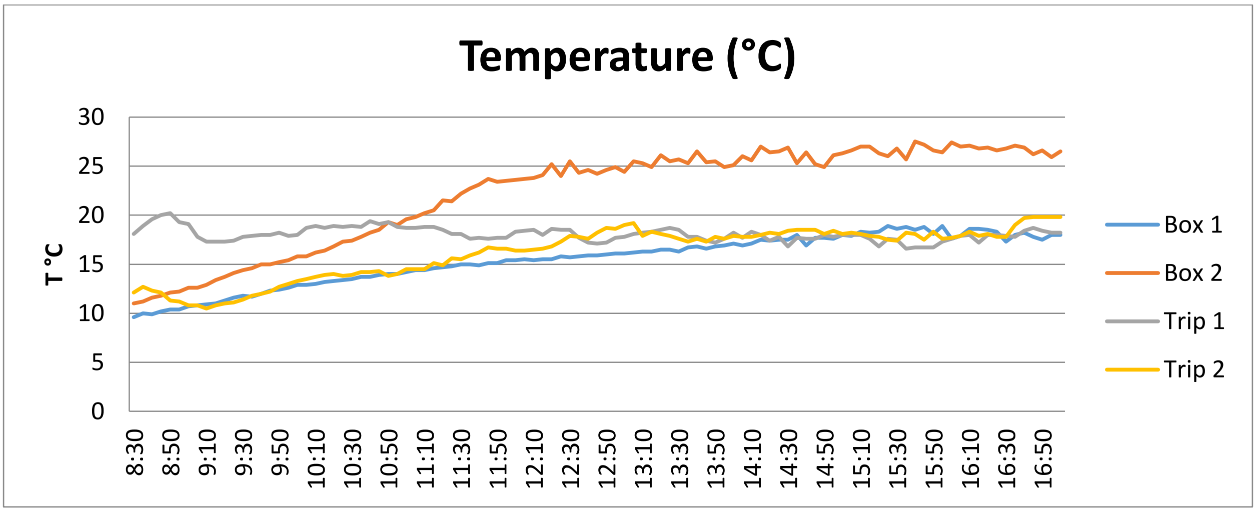
Figure 4. Journey route



Appendix 5

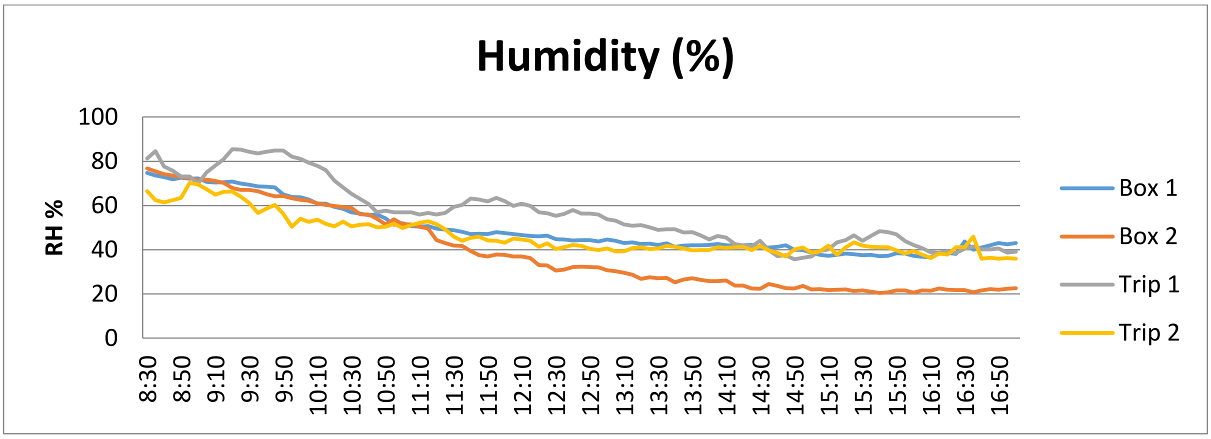
Figure 5. Exhaled breath condensate (EBC) collection

Figure 6. Temperature (°C) recorded inside the truck (Trip 1, Trip 2) and in the boxes (Box 1 and 2)



Appendix 5

Figure 7. Changes of humidity (%) inside the truck (Trip 1, Trip 2) and in the stable (Box 1 and 2)



Appendix 5

Figure 8. Bar chart produced for each TW collected from each horse at each time point (A: preloading, B: unloading, C: 24 hours AJ, D: 5 days AJ) at the family level. Pasteurellaceae expressed in blue-greenish.

