Detection of equine herpesvirus -4 and physiological stress patterns in young Thoroughbreds consigned to a South African auction sale

by

Marcha Badenhorst

A dissertation submitted in fulfilment of the requirements for the degree Magister Scientiae (Veterinary Science)

Faculty of Veterinary Science,

University of Pretoria,

South Africa

November 2014

Research conducted in the Departments of Companion Animal Studies,

Production Animal Studies and Equine Research Centre,

Faculty of Veterinary Science, University of Pretoria

Supervisor:

Professor Martin Schulman

Section of Reproduction, Department of Production Animal Studies

Co-supervisors:

Dr Patrick Page

Professor Alan Guthrie

Professor André Ganswindt

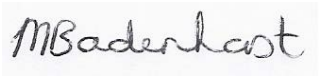
DECLARATION

I, Marcha Badenhorst, do hereby declare that the research presented in this dissertation was conceived and executed by myself, and apart from the normal guidance from my supervisor, I have received no assistance.

Neither the substance, nor any part of this dissertation has been submitted in the past, or is to be submitted for a degree at this University or any other University.

This dissertation is presented in fulfilment of the requirements for the MSc (Veterinary Science) degree in the Department of Companion Animal Clinical Studies, University of Pretoria.

I hereby grant the University of Pretoria free licence to reproduce this dissertation in part or as whole, for the purpose of research or continuing education.



21 November 2014

Marcha Badenhorst | Date

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to the following individuals and institutions, without whom this project would not have been possible.

My supervisor, Prof Martin Schulman, and co-supervisors, Prof Alan Guthrie, Prof André Ganswindt and Dr Patrick Page, for developing my interest in research and for guiding and supporting me throughout the process. It has been a privilege to work with each of you.

Racing South Africa (Pty) Ltd, the Equine Research Centre and Departments of Companion Animal Studies and Production Animal Studies for funding this project.

The South African Thoroughbred Breeders Association and the staff of the Gosforth Park sales complex for facilitating the research and the owners of participating farms for allowing the data collection.

Ms Shalaine Booysen and the staff of the participating farms for invaluable assistance with sample collection.

The staff of the Veterinary Genetics Laboratory, Endocrine Research Laboratory and Immunocontraception Laboratory for their assistance with sample processing. Particularly Mr Chris Joone, Ms Hettie Rossouw, Ms Anne Marie Human and Mr Ofentse Magoba.

Prof James Gilkerson and the Centre of Equine Virology, University of Melbourne, for assistance and expert advice on equine herpesvirus serology.

Dr Pete Laver and Dr Jannie Crafford for assistance with data analysis and interpretation.

Mr Gideon Van Der Merwe and Ms Estelle Mayhew for technical assistance and graphic design.

My parents, Frans and Jeanne Badenhorst, and my family for their continuous support.

My Heavenly Father for blessing me with this opportunity.

ABBREVIATIONS

AIC	Akaike's information criterion
Ct	Cycle threshold
DNA	Deoxyribonucleic acid
EAdV-1	Equine adenovirus-1
EHM	Equine herpes myeloencephalopathy
EHV-1, -2, -4, -5	Equine herpesvirus types-1, -2, -4, -5
EIA	Enzyme immunoassay
EIV	Equine influenza virus
EAV	Equine arteritis virus
ERAV	Equine rhinitis-A virus
ERBV	Equine rhinitis-B virus
FGM	Faecal glucocorticoid metabolites
gG	Glycoprotein G
GST	Glutathione-S-transferase
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IURD	Infectious upper respiratory tract disease
mRNA	Messenger ribonucleic acid
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RAO	Recurrent airway obstruction
S. equi subsp. *equi*	*Streptococcus equi* subspecies *equi*
USA	United States of America
VIF	Variance inflation factor

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

SUMMARY

Commingling of horses from various populations, together with physiological stress associated with transport and confinement at a sales complex, may be associated with detection and transmission of equine herpesvirus type-1 (EHV-1) and -4 (EHV-4). This prospective cohort study aimed to investigate the currently undefined prevalence of EHV-1 and -4 in young Thoroughbreds at an auction sale in South Africa, and associations between clinical signs, physiological stress and viral detection.

Ninety, two-year old Thoroughbreds (51 colts, 39 fillies) were consigned from eight farms and sampled at a South African auction sale. The horses were monitored for pyrexia and nasal discharge. Nasal swabs were collected for quantitative polymerase chain reaction (qPCR) assay to detect EHV-1 and -4 and faecal samples were collected for enzyme immunoassay (EIA) to determine faecal glucocorticoid metabolite (FGM) concentrations.

EHV-4 nucleic acid was detected in some and EHV-1 nucleic acid in none of the population. Pyrexia and nasal discharge were poor indicators of EHV-4 status. Variation in FGM concentrations was best explained by transportation and preparation for auction. Peaks in EHV-4 detection and increases in FGM concentrations were identified shortly post-arrival and on the first day of auction. Temporal changes in FGM concentrations of horses from individual farms showed two distinct patterns: Pattern A (biphasic peaks) and Pattern B (single peak).

It was concluded that sales consignment was associated with some EHV-4 nucleic acid detection and distinctive physiological stress patterns in this population of young Thoroughbreds.

Keywords: horse; equine herpesvirus; physiological stress; sales consignment; faecal glucocorticoid metabolites.

Chapter 1

General Introduction

Equine respiratory infection is a major cause of disease and economic loss resulting in significant wastage worldwide, particularly in the Thoroughbred racing industry (Bailey *et al* 1997, Carlson *et al* 2013). Anecdotal evidence suggests that up to 50% of veterinary call-outs made to horses in racing are for respiratory disease (Dynon *et al* 2007). Furthermore, a survey completed by Australian racehorse trainers listed viral respiratory infections as one of the three most significant causes of lost training days, regarded as even more significant than lameness (Bailey *et al* 1997).

Two of the pathogens most commonly associated with infectious upper respiratory tract disease (IURD) in horses, are equine herpesvirus type-1 (EHV-1) and -4 (EHV-4) (Pusterla *et al* 2011, Pusterla *et al* 2013). These closely related alphaherpesviruses are typically primary respiratory pathogens. Additional disease manifestations including abortion, neonatal foal disease and neurological disorders occur less frequently, but may all have major economic implications (Patel & Heldens 2005, Sonis & Goehring 2013).

EHV-1 and -4 are considered ubiquitous in equine populations worldwide (Patel & Heldens 2005, Ma *et al* 2013), however the prevalence of EHV-1 and -4 in South African Thoroughbreds is currently undefined. Key aspects in the pathogenesis of these viruses are latency and stress-related reactivation (Patel & Heldens 2005, Lunn *et al* 2009). EHV-1 and -4 are frequently transmitted to foals prior to weaning and have the ability to establish latent infections in their hosts (Gilkerson *et al* 1999b, Gilkerson *et al* 1999c). Viral reactivation, with respiratory tract replication and shedding, has been closely associated with various physiological, environmental and management-related stressors, concurrent disease and other forms of immunosuppression (Patel & Heldens 2005, Lunn *et al* 2009, Pusterla *et al*

2010). Spread to naive animals may occur by means of direct horse-to-horse contact, inhalation of aerosolised virus or contact with objects contaminated by respiratory secretions (Gilkerson *et al* 1999b, Harless & Pusterla 2006, Sonis & Goehring 2013).

Sales events provide opportune environments for commingling of horses from various populations. Disruption of established social groups, transport, routine and environmental changes, as well as confinement and handling at sales yards are some potential stressors inherent to the current worldwide consignment process, predisposing horses to shedding and transmission of respiratory pathogens (Gilkerson *et al* 1999a, Harless & Pusterla 2006, Pusterla *et al* 2009b, Carlson *et al* 2013, Schulman *et al* 2014). Age and season may further increase risk of respiratory infection (Matsumara *et al* 1992, Morley *et al* 2000, Lunn *et al* 2009, Pusterla *et al* 2011). Subsequent to sales events, the majority of young Thoroughbreds will enter pre-training or training facilities where similar combinations of risk factors may be encountered (Gilkerson *et al* 1999a).

The effects of sales consignment on viral prevalence and physiological stress responses among young Thoroughbreds have not been reported previously. During this prospective study, 90 young Thoroughbreds consigned from eight farms of origin across South Africa were monitored for the duration of their residence at a central sales complex. The study aimed to investigate the prevalence of EHV-1 and -4 in young Thoroughbreds at a South African auction sale, and any associations between detection of EHV-1 and -4 and clinical signs or physiological stress.

Chapter 2

Literature Review

2.1 Equine herpesvirus type-1 (EHV-1) and -4 (EHV-4)

2.1.1 Virology

Nine herpesviruses have been identified in equines, all of which belong to the family *Herpesviridae* within the order *Herpesvirales* (Davison *et al* 2009, Ma *et al* 2013). These nine herpesviruses are responsible for a variety of clinical disease manifestations primarily, but not exclusively, in equines. Arguably, the most clinically, economically and epidemiologically relevant of these pathogens are EHV-1 and -4, both of which belong to the genus *Varicellovirus* within the subfamily *Alphaherpesvirinae* (Patel & Heldens 2005, Davison *et al* 2009, Ma *et al* 2013). These closely related alphaherpesviruses show a high degree of genetic and antigenic similarity and were originally classified as subtypes 1 and 2 of EHV-1 (Sabine 1980, Ma *et al* 2013). It was only in 1981 that molecular evidence became available which allowed for differentiation between two distinct viruses (Studdert *et al* 1981). Both EHV-1 and -4 have linear double stranded deoxyribonucleic acid (DNA) genomes that have an approximate size of 150 kilobases and 146 kilobases, respectively (Ma *et al* 2013). The herpesvirus genome is contained within an icosahedral capsid, surrounded by a proteinaceous matrix and an outer lipid envelope containing membrane-associated proteins (Davison *et al* 2009). Herpesviruses are labile and easily inactivated by heat and disinfectants. Environmental persistence is estimated at less than seven days under most conditions, with a maximum survival of 35 days (Harless & Pusterla 2006, Slater 2007).

2.1.2 Pathogenesis

Despite the genetic and antigenic similarities of EHV-1 and -4, these viruses are markedly different with respect to their host range and pathogenicity. EHV-1 and EHV-1-related viruses

have a much broader host spectrum than EHV-4 and have been identified in a variety of non-equid species, including wild and domesticated ruminants, camelids, cervids and recently, in black bears and guinea pigs (Chowdhury *et al* 1988, Rebhun *et al* 1988, Wohlsein *et al* 2011). In contrast, EHV-4 infections are limited to equines (Ma *et al* 2013).

EHV-1 infection has a multi-organ, systemic distribution and may affect at least three distinct cell types in three organ systems (Osterrieder & Van de Walle 2010). Four disease manifestations are associated with EHV-1 infection in horses: upper respiratory tract disease, abortion, equine herpes myeloencephalopathy (EHM) and neonatal foal disease (Patel & Heldens 2005). The pathogenesis of EHV-1 has been well described. Initial viral replication occurs in the epithelial cells of the upper respiratory tract, resulting in cell death, epithelial erosions and viral shedding in respiratory secretions (Ma *et al* 2013). The virus spreads from epithelium to connective tissue below the basement membrane and uses migratory mononuclear leukocytes as vehicles for distribution (Ma *et al* 2013). Viral presence in the respiratory tract lymph nodes can be detected within 24 to 48 hours post-infection (Lunn *et al* 2009). Leukocyte-associated viraemia is established, which delivers EHV-1 to secondary sites of replication, including vasculature of the pregnant uterus and, or the central nervous system (Lunn *et al* 2009). Infection of endothelial cells triggers an inflammatory cascade and reactive immune response. Resulting vasculitis, microthrombosis, infarction and disseminated ischaemic damage may lead to abortion or central nervous system disorders (Harless & Pusterla 2006, Lunn *et al* 2009). Neonatal foal disease may result from near-term transplacental EHV-1 infection, resulting in birth of live foals that usually die within days due to progressive respiratory disease (Patel & Heldens 2005).

The pathogenesis of EHV-4 (“equine rhinopneumonitis virus”) has been reported to a much lesser extent than that of EHV-1. It has been suggested that differences in pathogenicity between EHV-1 and -4 can partly be attributed to the variation in cell tropism between the two viruses (Osterrieder & Van de Walle 2010). Primary EHV-4 infection is limited mainly to

the epithelial cells of the upper respiratory tract (Ma *et al* 2013). Replication of EHV-4 in endothelial cells has been described *in vivo* (Blunden *et al* 1995). Recent *in vitro* studies also demonstrated no significant difference in viral entry into endothelial cells between wild-type EHV-1 and -4 (Osterrieder & Van de Walle 2010). It has, however, been shown that EHV-4 has a low tropism for mononuclear leukocytes and is markedly less efficient at infecting these cells compared to EHV-1 (Osterrieder & Van de Walle 2010, Vandekerckhove *et al* 2011). Leukocyte-associated viraemia following EHV-4 respiratory infection is thus not a consistent feature of disease and neither is the resultant induction of abortion or nervous system disorders (Vandekerckhove *et al* 2011).

Key aspects in the pathogenesis of EHV-1 and -4 are latency and reactivation (Patel & Heldens 2005, Lunn *et al* 2009). Following primary infection, both EHV-1 and -4 have the ability to establish life-long latent infections in equine hosts. Primary latency sites have been identified as the trigeminal ganglia, lymphoid tissue, especially lymph nodes draining the respiratory tract, and peripheral blood leukocytes (Welch *et al* 1992, Slater *et al* 1994, Borchers *et al* 1997). Establishment of long term latency in the presence of an active host immune response is thought to be facilitated by various immunomodulatory strategies that have been developed by these viruses over time (Ma *et al* 2013). Latently-infected horses that may undergo periodic reactivation, resulting in shedding of infectious virus, form a reservoir which maintains these viruses within a horse population and most likely play an important role in the aetiology of herpesvirus-related disease outbreaks (Patel & Heldens 2005, Lunn *et al* 2009, Pusterla *et al* 2010, Ma *et al* 2013).

Physiological factors responsible for reactivation of latent virus remain undefined, although it is well recognised that spontaneous shedding may follow periods of physiological stress and other forms of immunosuppression (Patel & Heldens 2005, Lunn *et al* 2009, Pusterla *et al* 2010). It has been suggested that most reactivation events are neutralized by mucosal immune responses, thus preventing viral shedding and viraemia (Slater 2007). In the cases

of compromised host immune responses however, viral shedding of sufficient magnitude to permit transmission to susceptible horses may occur (Pusterla *et al* 2010). Reactivation is usually associated with mild to absent clinical signs (Edington & Bridges 1985, Slater *et al* 1994, Harless & Pusterla 2006, Pusterla *et al* 2010). Viraemia has been reported in association with nasal shedding of virus, but is not a consistent feature of reactivation (Edington & Bridges 1985, Slater *et al* 1994, Pusterla *et al* 2010). Reactivation also does not consistently result in increased antibody titres (Carr *et al* 2011). Following corticosteroid-induced reactivation of latent EHV-1 infection, increased antibody titres were reported in three of four horses (Pusterla *et al* 2010).

2.1.3 Epidemiology and transmission

EHV-1 and -4 infections are ubiquitous in horse populations throughout the world (Ma *et al* 2013). Latently-infected horses likely form the principal reservoir of infection, maintaining circulation of the viruses even within closed herds (Slater 2007). Epidemiological studies have suggested that foals frequently acquire EHV infections during their first few months of life. A study on a large Thoroughbred stud farm in Australia found the prevalence of EHV-4 antibodies in unvaccinated mares and their unweaned foals to be 99.1% and 99.6%, respectively (Gilkerson *et al* 1999b). Antibodies to EHV-1 were detected in 26.2% of mares and 11.4% of foals (Gilkerson *et al* 1999b) and evidence of EHV-1 infection in foals as young as 30 days of age was found (Gilkerson *et al* 1999c). Consequently, an annual cycle of EHV infection applicable to stud farms was proposed (Gilkerson *et al* 1999c). It was suggested that mare-to-foal transmission occurs early in life as a result of viral reactivation in the mares, following stressful events like parturition, lactation or oestrus activity (Gilkerson *et al* 1999b). Infection is propagated by means of foal-to-foal transmission prior to and after weaning (Gilkerson *et al* 1999c). Latently infected fillies may later become part of the broodmare herd, continuing the cycle of infection. EHV-1 and -4 continued to circulate even in populations where mares had been vaccinated with an inactivated whole virus EHV-1 and -4 vaccine (Foote *et al* 2006). An Australian study of the role played by EHV-1 and -4 in respiratory

disease in one- and two-year old racehorses in pre-training and training found the prevalence of EHV-1 antibodies to be between 46.3 to 68.2%, whereas the prevalence of EHV-4 antibodies approached 100% at every sampling (Gilkerson *et al* 1999a).

Due to the labile nature of herpesviruses, close contact between individuals is considered essential for spread (Gilkerson *et al* 1999b). Horizontal spread to naive animals may result from viral shedding in respiratory secretions of clinically-infected horses, as well as horses in which reactivation is not associated with overt clinical signs. Inhalation of aerosolised virus or contact with contaminated fomites are common routes of spread (Harless & Pusterla 2006, Sonis & Goehring 2013). Aborted foals, foetal membranes and placental fluids contain large amounts of infectious virus and are regarded as particularly hazardous (Slater 2007, Sonis & Goehring 2013). The possibility of horizontal transmission of EHV-1 and -4 by means of the venereal route has been the subject of several recent studies. EHV-1 DNA was detected by means of polymerase chain reaction (PCR) in the semen of 51 of 390 stallions (Hebia-Fellah *et al* 2009). No EHV-4 DNA was detected in these samples (Hebia-Fellah *et al* 2009). A study by Walter *et al* (2012) found PCR evidence of EHV-1 in the semen of two of three naturally infected stallions, while no evidence of EHV-4 shedding was detected. Viral isolation attempts on these samples were, however, unsuccessful (Walter *et al* 2012). Since no study has succeeded in demonstrating infectious virus in semen, it remains unclear whether the virus is capable of venereal spread (Ma *et al* 2013).

2.1.4 Clinical disease

Clinically there are no distinguishing characteristics between EHV-1 and -4 respiratory infections (Harless & Pusterla 2006). Clinical signs associated with the respiratory form of infection have been well described. The incubation period ranges between three to 10 days (Harless & Pusterla 2006, Slater 2007). Initial clinical signs reported in various combinations include lethargy, anorexia, pyrexia, profuse serous nasal discharge, ocular discharge, and lymphadenopathy of the mandibular lymph nodes, which may later progress to involve the

retropharyngeal lymph nodes (Patel & Heldens 2005, Harless & Pusterla 2006, Slater 2007). Coughing is not a consistent clinical feature and is more likely to occur in conjunction with aggravating conditions like poor stable hygiene or inadequate rest from training (Harless & Pusterla 2006, Slater 2007). Respiratory disease is usually self-limiting, however persistent pyrexia, mucopurulent nasal discharge and adventitial lung sounds may indicate secondary bacterial infection (Harless & Pusterla 2006).

A surveillance programme for equine infectious respiratory pathogens in the United States of America (USA) found nasal discharge and pyrexia to be the most commonly reported clinical signs among EHV-4-positive horses (Pusterla *et al* 2011). Similarly, pyrexia was most commonly reported among EHV-1-positive horses (Pusterla *et al* 2011). Naturally-infected Thoroughbred foals with copious amounts of serous or mucopurulent nasal discharge have been found more likely to shed EHV-4 than foals with no clinical signs or mild to moderate serous nasal discharge (Gilkerson *et al* 1994). The pyrexia associated with EHV-1 and -4 respiratory infection is characterised as biphasic (Harless & Pusterla 2006, Sonis & Goehring 2013). Pyrexia can be expected during the first 36 to 48 hours post-infection, corresponding with early upper respiratory tract viral replication (Sonis & Goehring 2013). A secondary pyrexia may occur between five to 10 days post-infection due to cell-associated viraemia (Sonis & Goehring 2013). A study by Sonis and Goehring (2013) aimed to detect shedding of EHV-1 and -4 in hospitalized, febrile horses by means of a single nasal swab for PCR collected within six hours of detecting pyrexia. Detection rates of 1.6% for EHV-4 and 0% for EHV-1 were surprisingly low, considering the hypothesis that more than 10% of pyrexias in hospitalized patients resulted from EHV-1 or -4 respiratory tract replication (Sonis & Goehring 2013). It was concluded that viral detection rates and sensitivity could be improved by increased sampling frequency and duration following detection of pyrexia (Sonis & Goehring 2013). It was further suggested that EHV-1 and -4 detection may be improved by including not only pyrexia, but also concurrent signs of respiratory tract disease in case selection criteria (Sonis & Goehring 2013). Twice-daily monitoring of rectal temperature in

combination with nasal swabbing for PCR analysis on two to four consecutive days was proposed for the detection of EHV-1 infection (Lunn *et al* 2009). Clinical findings recorded during an outbreak of EHV-1 supported twice-daily monitoring of rectal temperature (Walter *et al* 2013). During this EHV-1 outbreak, body temperatures of horses peaked mainly in the evenings, with normal temperatures recorded in the mornings (Walter *et al* 2013). Pyrexia was only recorded once in 11 of 71 horses and would have gone undetected without 12-hourly monitoring (Walter *et al* 2013).

Various durations of viral shedding in respiratory secretions and leukocyte-associated viraemia following primary infection have been reported. Infectious amounts of EHV-1 can be shed for 21 days or longer following infection (Lunn *et al* 2009). Experimental EHV-1 infection of eight Welsh ponies resulted in nasal shedding of virus for four to 10 days, starting between one to 12 days post-infection (Edington & Bridges 1985). Pusterla *et al* (2009a) detected nasal shedding of EHV-1 in four experimentally infected horses for between eight to 13 days. Following natural infection of 11 foals, the EHV-4 load in nasopharyngeal secretions remained high for at least four weeks after onset of clinical signs (Pusterla *et al* 2005). Leukocyte-associated viraemia was detected between three to 15 days post-infection in ponies experimentally infected with EHV-1 (Slater *et al* 1994). In contrast, almost no viral DNA could be detected in peripheral blood leukocytes of foals naturally infected with EHV-4 by day seven post-infection (Pusterla *et al* 2005). Unlike the case with EHV-1, EHV-4 leukocyte-associated viraemia is an inconsistent feature of infection, short in duration and thus its use for the purpose of diagnosing EHV-4 respiratory infection was not recommended (Pusterla *et al* 2005).

In cases of viral recrudescence, horses will actively shed virus in nasal secretions and show mild to absent clinical signs (Harless & Pusterla 2006, Pusterla *et al* 2009b). Leukocyte-associated viraemia is not a consistent feature of viral reactivation. Following corticosteroid-induced recrudescence of latent EHV-1 infection in four geldings, only one horse developed

pyrexia of three days' duration (Pusterla *et al* 2010). No other clinical signs were observed in these horses (Pusterla *et al* 2010). Viraemia of three to six days' duration was detected in all four horses, as well as nasal shedding of EHV-1 over a period of two to seven days (Pusterla *et al* 2010). Similarly, nasal shedding of EHV-1 was detected over a 10-day period in four ponies following corticosteroid-induced reactivation of latent infection (Slater *et al* 1994). No clinical signs and no viraemia were, however, detected in any of these horses (Slater *et al* 1994).

Different strains of EHV-1 vary in their abortogenic and neuropathogenic potential and simultaneous occurrence of abortion and EHM is rare (Walter *et al* 2013). Abortions occur principally during the last trimester of pregnancy and are generally sporadic events; abortion storms, particularly in vaccinated populations, are uncommon (Slater 2007, Lunn *et al* 2009). The incubation period prior to induction of abortion is highly variable and can range between nine days and four months post-infection to years later, as a result of reactivation of latent infection (Mumford *et al* 1987, Patel & Heldens 2005). Foetuses are usually aborted suddenly and spontaneously, including the foetal membranes (Van Maanen 2002). Depending on the degree of uterine vascular pathology, aborted foetuses may be virus-positive or virus-negative (Patel & Heldens 2005). Near-term transplacental EHV-1 infection could result in the birth of live, infected foals (Patel & Heldens 2005). Foals are diseased at birth or become ill within one to two days (Slater 2007). They show signs of rapidly-progressive lower respiratory disease due to viral pneumonia, which may later be complicated by secondary bacterial infection, and usually die within days (Slater 2007). Although rarely, EHV-4 has been associated with neonatal disease and mortality (O'Keefe *et al* 1995). EHM is a sporadic and relatively uncommon manifestation of EHV-1 infection with considerable variation in epidemiologic and clinical findings reported between outbreaks (Pusterla *et al* 2009c). As with the abortion-form of EHV-1 infection, the incubation period is usually between six and 10 days, but viral reactivation may result in clinical disease long after primary infection (Van Maanen 2002, Patel & Heldens 2005). Pyrexia has been

reported as the major clinical sign observed prior to the onset of neurological disease, but is frequently not present by the time neurologic deficits become apparent (Friday *et al* 2000, Henninger *et al* 2007). Neurological signs have a sudden onset and vary from mild hind limb ataxia and paresis, urinary incontinence and loss of tail and anal tone to complete recumbency with fore and hind limb paralysis (Friday *et al* 2000, Van Maanen 2002, Henninger *et al* 2007). Sudden onset of neurological signs, the involvement of multiple horses and a recent history of pyrexia, abortion or respiratory disease in a population are considered sufficient evidence for a tentative diagnosis of EHM (Pusterla *et al* 2009c). In contrast, EHV-4 is not commonly associated with abortion and evidence of its role in paresis is limited (Patel & Heldens 2005).

2.1.5 Differential diagnoses

The clinical signs described are not pathognomonic for EHV-1 or -4 respiratory infections, but can be seen in association with various other causes of IURD. Differential diagnoses include infection with *Streptococcus equi* subspecies *equi* ('strangles'), equine influenza virus (EIV), equine arteritis virus (EAV), equine herpesvirus type-2 (EHV-2), equine herpesvirus type-5 (EHV-5), equine rhinitis-A virus (ERAV), equine rhinitis-B virus (ERBV) and equine adenovirus-1 (EAdV-1) (Harless & Pusterla 2006, Pusterla *et al* 2011, Ko *et al* 2013, Pusterla *et al* 2013). The last cases of EIV and EAV reported in South Africa were in 2003 and 2001, respectively (World Organisation for Animal Health, 2014). A surveillance programme for important equine infectious respiratory pathogens in the USA detected PCR evidence of EHV-4 (10.7%), EIV (7.9%), *S. equi* subsp. *equi* (6.4%) and EHV-1 (3%) among 761 horses with acute febrile, upper respiratory and, or neurological disease (Pusterla *et al* 2011). An investigation into the role of lesser-characterised respiratory viruses associated with equine upper respiratory infections detected EHV-5, EHV-2, EAdV-1 and ERBV at variable frequencies in the nasal secretions of horses with IURD, and to a lesser frequency in healthy control horses (Pusterla *et al* 2013). The most commonly detected virus was EHV-5 (60%), followed by EHV-2 (49.3%), EAdV-1 (2.7%) and ERBV (2.4%) (Pusterla *et al* 2013). Several

cases of viral co-infections were detected and in some cases these viruses occurred in combination with more common infectious respiratory pathogens like EHV-1, EHV-4, EIV and *S. equi* subsp. *equi* (Pusterla *et al* 2013). The cited study concluded that it remains to be determined whether EHV-5 and -2 have primary pathogenic effects in horses with IURD, whether these viruses are present as a result of reactivation of latent infections and whether they played secondary roles in host immunomodulation, predisposing horses to co-infections (Pusterla *et al* 2013). ERBV was speculated to be an important respiratory pathogen, whereas EAdV-1 was found likely to be an incidental finding (Pusterla *et al* 2013).

2.1.6 The Thoroughbred industry and risk factors

Consequences of respiratory disease are of major importance in performance horses, particularly in racehorses. Coughing and nasal discharge were perceived as the second and fourth most important causes of lost training days and prolonged absence from racing by Australian racehorse trainers (Bailey *et al* 1997). Participants in this survey also identified viral respiratory disease as a significant cause of wastage in the Australian Thoroughbred racing industry, regarded as even more significant than lameness (Bailey *et al* 1997). Despite the usual characterisation EHV-associated respiratory disease as self-limiting, it has been suggested that horses may develop a 'poor performance syndrome', resembling recurrent airway obstruction (RAO), which could negatively affect long-term athletic performance (Slater 2007).

Identification of factors that determine the risk of infection and influence disease expression in susceptible horses, provide a foundation on which to base disease prevention and control programs (Morley *et al* 2000). Risk associated with equine infectious respiratory disease is multifactorial and involves host, environmental, management as well as pathogen-specific factors (Lunn *et al* 2009). Age as a risk factor for infectious respiratory disease in juvenile horses has been well recognised (Matsumara *et al* 1992, Morley *et al* 2000, Lunn *et al* 2009, Pusterla *et al* 2011, Cardwell *et al* 2013, Carlson *et al* 2013). Most horses affected by the

respiratory form of EHV-1 or -4 infection are less than three years of age (Harless & Pusterla 2006). Horses aged two years and younger have been found to be approximately five to eight times more likely to develop IURD than horses aged five years and older (Morley *et al* 2000).

Another frequently reported risk factor is season. For each of the four respiratory pathogens EHV-1, EHV-4, EIV and *S.equi* subsp. *equi*, a higher detection rate of quantitative polymerase chain reaction (qPCR)-positive horses has been observed during the winter months (Pusterla *et al* 2011). These findings were postulated to be associated with management practices, such as keeping a higher density of young animals indoors in winter for training and sales purposes (Pusterla *et al* 2011). Matsumara *et al* (1992) concluded that EHV-1 infections occurred predominantly in winter, whereas infections by EHV-4 occurred throughout the year.