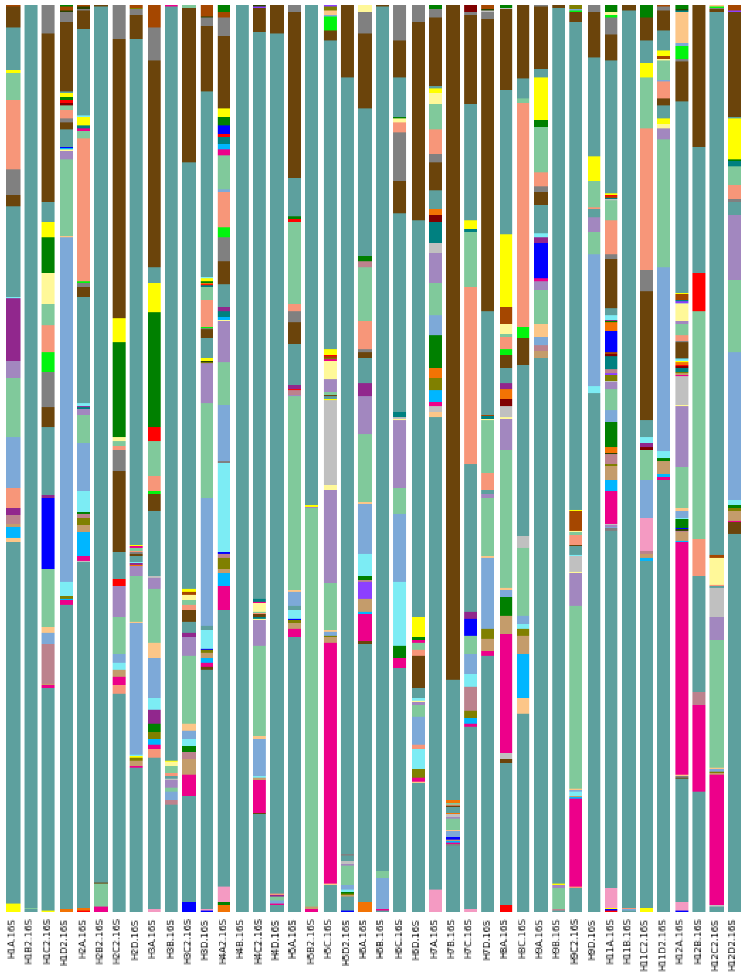


Figure 9: Pilot bar for *Pasteurellaceae* for each horse at the different times obtained in phyloseq (McMurdie and Holmes, 2013). No enough Dna was extracted for the sample of H8 at unloading and 5 days AJ. In the legend, if there is no name for a colour, it indicates that for that OTU the identification did not progress to genus, thus family = *Pasteurellaceae* but no further information for that particular sequence. H: horses. H1, H2, H3, H4, H11 and H12 formed group A (abnormal auscultation at unloading) and H5, H6, H7, H8, H9 formed group B (normal auscultation at unloading). H2, H3 and H4 retained high levels of *Aggregatibacter spp*. 5 days post transport.