Arguably, one of the most important risk factors associated with respiratory infections is physiological stress. Not only has stress been closely associated with recrudescence and shedding in cases of latent EHV-1 and -4 infections, but the immunosuppressive influence of stress on the body may increase the susceptibility of naive animals to new infections (Gilkerson *et al* 1999a, Patel & Heldens 2005, Carr *et al* 2011). Stressors such as weaning, castration, poor nutrition, parasite infestation, concurrent disease and administration of immunosuppressive drugs are frequently cited as risk factors (Edington & Bridges 1985, Patel & Heldens 2005, Harless & Pusterla 2006, Lunn *et al* 2009, Pusterla *et al* 2010).

Thoroughbred horses in their second year of life are exposed to various environmental factors and management practices that may predispose them to infections such as EHV-1 and -4. Transport to, as well as confinement, handling and management at sales events may contribute greatly to the physiological stress experienced by these young animals (Gilkerson *et al* 1999a, Harless & Pusterla 2006, Pusterla *et al* 2009b, Carlson *et al* 2013). Large,

intermingled assemblages of horses from diverse sources not only provide an opportune environment for viral shedding and transmission, but also result in potentially stressful disruption of established social groups (Harless & Pusterla 2006, Lunn *et al* 2009, Schulman *et al* 2014). The sales environment has further been associated with suggested risk factors like high density housing, frequent handling of horses and random mixing and contact between horses at communal facilities (Harless & Pusterla 2006, Slater 2007).

The majority of juvenile horses attending Thoroughbred bloodstock sales will enter training or pre-training facilities shortly after such an event. Yearling and two-year old Thoroughbreds are unaccustomed to the pre-training and racing stable environment. Consequently, these horses may be more likely to be stressed and hence susceptible to primary infection or reactivation of EHV-1 and -4 (Gilkerson *et al* 1999a). Similar to the sales environment, transport, mixing, housing and handling of these young horses may act as stressors singly or in combination, in addition to the physiological demands of training.

2.1.7 EHV vaccination

EHV-1 and -4 infections are not prevented by inactivated vaccines such as those currently available in South Africa (Harless & Pusterla 2006). EHV-1 and -4 continued to circulate in populations where mares had been vaccinated with an inactivated whole virus EHV-1 and -4 vaccine (Foote *et al* 2006). Furthermore, serologic responses to inactivated vaccines in foals appear to be limited by maternal antibodies until at least five months of age, at which point the majority of foals are likely to have encountered field exposure to these viruses already (Harless & Pusterla 2006). Consequently, the aims of a vaccination strategy with inactivated vaccines would not include infection prevention, but simply the reduction of severity of clinical disease and viraemia, as well as induction of short-lived mucosal immunity to reduce nasopharyngeal shedding and limit viral transmission (Harless & Pusterla 2006, Lunn *et al* 2009). Although booster vaccination of exposed pregnant mares in the face of abortigenic EHV-1 outbreaks is common practice (Harless & Pusterla 2006), the value of prophylactic

vaccination of horses shortly prior to exposure to high-risk environments such as auction sales, has not been investigated.

2.1.8 Diagnosis and sampling

2.1.8.1 Direct evidence of infection

Virus isolation provides unequivocal evidence of the presence of infectious virus in samples and has traditionally been regarded as the gold standard for diagnosing EHV-1 and -4 shedding and viraemia (Harless & Pusterla 2006, Slater 2007, Lunn *et al* 2009, Pusterla *et al* 2009a). This time-consuming and relatively expensive process is still of use, as it is the only method to secure an isolate for any further comparative analysis (Patel & Heldens 2005). In practice however, PCR offers several advantages over virus isolation and has become the diagnostic test of choice (Lunn *et al* 2009, Pusterla *et al* 2009a). In order to investigate the epidemiology of EHV-1 and -4 infection in equine populations and determine the association of these viruses with respiratory disease, a rapid, sensitive, discriminatory test which can be applied to samples collected from horses in the field, is required (Gilkerson *et al* 1994). PCR is relatively economical and has the potential to offer rapid results with high sensitivity and specificity (Pusterla *et al* 2005, Lunn *et al* 2009, Pusterla *et al* 2009a). In cases of low EHV viral load, positive PCR results may be obtained when virus isolation is negative (Lunn *et al* 2009). Following corticosteroid-induced recrudescence of latent EHV-1 infection in four horses, low viral loads were detected by means of PCR in the blood and nasal swabs of all four horses, while virus isolation attempts yielded negative results for all the samples (Pusterla *et al* 2010).

Earlier PCR assays were non-quantitative, thus capable of detecting the presence of viral DNA in a sample, but incapable of determining the amount of viral DNA or distinguishing between lytic, latent and dead virus (Harless & Pusterla 2006, Lunn *et al* 2009, Pusterla *et al*

2009a). Real-time PCR analysis is quantitative and has enabled the study of EHV viral kinetics in respiratory secretions, blood and tissues (Pusterla *et al* 2006, Pusterla *et al* 2009a). Real-time PCR accurately distinguishes and measures specific nucleic acid sequences in a sample, even when as little as five or less copies of the target sequence are present (Klein 2002, Valasek & Repa 2005). Fluorogenic probes are used to monitor each cycle of target sequence amplification in real-time. The number of cycles required for the fluorescent signal to reach a threshold level correlates with the amount of original target sequence, thereby enabling quantification (Valasek & Repa 2005). Nucleic acid amplification and detection steps are combined into one homogenous assay in a closed reaction vessel, eliminating the need for post-PCR processing and minimizing chances of laboratory cross-contamination (Bustin *et al* 2005, Valasek & Repa 2005, Espy *et al* 2006). The combination of excellent sensitivity and specificity, low contamination risk, ease of performance and speed, has made real-time PCR an appealing alternative to most conventional diagnostic techniques (Espy *et al* 2006). An additional advantage of real-time PCR is the possibility of a multiplex approach (Klein 2002). In multiplexing, multiple primer pairs are used to amplify different DNA sequences in a single reaction, allowing simultaneous detection of two or more pathogens and shorter processing times of large sample quantities (Mackay *et al* 2002, Diallo *et al* 2007). A sensitive and specific multiplex real-time PCR for detection and differentiation of EHV-1 and -4 has been described (Diallo *et al* 2007).

The application of qPCR analysis at both the DNA and mRNA levels has diagnostic implications. Establishing the viral load of EHV-1 in nasal swabs from infected horses allows the assessment of the infectious risk to other horses, characterization of the disease stage and monitoring of the response to antiviral therapy (Lunn *et al* 2009, Pusterla *et al* 2009a). A study of EHV-4 kinetics in the peripheral blood leukocytes and nasopharyngeal secretions of foals, made use of real-time PCR results to distinguish between lytic and latent infections (Pusterla *et al* 2005).

2.1.8.2 Indirect evidence of infection

Following EHV infection, an increase in immunoglobulin M (IgM) may be detected within four to five days, peaking after 20 to 30 days and decreasing to baseline levels by 60 to 80 days (Slater 2007). A delayed increase in immunoglobulin G (IgG) may occur eight to 10 days post-infection, peaking after 30 to 40 days and remain detectable for more than nine months (Slater 2007). Serology primarily provides retrospective information (Harless & Pusterla 2006, Slater 2007). Historical viral exposure may be confirmed from antibody titres of single serum samples (Diallo *et al* 2007). A significant increase in EHV-1 or -4 antibody titres, as defined by the specific laboratory, between acute and convalescent serum samples collected seven to 21 days apart, provided presumptive evidence of viral infection (Harless & Pusterla 2006, Lunn et al 2009, Diallo *et al* 2007).

A major drawback of conventional antibody assays such as complement fixation, for the short-lived IgM response, and virus neutralisation, for the longer-lived IgG response, is the inability to differentiate between EHV-1 and -4 infection, due to extensive antigenic cross-reactivity between these closely-related, but distinct viruses (Crabb & Studdert 1993, Patel & Heldens 2005, Kydd *et al* 2006, Ma *et al* 2013). Discovery of type-specific epitopes contained within the viral glycoprotein G (gG) of EHV-1 and EHV-4 provided the basis for the development of a single-well, type-specific ELISA to differentiate EHV-1 antibodies from those of EHV-4 and identify horses that have been infected with either or both viruses (Crabb & Studdert 1993, Crabb *et al* 1995). This discovery enabled the epidemiology of these viruses to be studied in greater depth than previously possible (Gilkerson *et al* 1999b, Gilkerson *et al* 1999c). Serological studies utilising a type-specific ELISA for EHV-1 and -4 antibodies have been reported in several equine populations (Gilkerson *et al* 1999a, Gilkerson *et al* 1999b, Gilkerson *et al* 1999c, Foote *et al* 2006, Dynon *et al* 2007, Pusterla *et al* 2009b). Positive antibody titres have been routinely detected following vaccination with monovalent or bivalent herpesvirus vaccines, which may confound interpretation of serological results (Pusterla *et al* 2009b).

2.1.8.3 Respiratory secretion sampling

Respiratory secretion samples for PCR detection of EHV may be retrieved by means of either nasopharyngeal or nasal swabs. Less compliant horses frequently resist collection of nasopharyngeal secretions with traditionally recommended 40 cm swabs (Pusterla *et al* 2008). Lack of compliance may be exacerbated by repeat samplings. Similar recovery of EHV-1 DNA between nasopharyngeal and nasal swabs has been demonstrated (Pusterla *et al* 2008). It was speculated that nasal swabs were in fact more likely than nasopharyngeal swabs to contain target EHV-1 DNA from mucus and exfoliated upper airway cells drained via the ventral meatus (Pusterla *et al* 2008). The cited study concluded that nasal swabbing represented a sensitive and better tolerated alternative to nasopharyngeal swabbing for molecular detection of EHV-1 shedding in horses (Pusterla *et al* 2008).

Viral respiratory pathogens may be found in respiratory epithelial cells as well as in free mucus (Pusterla *et al* 2008). It is therefore desirable that samples containing both mucosal cells and mucus are collected for molecular diagnostic techniques (Pusterla *et al* 2008). Factors that may influence the retrieval of nasal secretions, thus affecting cell number and amount of nucleic acid collected, include variation in collection technique and size and composition of the swab tip (Pusterla *et al* 2009a). Rayon is a material shown to not negatively influence PCR results (Pusterla *et al* 2008). Rayon-tipped nasal swabs have been successfully used for PCR-detection of EHV-1 and -4 in numerous studies (Pusterla *et al* 2008, Pusterla *et al* 2009a, Pusterla *et al* 2010, Pusterla *et al* 2011, Sonis & Goehring 2013).

2.2 Faecal glucocorticoid metabolites (FGM's)

Stimuli that lead to an imbalance in homeostasis act as 'stressors' and the corresponding defence reaction of an animal is a 'stress response' (Möstl & Palme 2002). The primary hormones involved in a stress response to an adverse situation are glucocorticoids and catecholamines. The levels of these hormones can be determined as an indicator of adrenal activity and thus as a measure of homeostatic disturbance (Möstl & Palme 2002).

The concentrations of glucocorticoids or their metabolites can be measured in various body fluids and excreta, including blood, saliva, milk, urine or faeces (Möstl & Palme 2002). The cortisol concentration in blood is affected by time of day, occurrence of short-term fluctuations and how accustomed a horse is to its environment and routine (Irvine & Alexander 1994). Stress associated with frequent handling and blood collection by means of jugular venipuncture may further confound results (Möstl & Palme 2002). Although less invasive, urine, saliva and milk collection still involve a degree of handling, while milk can also only be collected from lactating animals (Möstl & Palme 2002).

Measurement of glucocorticoid metabolite concentrations in faeces provides a practical, non-invasive and feedback-free alternative. A delayed, time-averaged response to a stressor is provided dependant on species-specific gut-passage times, with an interval of approximately 24 hours in horses (Palme *et al* 1996). An enzyme immunoassay (EIA) that detects 11,17-dioxoandrostanes, a group of cortisol metabolites, has previously been validated for use in horses (Merl *et al* 2000). It is recommended that fresh faecal samples be collected for this purpose and stored at -20*C immediately after collection until analysis (Schmidt *et al* 2010a). Fluctuations in FGM concentrations are reported for determining stress responses in horses consigned to sales, horses subjected to painful incidents as well as horses transported by road over short, medium and long distances (Schulman *et al* 2014, Merl *et al* 2000, Schmidt *et al* 2010a, Schmidt *et al* 2010b).

Chapter 3

Detection of equine herpesvirus -4 and physiological stress patterns in young Thoroughbreds consigned to a South African auction sale

3.1 Introduction

Equine respiratory infection is a major cause of disease and economic loss worldwide, resulting in significant wastage, particularly in the Thoroughbred racing industry due to lost training days, prolonged absence from racing and possible negative effects on long-term athletic performance (Bailey *et al* 1997, Slater 2007). EHV-1 and -4 are amongst the most commonly identified agents associated with IURD (Pusterla *et al* 2011). Few surveillance studies to detect these viruses are reported in healthy horse populations (Foote *et al* 2004, Wang *et al* 2007, Schulman *et al* 2014).

Risks associated with IURD are multifactorial, including host, environmental, management and pathogen-specific factors (Morley *et al* 2000, Lunn *et al* 2009). Age as a risk factor in juvenile horses has been well recognised with higher detection rates of EHV-1 and -4 reported during the colder winter months (Matsumara *et al* 1992, Morley *et al* 2000, Harless & Pusterla 2006, Lunn *et al* 2009, Pusterla *et al* 2011, Cardwell *et al* 2013, Carlson *et al* 2013). Physiological stress, arguably one of the more important risk factors associated with respiratory infections, is closely associated with recrudescence and shedding in cases of latent EHV-1 and -4 infections (Gilkerson *et al* 1999a, Patel & Heldens 2005, Pusterla *et al* 2010). The immunosuppressive influence of stress may further increase the susceptibility of naive animals to new infections (Gilkerson *et al* 1999a). Transport and the subsequent confinement, handling and management at auction sales may contribute markedly to physiological stress (Gilkerson *et al* 1999a, Harless & Pusterla 2006, Pusterla *et al* 2009b, Carlson *et al* 2013). Large, intermingled assemblages of horses from diverse sources

provide an opportune environment for viral shedding and transmission, exacerbated by potentially stressful disruption of established social groups (Harless & Pusterla 2006, Lunn *et al* 2009, Schulman *et al* 2014). Detection of EHV-1 from nasal secretions following long distance transport of horses and both EHV-1 and -4 upon horses' arrival and shortly thereafter at North American sales and show events, have been reported (Pusterla *et al* 2009b, Carlson *et al* 2013).

Measurement of FGM concentrations to monitor adrenocortical endocrine function in horses provides a practical, non-invasive and feedback-free alternative to glucocorticoid (e.g. cortisol) determination in blood, saliva, urine or milk (Möstl & Palme 2002). FGM concentrations, unlike rapidly-fluctuating blood cortisol levels, reflect cumulative secretion and elimination of hormones over an extended interval. A delayed, time-averaged response to a stressor is provided dependant on species-specific gut-passage times, with an interval of approximately 24 hours in horses (Möstl & Palme 2002, Touma & Palme 2005). Fluctuations in FGM concentrations are reported for determining stress responses in horses consigned to sales and following short, medium and long-distance road transport (Schmidt *et al* 2010a, Schmidt *et al* 2010b, Schulman *et al* 2014).

The effects of sales consignment on viral prevalence and physiological stress responses among young Thoroughbreds have not been reported previously. This study aimed to investigate the prevalence of EHV-1 and -4 in young Thoroughbreds at an auction sale in South Africa, and associations between EHV-1 and -4 detection and clinical signs or physiological stress.

3.2 Materials and methods

3.2.1 Study population and design

A prospective cohort study was performed during the late southern hemisphere winter at the Thoroughbred Breeders' Association National Two Year Old Sales hosted at the Gosforth Park sales complex in Germiston, South Africa. Horses travelled by road transport and arrived at the sales complex on various dates. Their residence period varied between four and nine days. The auction occurred over two days (15 and 16 August 2013), with these dates regarded as the end-points of data collection, immediately prior to departure of the horses. The period from day of arrival until the third day post-arrival ($D_{arrival}$ to $D_{arrival+3}$) was defined as the 'adaptation phase'. The period from two days prior to auction until the second day of auction ($D_{auction-2}$ to $D_{auction\ 2}$) was defined as the 'auction phase'.

The study population included 90 (51 colts, 39 fillies) of the 358 two-year old Thoroughbreds catalogued for the sales auction. The horses enrolled were pre-selected from the sales catalogue based on owners' consent to participate and the availability of accurate records from the particular farms. Selected horses originated from eight farms situated in three provinces: 30 from three (Farms 1, 2 and 3) in the Western Cape Province, 18 from two (Farms 4 and 5) in the Eastern Cape Province and 42 from three (Farms 6, 7 and 8) in KwaZulu-Natal Province (Figure 1).

Housing at the complex consisted of 44 barn-style buildings, subdivided into 771 individual stables and separated by walkways. Each farm's consigned horses were allocated a unique stable number and housed in adjacent stables, although often within the same barn as horses from other farms. The buildings' design allowed for free movement of air between stables and windows covered with metal grids enabled nose-to-nose contact between horses in adjacent stables (Figure 2A). Horses were periodically removed from their stables into adjacent walkways for showing or grooming (Figure 2B) and mixed randomly at communal

facilities during routine daily activities including in-hand exercise (Figure 2C) and washing (Figure 2D). Daily care activities of horses, including feeding, were performed by staff of the respective farms according to each farm's protocol. All enrolled horses were vaccinated against EIV, African horse sickness and tetanus, but none against EHV-1 or -4. Informed, written consent for participation was obtained from the owners of each participating farm. The study was approved by the Animal Ethics Committee of the University of Pretoria (Study V040-13).

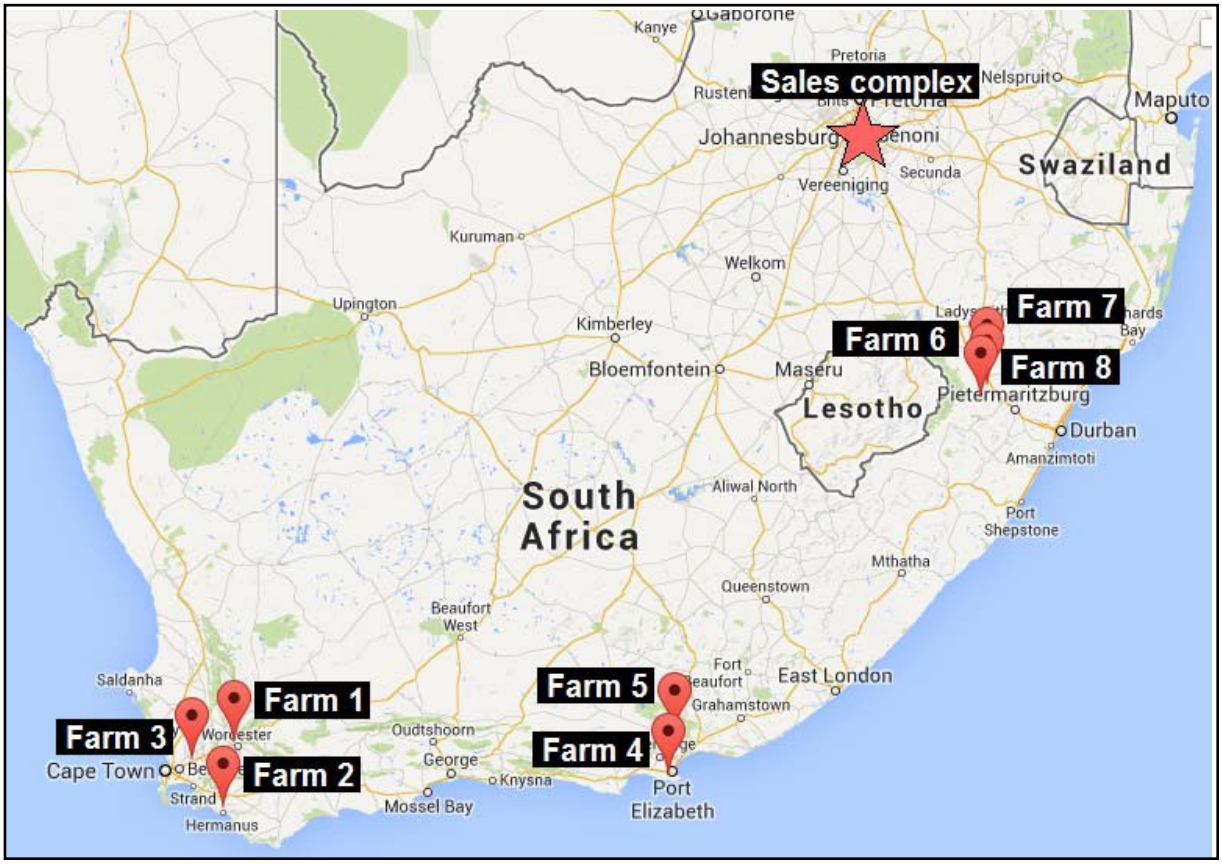


FIGURE 1: A map of South Africa indicating locations of the Gosforth Park sales complex and the farms of origin (n=8) from which the 90 Thoroughbreds in the study population were consigned.

FIGURE 2: Facilities at the Gosforth Park sales complex. Buildings' design allowed for free movement of air between stables and windows covered with metal grids enabled nose-to-nose contact between horses (A). Horses were periodically removed from their stables into adjacent walkways for showing (B) and mixed randomly at communal facilities during routine daily activities including in-hand exercise (C) and washing (D).

3.2.2 Daily monitoring

From arrival until departure horses were monitored twice daily, between 06h00-09h00 and 15h00-18h00 with rapid digital thermometers (Thermoval® [a]) for pyrexia, defined as a rectal temperature ≥38.5˚C (Figure 3). Horses were additionally monitored once daily, between 06h00-09h00, for the presence of a nasal discharge. Degree of nasal discharge was subjectively classified as normal-mild, obvious-moderate or obvious-severe (Figure 4).

FIGURE 3: Rectal temperature monitoring. Horses were monitored twice daily, between 06h00-09h00 and 15h00-18h00 with rapid digital thermometers (Thermoval® [a]) for pyrexia, defined as a rectal temperature ≥38.5˚C.

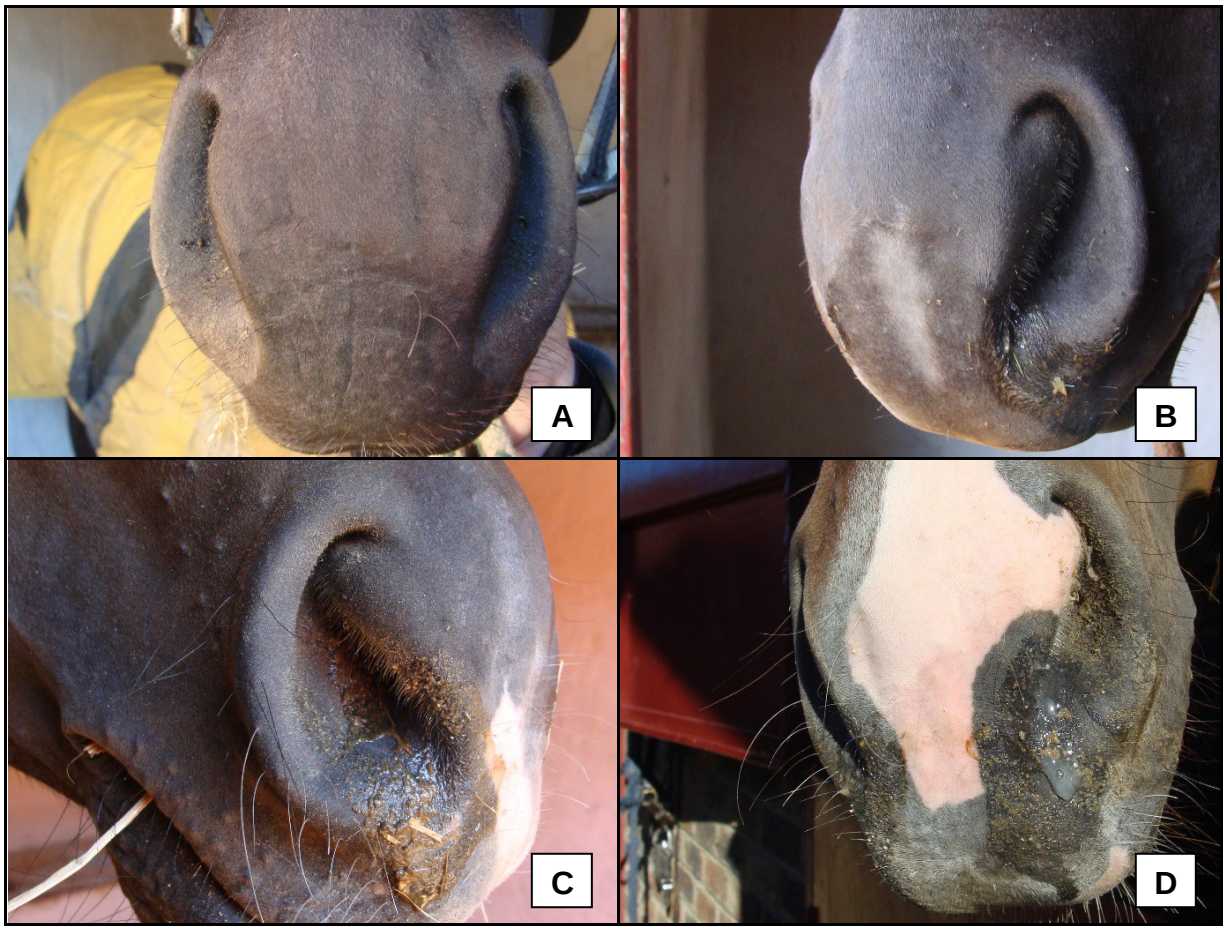


FIGURE 4: Subjective classification of degree of nasal discharge observed in 90 Thoroughbreds consigned to an auction sale: normal - none (A); normal - mild (B); obvious - moderate (C); obvious - severe (D).

3.2.3 Sample collection

At arrival. Duplicate nasal swabs, an 8.5 ml serum tube of blood and a faecal sample were collected from each horse within 24 hours of arrival. Nasal secretion samples were collected using two 15 cm metal shaft rayon-tipped swabs[b] advanced simultaneously into either of the horse's nostrils and rotated against the mucous membranes for collection of nasal secretion and epithelial cells (Figure 5A). Following collection each swab was placed in a sterile, dry, 15 ml plastic tube and refrigerated at 4-6°C until delivery to the Veterinary Genetics Laboratory, University of Pretoria (Figure 5B). One 8.5 ml BD Vacutainer® SSTTM II Advance Plus serum tube[c] was collected from each horse by means of jugular venipuncture (Figure 5C and D). Blood samples were refrigerated at 4-6°C after collection, until delivery to the Immunocontraception Laboratory, University of Pretoria. A fresh faecal sample was collected from each horse's stable between 06h00-09h00 in a 25 ml plastic specimen container, frozen at -20˚C within 2 hours of collection and kept frozen until delivery to the Endocrine Research Laboratory, University of Pretoria (Figure 5E).

Daily sampling of horses with pyrexia and, or nasal discharge. Subsequent to recording a pyrexia and, or nasal discharge in any horse, serial nasal swabs were collected daily as described until the day of departure.

Daily sampling of study population. Faecal samples were collected daily as described.

Prior to departure. Duplicate nasal swabs, an 8.5 ml serum tube of blood and a faecal sample were collected as described from each horse following their auction, within 24 hours prior to their departure (Figure 5F).

Sampling and monitoring schedules are summarised in Figure 6.

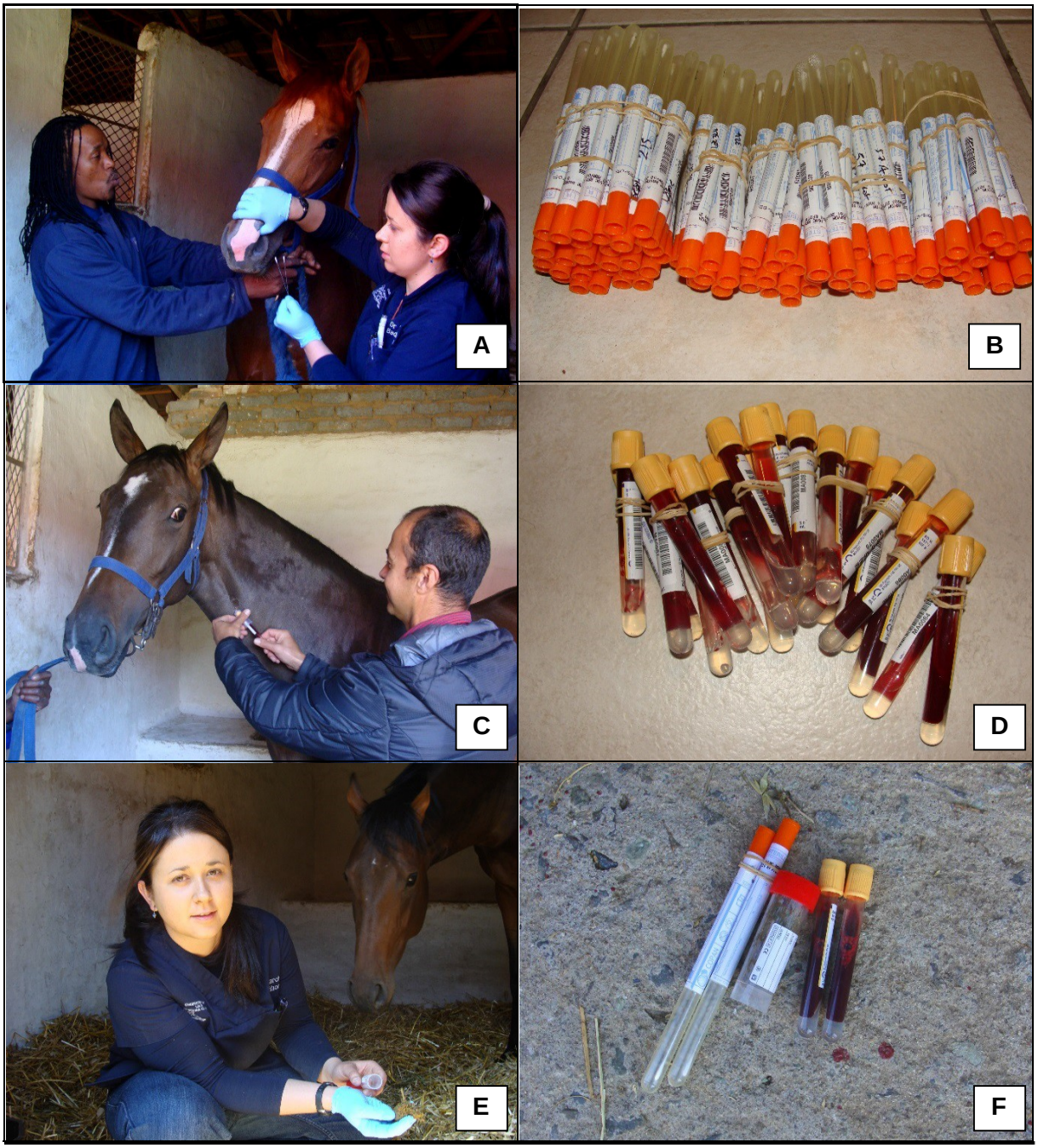


FIGURE 5: Sample collection. Collection of duplicate nasal swabs (A & B), serum samples (C & D) and faecal samples (E). Complete sample sets were collected from study horses upon sales arrival and departure (F).