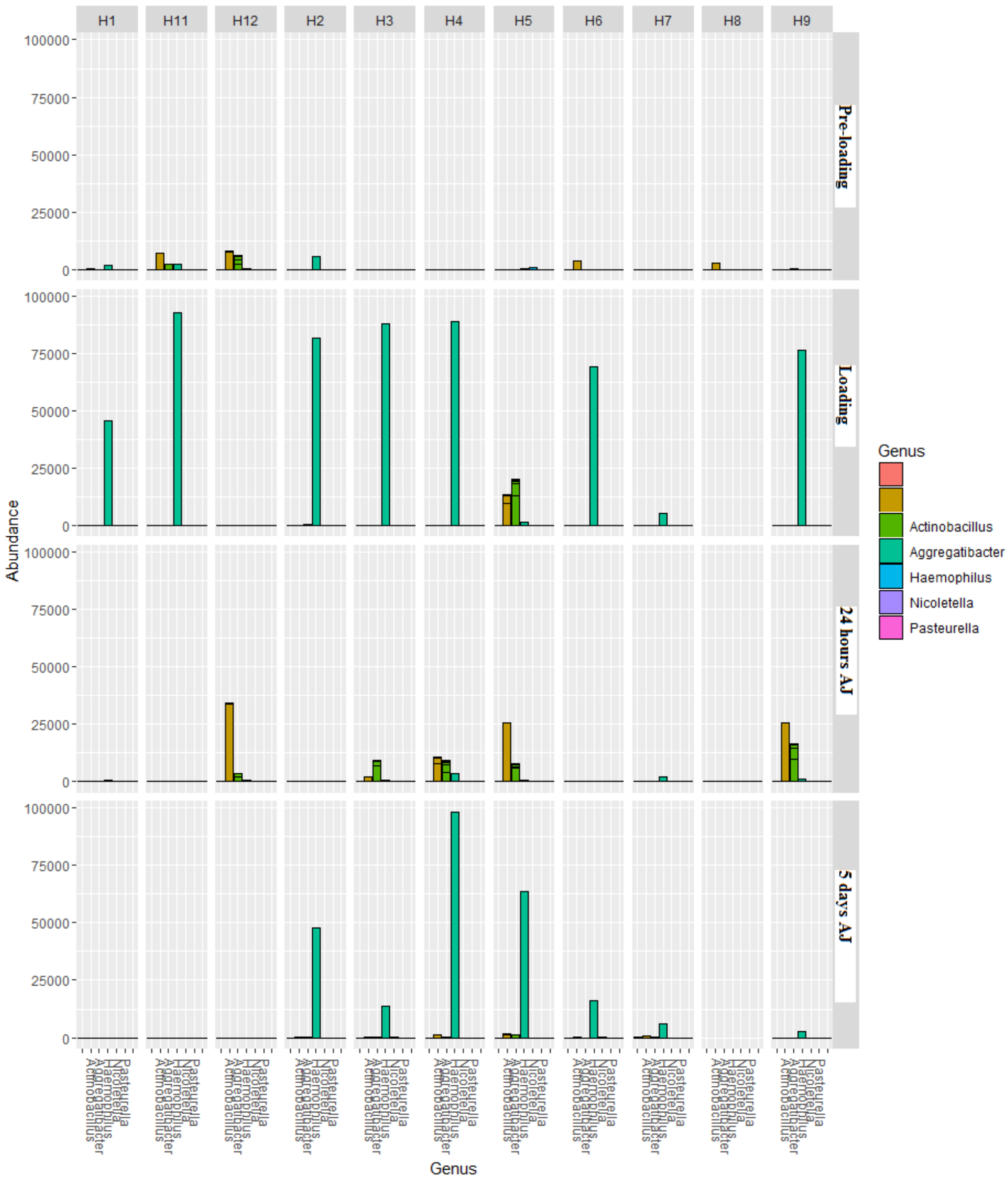


Figure 10. Pilot bar for *Streptococcaceae* for each horse at the different times obtained in phyloseq(McMurdie and Holmes, 2013). No enough Dna was extracted for the sample of H8 at unloading and 5 days AJ In the legend, if there is no name for a colour, it indicates that for that OTU the identification did not progress to genus, thus family = *Streptococcaceae*, but no further information for that particular sequence.