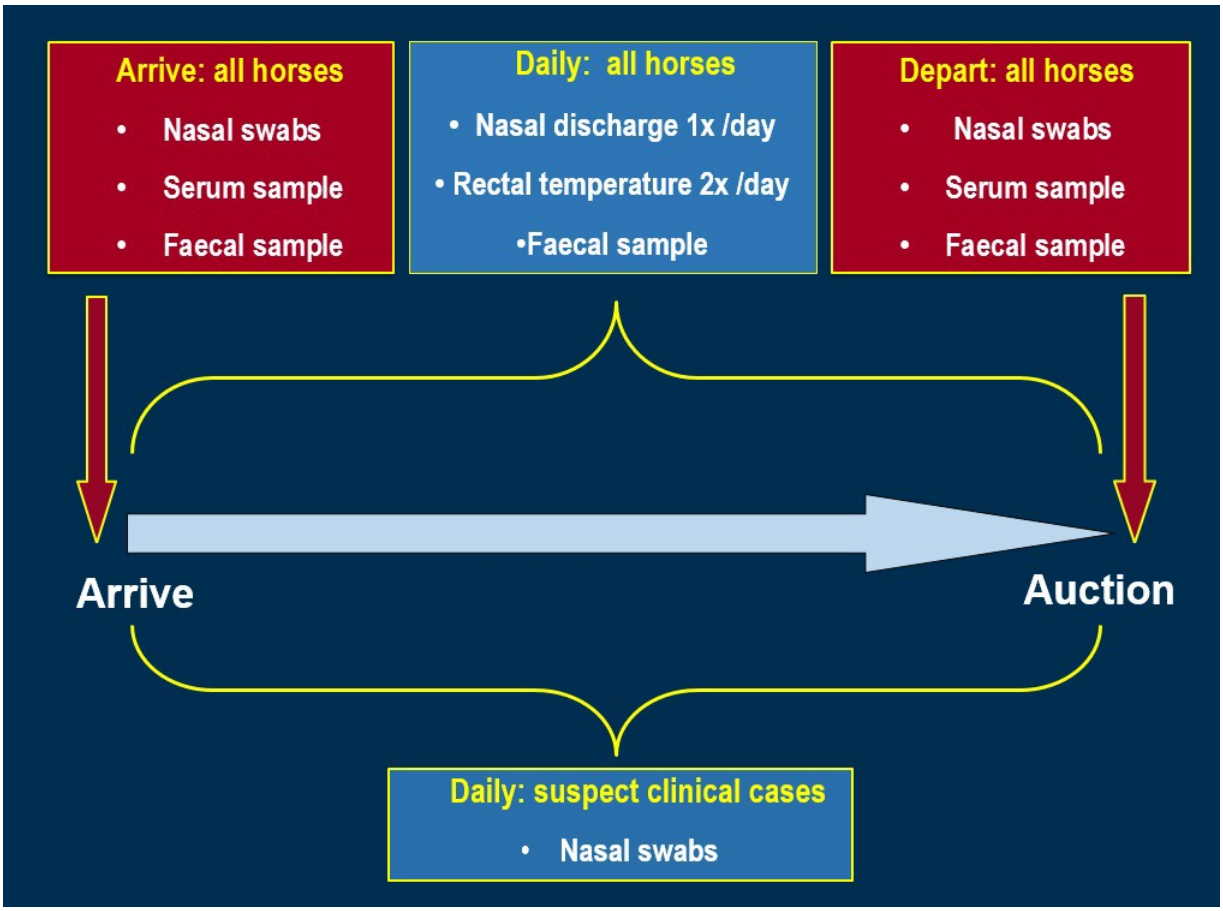


FIGURE 6: Summary of the sampling and monitoring schedule for the 90 Thoroughbreds in the study population during their period of residence at the Gosforth Park sales complex.

3.2.4 Laboratory analyses

Multiplex qPCR for EHV-1 and -4. Nasal swabs were agitated for 5 s in 0.5 ml of 0.1 M phosphate buffered saline (pH 7.4) in a 1.5 ml Eppendorf tube. Nucleic acid was extracted from 100 µl of the preparation using MagMax™ Pathogen DNA/RNA kit[d] and a Kingfisher 96 Magnetic Particle Processor[e] according to manufacturer's protocols. Subsequently, a duplex PCR for EHV-1 and -4 was performed using previously described primers and probes (Diallo *et al* 2007). Briefly, 17 µl of a master mix consisting of 1 µl of each primer/probe mix, 5 µl of nuclease-free water and 10 µl of Kapa Probe Fast ABI Prism® 2X PCR master mix[f] was added to each well of a PCR plate and 3 µl of the extracted template was added. Positive

and negative template controls were included on each plate. The PCR was performed according to the manufacturer's protocol with the assignment of a cut-off value of < 40 cycles (Ct) for positive detection of viral DNA. The Veterinary Genetics Laboratory's standard operating procedure for EHV-1 and -4 real-time PCR is included as Appendix 1.

ELISA for EHV-1 and -4 antibodies. Each serum sample was tested against the glutathione-S-transferase (GST) fusion proteins of EHV-1 gG, EHV-4 gG and against GST only, essentially as previously described by Crabb *et al* (1995) and validated by Gilkerson *et al* (1999c). To increase the sensitivity of the assay and assess inter-sample variation, all samples were tested against each antigen in duplicate and the mean absorbance of the two tests was used as the test result (Gilkerson *et al* 1999c). The following cut-off levels were used for interpretation of the absorbance values: >0.2 for antibody-positive; 0.1-0.2 for indeterminate; and <0.1 for antibody-negative. Positive control samples with EHV-1 and EHV-4 antibodies were included on each plate. Assays were performed in Nunc MaxiSorp® flat-bottom 96 well plates[g]. All antigens and positive control samples used in these assays were obtained from the Centre for Equine Virology, University of Melbourne. The University of Melbourne's standard operating procedure for the EHV-1 and -4 ELISA is included as Appendix 2.

Faecal extraction and hormone analysis. Frozen faecal samples were lyophilized, pulverized and sifted using a metal mesh strainer to remove fibrous material (Ganswindt *et al* 2010). Between 0.10-0.11 g of the faecal powder was then extracted by vortexing for 15 min with 80% ethanol in water (3 ml). Following centrifugation for 10 min at 1500 g, supernatants were transferred into micro-centrifuge tubes and stored at -20˚C until analysis. Extracts were measured for immunoreactive FGM concentrations using an EIA that detects 11,17-dioxoandrostanes, previously shown to provide reliable information on adrenocortical function in various mammals, including horses (Merl *et al* 2000, Schatz & Palme 2001, Heistermann *et al* 2006). Serial dilutions of extracts gave displacement curves parallel to the standard curve of the assay. Sensitivity of the assay at 90% binding was 1.8 ng/g faeces.

Intra- and inter-assay coefficients of variation, determined by repeated measurement of high- and low-value quality controls, ranged between 1.9% and 16.5%. Cross-reactivities of the antibody and assay methods were as previously described (Palme & Möstl 1997, Ganswindt *et al* 2002). The Endocrine Research Laboratory's standard operating procedure for faecal extraction and hormone analysis is included as Appendix 3.

3.2.5 Owner questionnaire

Historical data was obtained from individual farm owners by means of a questionnaire (Appendix 4).

3.2.6 Environmental data and sales prices

The environmental temperatures at the sales complex and at the eight farms of origin were investigated by reference to a web-based source reporting climatic data (www.worldweatheronline.com). Sales prices of study horses were obtained from the website of the Thoroughbred Breeders' Association of South Africa (www.tba.co.za).

3.2.7 Statistical analyses

The natural-log-transformed FGM concentrations obtained from 655 samples were modelled in linear mixed models (R Core Team 2012). All global model subsets were used (all were plausible), but interaction terms were omitted, allowing for balanced-design model averaging. The global model included five standardized fixed effects: days since transport; days since start of auction preparation; pyrexia; nasal discharge; EHV-4 status. Repeated measures were modelled as random effects: horse identity (1|horse); farm identity (1|farm).

Variables were standardized between categories (Gelman 2008). Variance inflation factors (VIF) were used to assess multicollinearity and candidate models were evaluated with Akaike's Information Criterion with small sample size correction (AIC_c) (Akaike 1974). Multimodel inference and model averaging were performed using Akaike weights (w_i) of all candidate models (Burnham & Anderson 2002). Goodness of fit of parameter estimates was assessed using 85% confidence intervals and the variation explained by the global model assessed using Ω_0^2 (Xu 2003, Arnold 2010).

Pearson product-moment correlation coefficient was calculated for the relationship between median FGM values of the adaptation phase and travel time (from farm of origin to the sales complex). Mann-Whitney rank sum test was used to determine if differences in travel time were reflected by a higher probability of identified EHV-4 positive horses.

3.3 Results

3.3.1 EHV detection

No EHV-1 was detected, however EHV-4 nucleic acid was detected in 13 of 90 (14.4%) horses originating from 7 of 8 participating farms. In total, 21 EHV-4-positive swabs were obtained from the 13 horses. Repeated incidents were detected in 4 of 13 (30.8%) horses. Both the longest period of continuous EHV-4 viral detection and the longest interval between consecutive events by a horse, was four days. Nasal swabs from 1 of 90 (1.1%) and 7 of 90 (7.8%) horses were positive for EHV-4 on days of arrival and departure, respectively. Details of the temporal pattern of EHV-4 detection during the observation period are shown in Table 1. The prevalence of EHV-4 amongst horses consigned by Western Cape and Eastern Cape farms ranged between 23.1-40%, except in the case of Farm 1, where EHV-4 was detected in only 7.7% of consigned horses (Table 1). The prevalence of EHV-4 amongst horses

consigned by KwaZulu-Natal farms ranged between 0-10% (Table 1). Aerial views of the sales complex indicating the temporal pattern of EHV-4 detection by date are shown in Figure 7A to I.

3.3.2 Environmental data

The mean minimum and maximum temperatures recorded at the eight farms of origin during the two weeks preceding travel (24 July to 7 August 2013) and at the sales complex during the horses' period of residence (8 to 16 August 2013) are shown in Table 2.

3.3.3 EHV-4 detection and clinical signs

Pyrexia and nasal discharge were poor indicators of EHV-4 status. Although all EHV-4-positive horses showed either a nasal discharge alone or both pyrexia and nasal discharge, the majority of the EHV-4-negative horses also showed one or both of these signs (Table 3). Duration of pyrexia was <24 hours in 7 of 8 horses with concurrent EHV-4 detection.

3.3.4 EHV serology

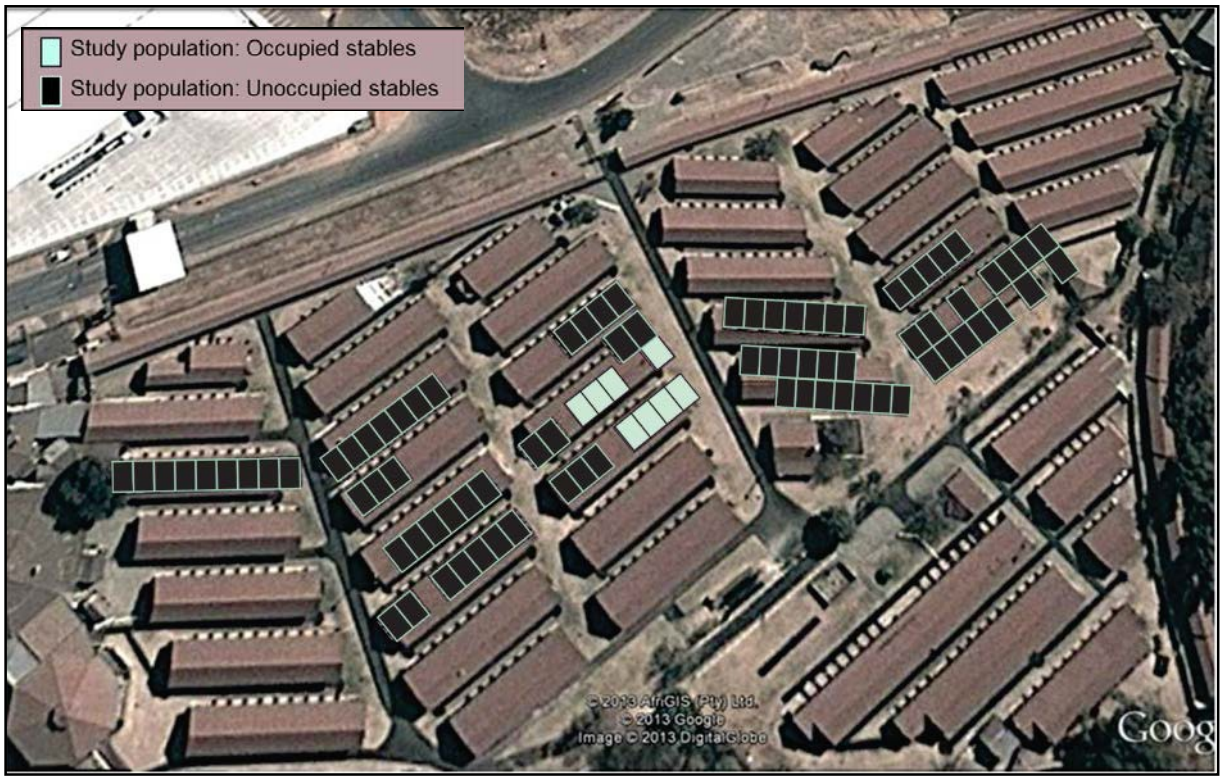
Serum samples were collected from 89 and 88 of the study population horses upon sales arrival and departure, respectively. Results of the ELISA-tests for EHV-1 and -4 antibodies are summarized in Table 4. ELISA results for all arrival and departure serum samples are included as Appendix 5. Horse 3-A, one of the 13 horses from which EHV-4 nucleic acid was retrieved, was EHV-4 antibody-negative both on arrival and departure.

TABLE 1: Proportion of EHV-4 PCR-positive nasal swabs obtained from 90 Thoroughbreds by their farm of consignment at an auction sale.

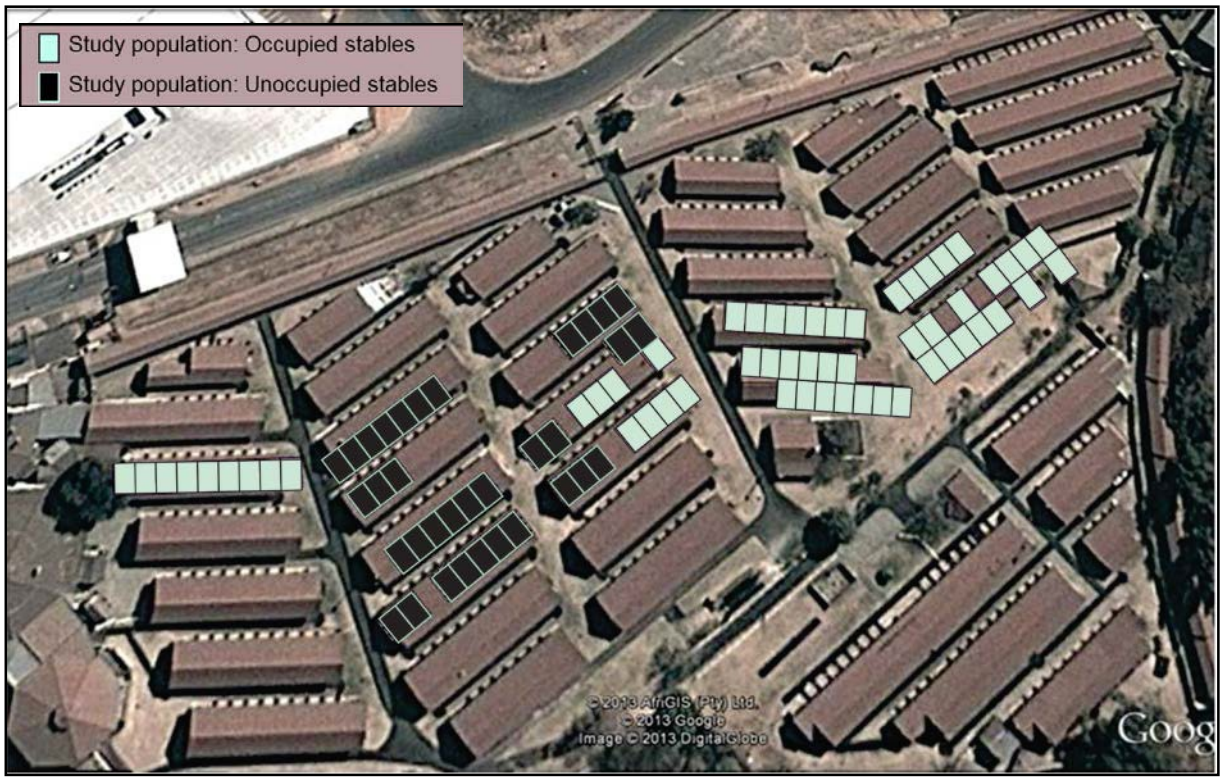
Farm of consignment	Province of origin	Consigned horses (n)	Number (%) of consigned horses that shed EHV-4	Sample dates (August 2013)								
				8	9	10	11	12	13	14	15 *	16 *
1	Western Cape	13	1 (7.7)		0/13	1/8	0/9	0/9	0/9	0/9	0/12	0/5
2	Western Cape	9	3 (33.3)		0/9	2/5	0/5	0/5	0/6	1/7	1/8	1/5
3	Western Cape	8	2 (25)	0/8	0/5	0/5	0/5	1/6	1/7	1/7	1/8	1/5
4	Eastern Cape	13	3 (23.1)			1/13	0/4	0/4	0/7	0/7	3/12	0/4
5	Eastern Cape	5	2 (40)			0/5	0/1	0/3	1/3	2/4	1/5	0/1
6	KwaZulu-Natal	10	1 (10)				0/10	0/0	1/3	0/5	0/8	0/3
7	KwaZulu-Natal	26	1 (3.8)		0/26	0/16	0/16	0/17	0/18	0/19	1/25	0/9
8	KwaZulu-Natal	6	0 (0)						0/6	0/1	0/6	0/2
Number (%)		90	13	0/8 (0)	0/53 (0)	4/52 (7.7)	0/50 (0)	1/44 (2.3)	3/59 (5.1)	4/59 (6.8)	7/84 (8.3)	2/34 (5.9)

(*) = auction dates

FIGURE 7: Aerial views of the Gosforth Park sales complex by date (A-I), indicating the temporal pattern of EHV-4 detection in 90 Thoroughbreds consigned to an auction sale.



A: 8 August 2013



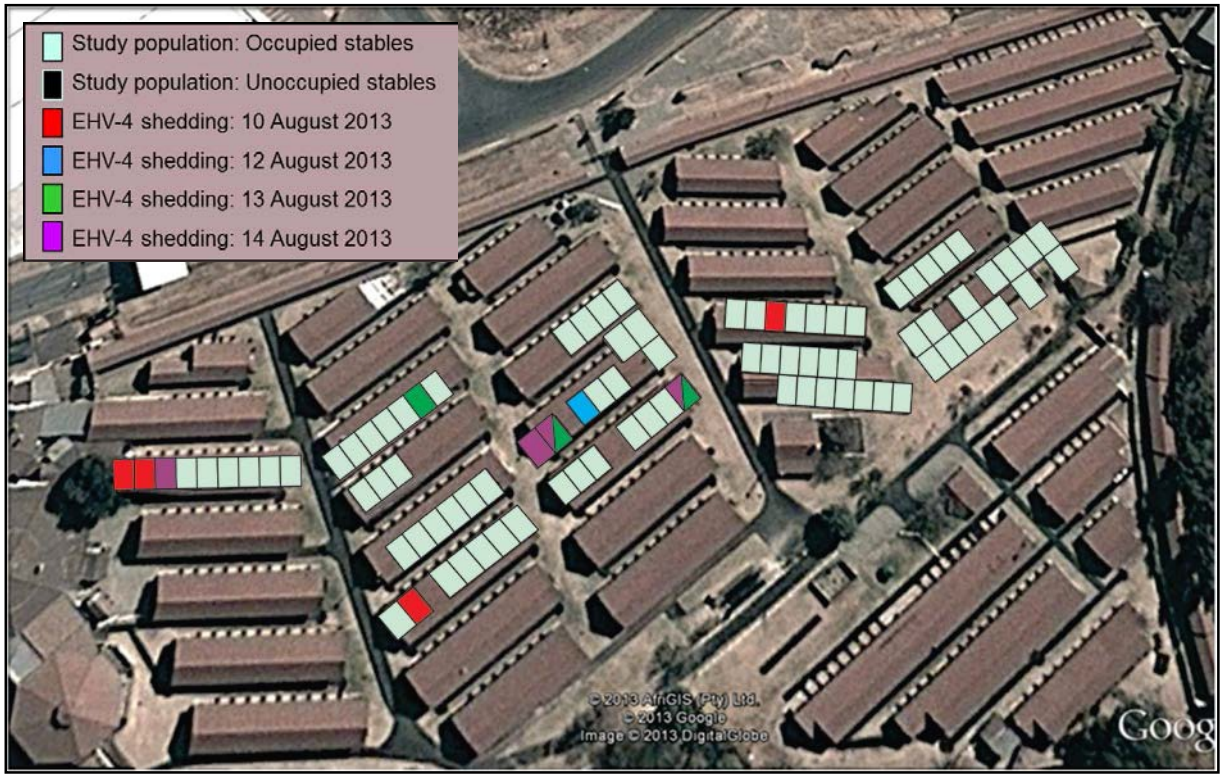
B: 9 August 2013

C: 10 August 2013

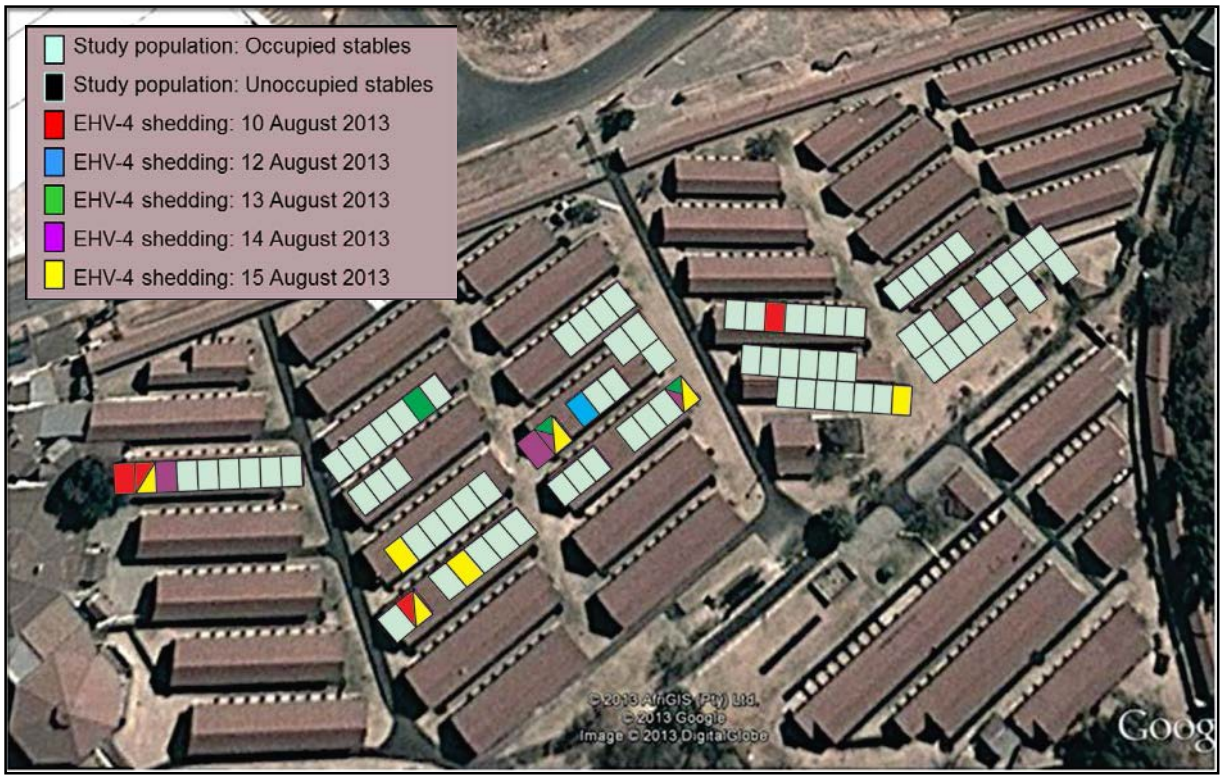
D: 11 August 2013

E: 12 August 2013

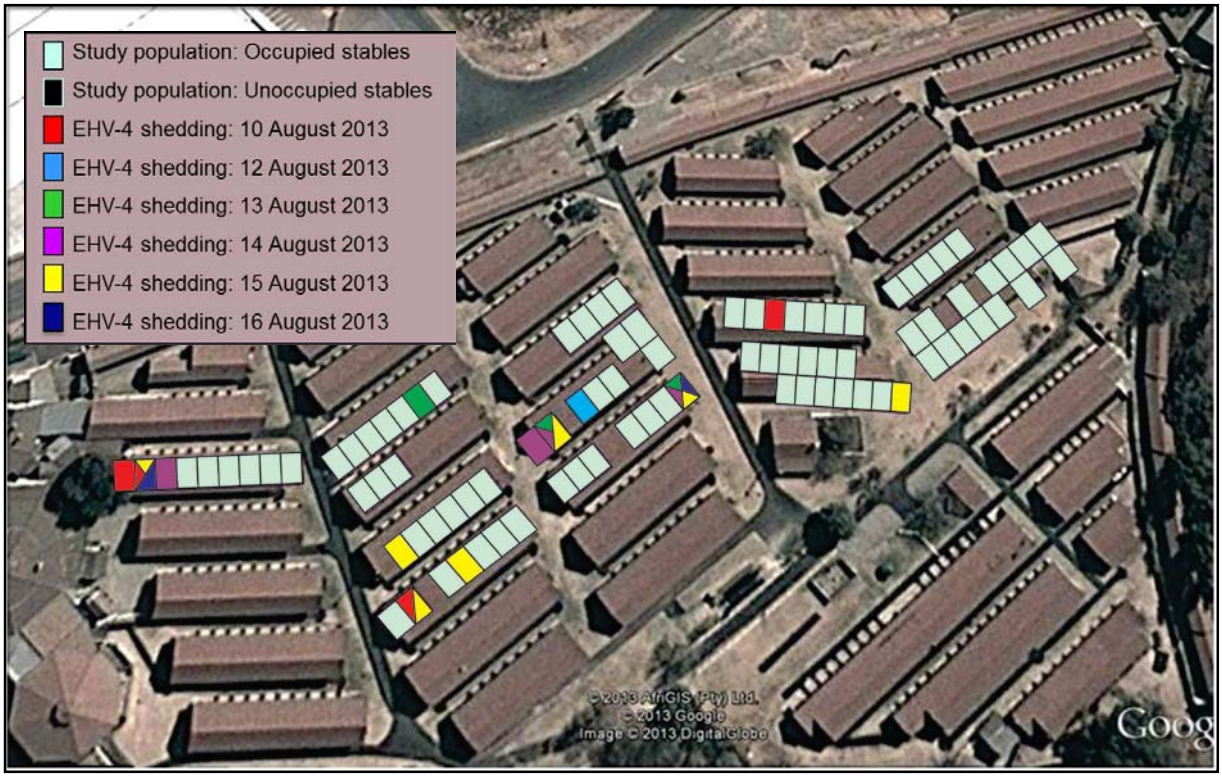
F: 13 August 2013



G: 14 August 2013



H: 15 August 2013



I: 16 August 2013

TABLE 2: Mean minimum and maximum environmental temperatures recorded at the farms of origin during the two weeks preceding travel to the auction sale (24 July to 7 August 2013) and at the sales complex during the horses' period of residence (8 to 16 August 2013)*.