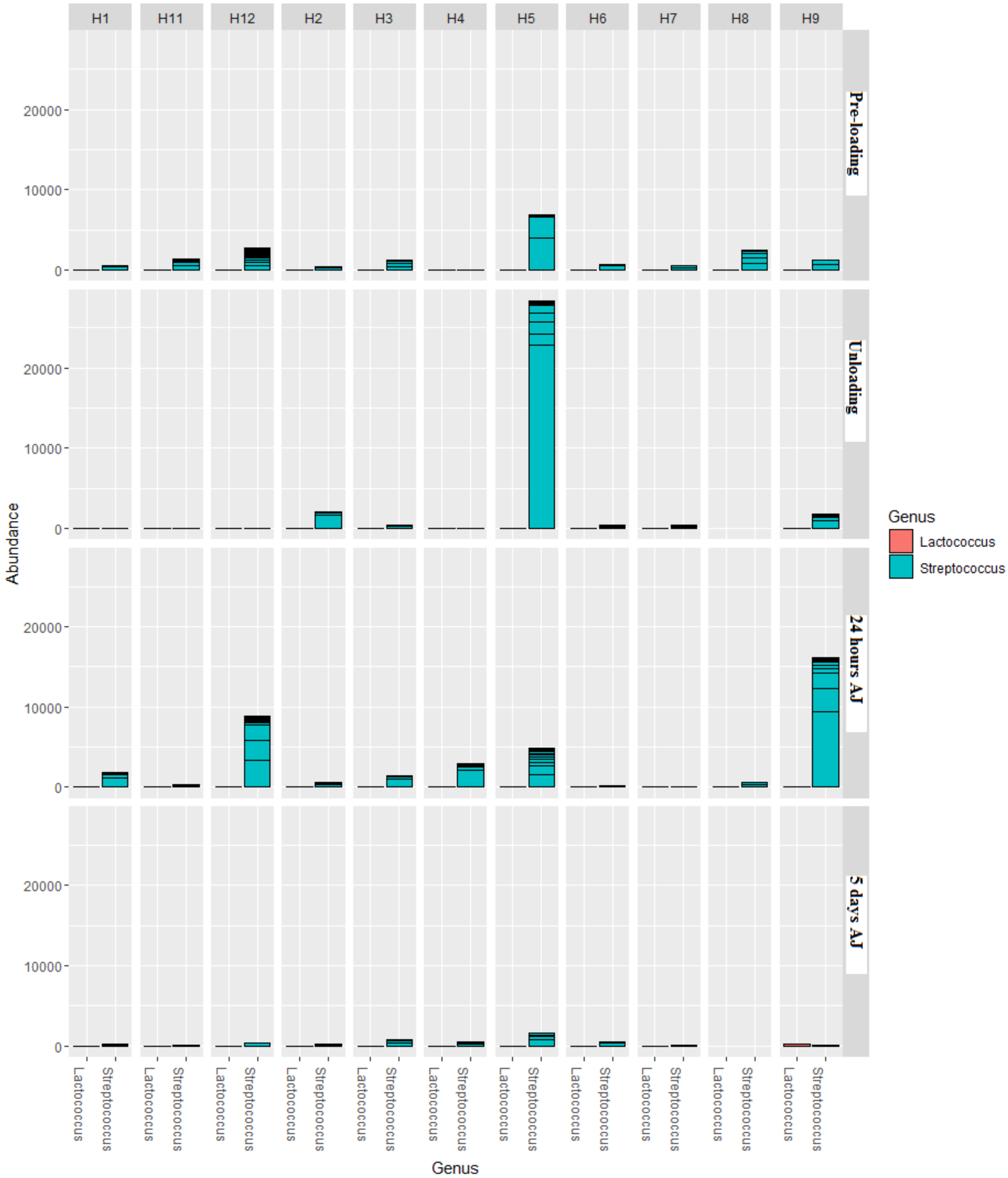


Table 1. Animal details

Identification Number	Sex	Age (y)	Breed	BCS	Body Weight (Kg)
H1	mare	4	Standardbred	2	395
H 2	gelding	7	Thoroughbred	3	477
H 3	gelding	8	Thoroughbred	2	468
H 4	mare	6	Standarbred	2	512
H 5	gelding	4	Standarbred	2	490
H 6	mare	3	Thoroughbred	3	494
H 7	mare	3	Standarbred	2	432
H 8	gelding	4	Standarbred	2	380
H 9	gelding	6	Standarbred	3	497
H 10	mare	8	Thoroughbred	2	418
H 11	gelding	3	Standarbred	2	389
H 12	gelding	3	Standarbred	2	344

BCS: Body condition score (Carroll and Huntington, 1988)

Table 2. Results of the Ultrasounds.