Location	Province	Mean min temp (ºC)	Mean max temp (ºC)
Farm 1	Western Cape	3	18.1
Farm 2	Western Cape	10.1	18.7
Farm 3	Western Cape	6.9	17.9
Farm 4	Eastern Cape	13.7	23.5
Farm 5	Eastern Cape	11.8	23.1
Farm 6	Kwa-Zulu Natal	4.3	19.2
Farm 7	Kwa-Zulu Natal	4.3	19.2
Farm 8	Kwa-Zulu Natal	4.3	19.2
Germiston	Gauteng	8.8	18.1

**www.worldweatheronline.com*

TABLE 3: Summary of the associations between EHV-4 detection, pyrexia and nasal discharge in 90 Thoroughbreds consigned to an auction sale.

EHV-4 detection	Pyrexia	Nasal discharge	Number (%) of study population
-	-	-	12 (13.3)
-	+	-	13 (14.4)
-	-	+	25 (27.8)
-	+	+	27 (30)
+	-	-	0 (0)
+	+	-	0 (0)
+	-	+	5 (5.6)
+	+	+	8 (8.9)

(+) = detected; (-) = not detected

TABLE 4: Summary of the EHV-1 and -4 ELISA results for arrival (n=89) and departure (n=88) serum samples obtained from Thoroughbred horses consigned to an auction sale.

ELISA result	EHV-1		EHV-4	
	Arrival Proportion (%)	Departure Proportion (%)	Arrival Proportion (%)	Departure Proportion (%)
Positive	1/89 (1.1)	0/88 (0)	83/89 (93.3)	81/88 (92)
Indeterminate	1/89 (1.1)	1/88 (1.1)	3/89 (3.4)	3/88 (3.4)
Negative	87/89 (97.8)	87/88 (98.9)	3/89 (3.4)	4/88 (4.5)

3.3.5 Measurement of physiological stress

Based on model analysis, the covariates that explained variation in FGM concentrations were days since transport and days since start of auction preparation (Table 5, Figure 8). These two variables as well as EHV-4 status and nasal discharge were selected in the best candidate models ($\Delta AIC_c < 2$, Table 5). However, only days since transport and days since start of auction preparation had large standardized effect sizes, which differed from zero, after model averaging (Figure 8). EHV-4 status and the two clinical signs had either high variability in parameter estimates or a small effect size (Figure 8). The global model explained 43% of variation in FGM concentrations, with R^2_{adj} = 0.43. VIF's for all covariates were below 4, suggesting that multicollinearity was not problematic in these models.

Post hoc graphical analysis of the FGM data confirmed the results of the model averaging and suggested that during the adaptation phase, median FGM concentrations were on average 93% higher at 24 hours when compared to 72 hours after arrival (Figure 9A). A second more moderate increase in FGM concentrations was associated with the auction phase, with overall 31-48% higher median FGM concentrations on the first day of auction compared to both 24 hours prior to auction and the second day of auction (Figure 9B). Further analysis of the FGM data of individual farms revealed two distinct patterns. Pattern A (Farms 1, 2, 4 & 5) was biphasic with distinct increases in median FGM concentrations

during both the adaptation and auction phases (Figure 10A). Pattern B (Farms 3, 6 & 7) showed a distinct increase in median FGM concentrations during the adaptation phase, without a distinct change during the auction phase (Figure 10B). FGM data from Farm 8, the farm with the shortest duration of stay, did not conform to either pattern. Individual patterns of FGM alterations of horses from the eight consigning farms are shown in Figure 11.

The mean (range) travel times from the Western and Eastern Cape Provinces were 17.4 hours (15-22 hours) and from KwaZulu-Natal Province were 6.8 hours (6-7.5 hours). During the adaptation phase, peak FGM concentrations measured for horses from KwaZulu-Natal Province were on average 40% and 31% lower than those from the Western Cape and Eastern Cape Provinces, respectively. There was a weak ($r = 0.383$), but significant correlation between travel time and subsequent individual median FGM concentrations during the adaptation phase. Differences in individual travel time were not reflected in a higher probability of identified EHV-4-positive horses ($P = 0.63$).

The sales prices of 76 study horses were obtained, while the other 14 horses were bought back by their vendors for undisclosed amounts. The 76 horses were categorized according to sales price: <R40 000; R40 000-R69 000; R70 000-R99 000 and ≥R100 000. The auction phase median FGM concentrations of horses in the four categories were compared (Figure 12). The overall median FGM concentration of each of the four categories did not differ significantly.

TABLE 5: Models with Akaike weights (w_i) > 0, modelling FGM levels in horses. Individual horses (1|horse) and farms (1|farm) were random effects in all models. Fixed effects were days since transport (trans), days since start of auction preparation (prep), pyrexia, nasal discharge and EHV-4 detection.

Model: log(FGM) ~	log*L*	*K*	AICc	Δ	w_i
pyrexia + nasal discharge + EHV-4 + prep + trans + (1|farm) + (1|horse)	-287.9	9	594.3	0.0	0.67
nasal discharge + EHV-4 + prep + trans + (1|farm) + (1|horse)	-290.0	8	596.3	1.9	0.25
pyrexia + EHV-4 + prep + trans + (1|farm) + (1|horse)	-291.6	8	599.5	5.2	0.05
EHV-4 + prep + trans + (1|farm) + (1|horse)	-293.6	7	601.5	7.1	0.02

.

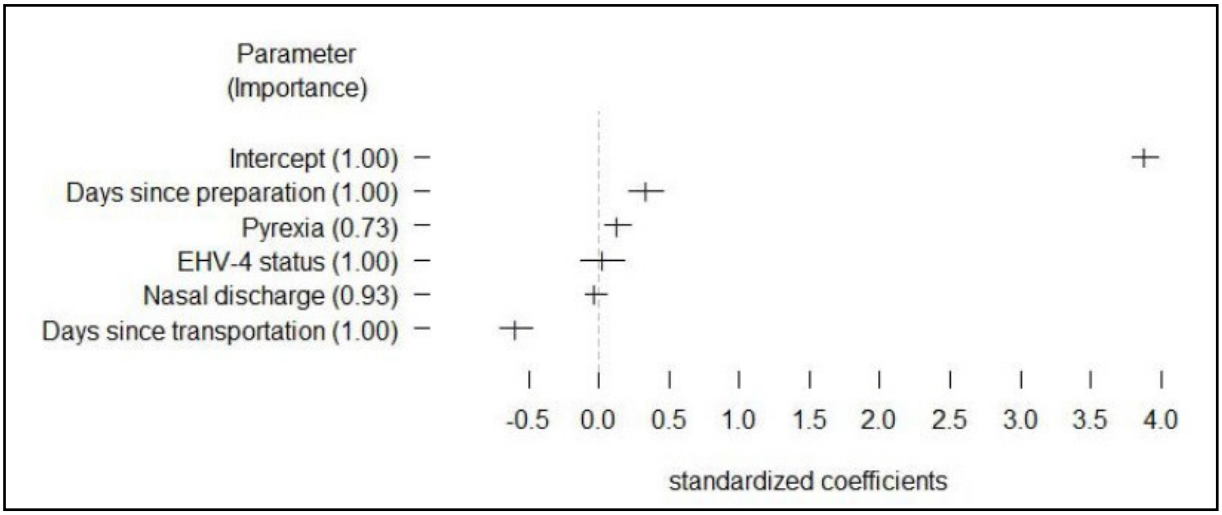


FIGURE 8: Model averaging of covariates of FGM levels in horses. Vertical lines indicate the intercept and effect sizes for standardized parameters. Horizontal lines indicate 85% confidence intervals. Values in parentheses indicate relative importance.

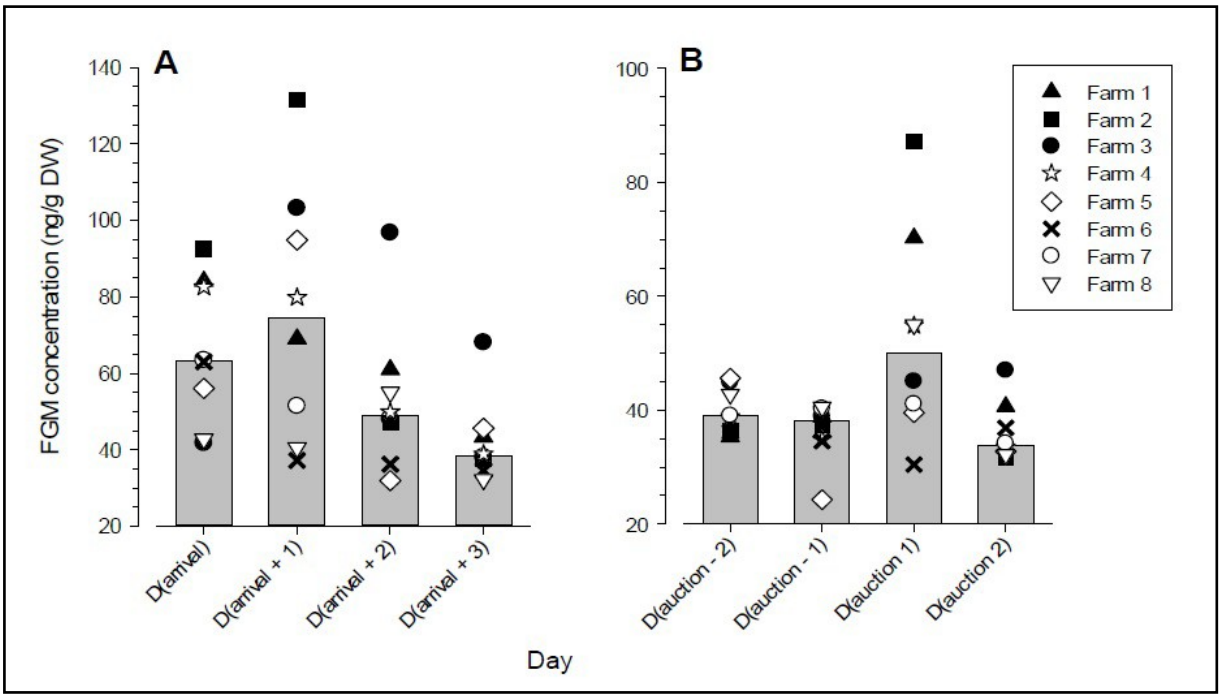


FIGURE 9: FGM concentrations of consigning farms (n=8) during the adaptation (A) and auction (B) phases. Different symbols represent median FGM concentrations for horses from different farms. Bars represent daily overall median FGM values. Farms 1, 2 & 3 (Western Cape Province); Farms 4 & 5 (Eastern Cape Province); Farms 6, 7 & 8 (KwaZulu-Natal Province).

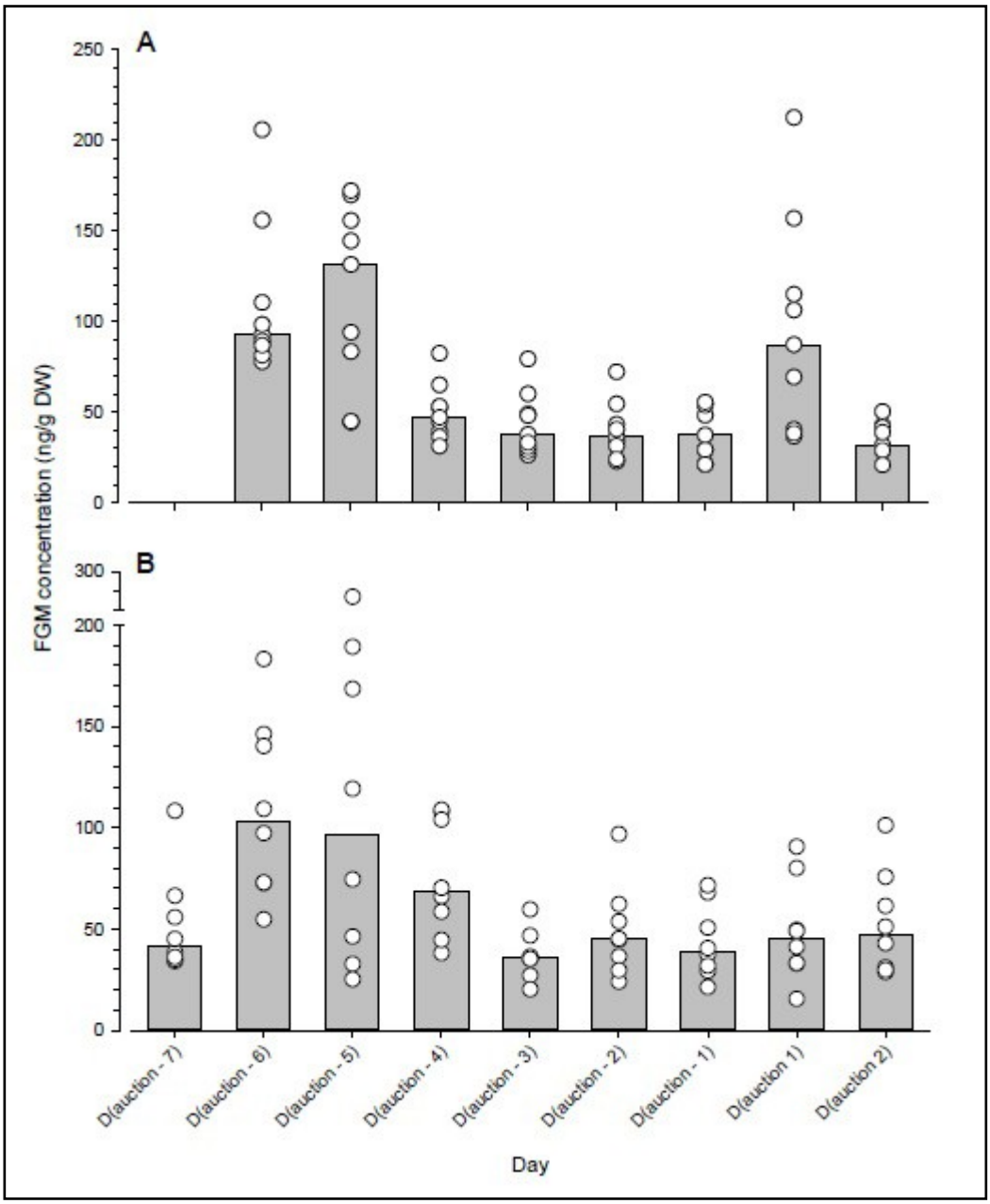


FIGURE 10: Examples of the two distinct patterns of FGM concentrations observed in Thoroughbred horses during residence at a sales complex, with elevated FGM concentrations during the adaptation and auction phases (Pattern A) or during the adaptation phase only (Pattern B). Dots represent FGM concentrations of individual horses and bars represent the median FGM concentration for horses of a consigning farm on a given day.