			ICS 4th			ICS 5th			ICS 6th		
Group	Horse	Time	Fluid (cm)	Loss of aeration	Pleural changes	Fluid (cm)	Loss of aeration	Pleural changes	Fluid (cm)	Loss of aeration	Pleural changes
	H1	T1									
	H2	T1									
A	H3	T1	0	0	0	0	0	0	0	0	0
A	H4	T1	0	0	0	0	0	0	0	0	0
A	H11	T1	0	0	0	0	0	0	0	0	0
A	H12	T1	0	0	0	0	+	++	0	0	0
A	H1	T2	0	0	0	**0.8**	+	++	**0.5**	0	+
A	H2	T2	**1**	0	0	**0.1**	0	+	0	0	0
A	H3	T2	0	0	+	0	0	0	0	0	0
A	H4	T2	**0.25**	+	+	**0.5**	0	+	0	0	0
A	H11	T2	0	0	0	0	+	++	**0.25**	+	++
A	H12	T2	**2.5**	+	+	**0.5**	0	+	0	0	0
A	H1	T3	0	0	+	**0.25**	0	+	0	0	+
A	H2	T3	**1**	0	+	0	0	0	0	0	+
A	H3	T3	0	0	+	0	0	+	0	0	+
A	H4	T3	0	0	0	0	0	0	0	0	0
A	H11	T3	**1.5**	0	+	0	0	0	0	0	0
A	H12	T3	**1**	0	+	0	0	+	0.1	0	+
A	H1	T4	0	0	+	**1.2**	0	++	0	0	+
A	H2	T4	0	0	+	**0.5**	+	++	0	0	+
A	H3	T4	0	0	0	0	0	0	0	0	0
A	H4	T4	0	0	0	**0.25**	+	+	0	0	0

Appendix 5

A	H11	T4	**0.25**	+	+	**0.4**	+	0	0	0	0
A	H12	T4	0	+	+	0	0	0	0	0	0
	H10	T1	0	0	+	0	0	0	0	0	0
	H10	T2	0	0	0	0	0	0	0	0	+
	H10	T3	0	0	0	0	0	0	0	0	+
	H10	T4	0	0	+	0	0	0	0	0	0
B	H5	T1	**2**	+	+	0	0	+	0	0	0
B	H6	T1	**0.2**	0	0	**0.5**	0	0	0	0	0
B	H7	T1	0	0	0	**0.2**	+	+	0	0	0
B	H8	T1	**0.5**	+	++	0	0	+	0	0	0
B	H9	T1	**1.5**	+	+	0	0	+	0	0	0
B	H5	T2	0	0	+	0	0	+	0	0	0
B	H6	T2	**1**	0	+	0	+	0	0	0	0
B	H7	T2	0	0	0	0	0	+	0	0	++
B	H8	T2	0	0	++	**1**	0	+	0	0	0
B	H9	T2	**0.5**	0	+	**0.5**	0	+	0	0	+
B	H5	T3	0	0	0	0	0	0	**0.1**	0	0
B	H6	T3	0	0	0	**0.4**	+	+	**1**	0	++
B	H7	T3	0	0	0	0	0	+	0	0	++
B	H8	T3	0	0	+	0	0	+	0	0	0
B	H9	T3	**1.2**	0	+	**1.5**	0	+	0	0	0
B	H5	T4	**0.25**	0	0	**0.8**	0	0	**1**	+	+
B	H6	T4	0	0	+	0	0	+	0	0	0
B	H7	T4	0	0	+	0	0	+	0	0	0

B	H8	T4	0	0	+	0	0	0	0	0	0
B	H9	T4	**0.25**	0	0	**0.25**	0	0	0	0	0