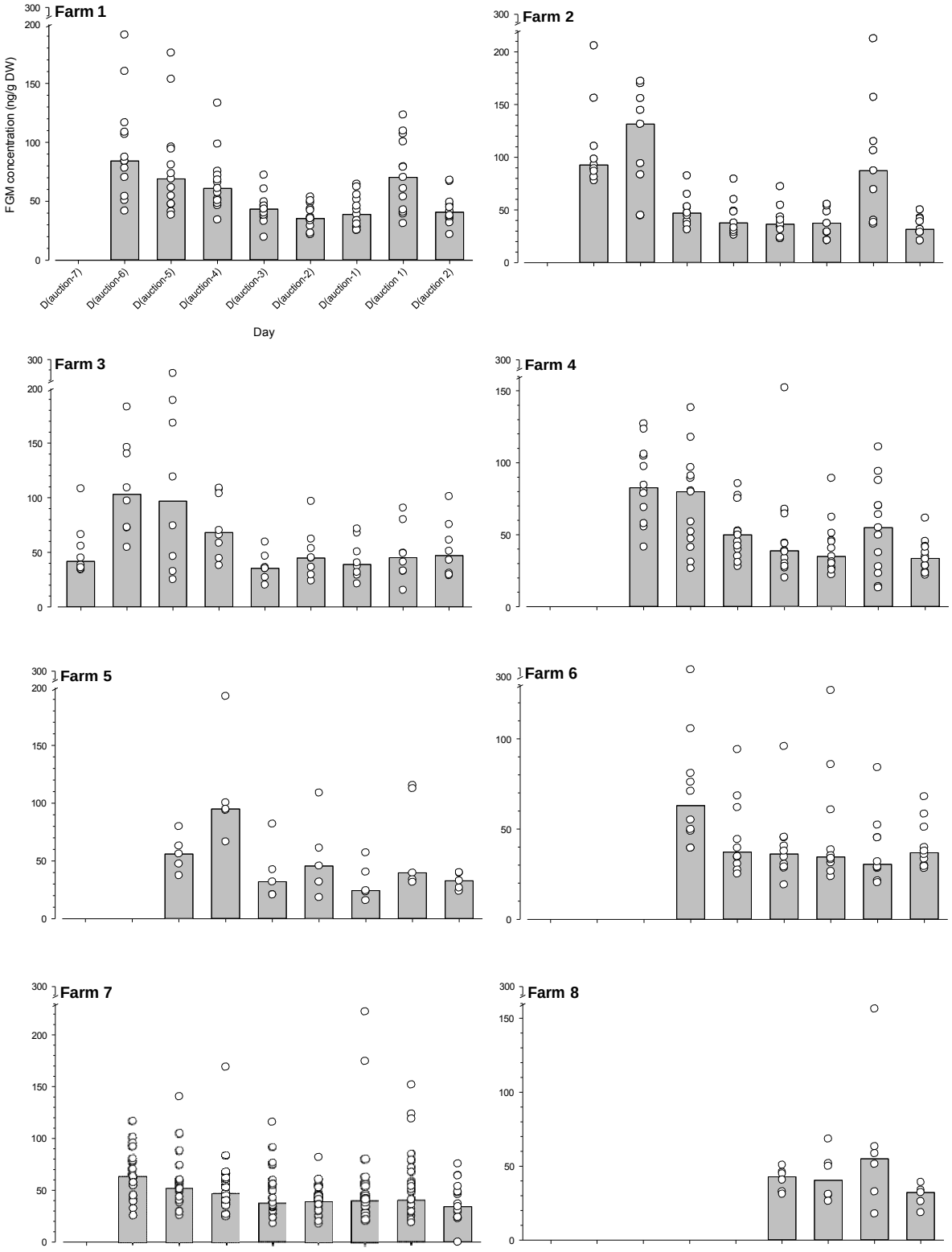


FIGURE 11: Patterns of FGM alterations in Thoroughbred horses from individual consigning farms (n=8) during residence at a sales complex. Farms 1, 2, 4 and 5 conformed to Pattern A (biphasic peaks: shortly post-arrival and on first day of auction). Farms 3, 6 and 7 conformed to Pattern B (single peak: shortly post-arrival). Farm 8 did not conform either pattern.

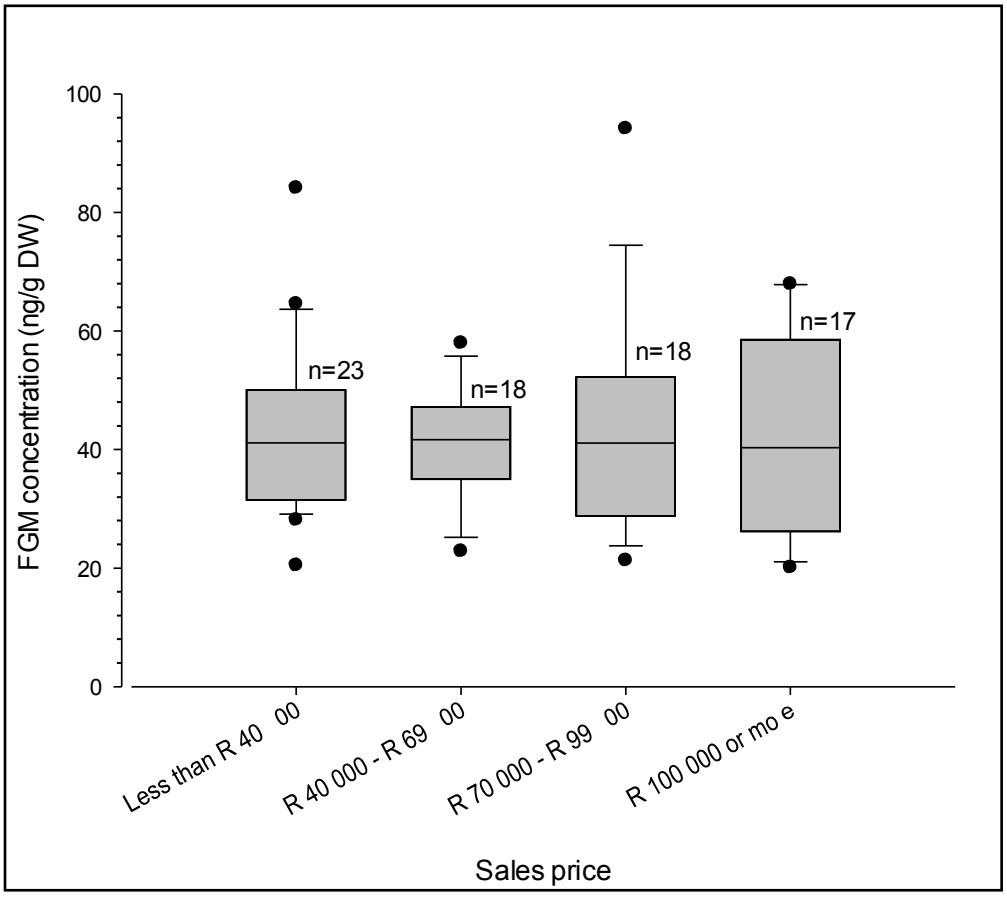


FIGURE 12: Auction phase median FGM concentrations of 76 Thoroughbred horses categorized according to their sales prices.

3.4 Discussion

This study population's marked difference in the prevalence of EHV-4 and EHV-1 correlated with the markedly higher seroprevalence of EHV-4 compared to EHV-1 reported in other populations worldwide and the low detection rate of EHV-1 reported in populations exposed to stressors (Gilkerson *et al* 1999a, Gilkerson *et al* 1999b, Pusterla *et al* 2009b, Carr *et al* 2011, Aharonson-Raz *et al* 2014). It was previously suggested that EHV-1 rarely circulates outside breeding populations inclusive of young foals (Carlson *et al* 2013). In the present study, EHV-4 detection included single and repeated, continuous or interrupted events and the relatively low prevalence upon arrival was similar to a previous report (Carlson *et al* 2013). A distinct biphasic pattern of EHV-4 detection, with most events during the auction

phase (peaking on the first day) was observed. The second, lower peak apparent shortly post-arrival possibly reflected cumulative events associated with transport and initial adaptation stressors. Both viral recrudescence and horizontal spread of primary infection may have contributed to the observed increased detection of EHV-4 between arrival and departure (Gilkerson *et al* 1999a, Patel & Heldens 2005). The EHV status of horses subsequent to sales departure was unknown. The implications of EHV-1 and -4 positive status at departure and associated risks of viral shedding and transmission at subsequent destinations, including training facilities, warrant further investigation. The current study supported the application of risk mitigation strategies for equine herpesviruses at Thoroughbred sales complexes and training facilities in South Africa and other countries with similar populations (Lunn *et al* 2009).

Climatic conditions on the farms of origin may have biased the prevalence of EHV-4 observed amongst horses from individual farms. Compared to the sales complex, the four farms with the highest detected prevalence had similar or higher mean minimum environmental temperatures. The four farms with the lowest detected prevalence all had markedly lower mean minimum environmental temperatures compared to the sales complex. These horses were possibly better adapted to more severe environmental conditions and less affected by the sudden climatic change.

A limitation of the study design was that the study population selection was not randomized and included pre-selection of horses from the sales catalogue based on farm of origin. The selection was, however, representative of the South African Thoroughbred breeding demographic and more than one farm per province was included to balance selection bias.

The prevalence of pyrexia recorded among EHV-4-positive horses in this study was similar to and nasal discharge higher than previously reported (Pusterla *et al* 2011). Pyrexia duration of <24 hours in the majority of EHV-4-positive horses supports the utility of twice-daily rectal temperature monitoring for suspected clinical cases of EHV-infection (Lunn *et al* 2009,

Walter *et al* 2013). Based on published recommendations, daily sampling of respiratory secretions following detection of pyrexia and, or nasal discharge was performed to improve viral detection rates (Sonis & Goehring 2013). A study limitation was the reliance of nasal swabbing on observation of clinical signs, potentially resulting in lower detection rates of sub-clinical EHV-1 and -4 infections. Neither pyrexia nor nasal discharge, nor a combination of these signs predicted EHV-4 status in the current study, contrasting with a reported association between clinical signs of respiratory disease and EHV-4 detection in foals (Bell *et al* 2006). Although pyrexia and nasal discharge have been cited as the most common clinical signs among EHV-4-positive horses (Pusterla *et al* 2011), the combination of these non-specific signs has also been associated with molecular evidence of lesser-characterised respiratory viruses, including EHV-2 and -5, EAdV-1 and ERBV (Pusterla *et al* 2013). The current study's discrepancy in the prevalence of clinical signs and detection of EHV-1 and EHV-4 nucleic acid warrants further investigation of the association of IURD with alternative infectious agents in similar populations during sales consignment.

EHV-1 and -4 antibody detection rates in the current study's unvaccinated population were similar to the seroprevalence reported for an equine population in Israel (Aharonson-Raz *et al* 2014). Australian reports have cited similar and higher prevalence of antibodies against EHV-4 and EHV-1, respectively (Gilkerson *et al* 1999a, Gilkerson *et al* 1999b). EHV-4 antibody-negative serum was collected on arrival and departure, seven days apart, from one horse which tested EHV-4 PCR-positive on nasal swabs. This most likely resulted from a recent primary EHV-4 infection and nasal shedding of virus, with serum sample collection prior to establishment of a detectable antibody response. Similar to a previous report (Dynon *et al* 2007), EHV-4 nucleic acid was detected in the nasal secretions of 12 other horses despite the presence of EHV-4 antibodies in the serum samples of all of these animals. Although EHV-1 and -4 infections are not prevented by inactivated vaccines currently available in South Africa, these vaccines may reduce the severity of clinical disease and viraemia, as well as induce short-lived mucosal immunity to reduce nasopharyngeal

shedding and limit viral transmission (Harless & Pusterla 2006, Lunn *et al* 2009). The value of prophylactic vaccination of horses shortly prior to exposure to high-risk environments such as auction sales, has not been investigated.

Two distinctive patterns in FGM alterations associated with sales consignment were identified in the present study. The initial peak observed soon after arrival, presumably reflected a cumulative series of potentially stressful events associated with transport, and gradually decreased during a subsequent adaptation period as horses became accustomed to environmental and routine changes. The second peak arguably reflected events shortly prior to auction. This is a period typically associated with increased buyer activity at the sales complex and intensive preparation of horses for their sales-ring appearance. Notably, the auction process itself did not appear to initiate any prolonged physiological stress, with FGM concentrations returning to pre-auction levels within 24 to 48 hours. The farm with the shortest duration of residence was the single exception to the patterns observed, probably due to an overlap of the adaptation and auction phases in this case.

Transport-associated elevation in FGM concentrations occurred in horses from all farms, independent of distance and travel time, although the travel time apparently affected the magnitude of the subsequent stress response. Physiological stress levels increased in approximately half of the farms' horses during the auction phase, suggesting that future management practices may be adapted to minimize physiological stress during auction preparation. It was hypothesized that horses' sales value may play a role in physiological stress, especially during the auction phase. High-value horses particularly attract more attention from potential buyers and are intensively handled during the days leading up to the auction. This hypothesis was, however, not proven.

Although not necessarily associated, the similarities between the distinctly biphasic patterns of both physiological stress and nasal detection of EHV-4 was notable. Practicalities

precluded monitoring of case-matched horses on the farms of origin for similar, concurrent EHV-1 and -4 and FGM patterns, not associated with transport and sales consignment.

Manufacturers' details

[a] Paul Hartmann AG, Heidenheim, Germany.

[b] Copan Diagnostics Inc., Murrieta, California, United States of America.

[c] BD (Becton, Dickinson and Company), Plymouth, United Kingdom.

[d] Life Technologies, Carlsbad, California, United States of America.

[e] Thermo Fisher Scientific Inc., Waltham, Massachusetts, United States of America.

[f] Kapa Biosystems, Cape Town, South Africa.

[g] Thermo Fisher Scientific Inc., Waltham, Massachusetts, United States of America.

Chapter 4

General Conclusions

EHV-4 was detected in the respiratory secretions of some of the two-year old Thoroughbreds consigned to an auction sale in South Africa. Peaks in viral detection occurred shortly post-arrival and on the first day of auction and coincided with periods of maximal physiological stress. Pyrexia and nasal discharge were found to be non-specific clinical signs that were unreliable predictors of EHV-4 status. Serology results indicated that almost the entire population had previously been exposed to EHV-4, as opposed to almost no previous exposure to EHV-1. EHV-4 nucleic acid was detected in respiratory secretions of horses despite the presence of EHV-4 antibodies. The initial transport and arrival and later auction preparation phases were key periods in terms of sales consignment-associated physiological stress, resulting in the majority of study population horses showing either of two distinctive stress response patterns. Transport-associated stress occurred independent of travel distance and duration, but travel duration did affect the magnitude of the subsequent stress response. Variation in management practices between farms appeared to affect physiological stress responses of consigned horses, particularly during sales preparation.

Sales consignment and its associated stressors are inherent to the Thoroughbred breeding and racing industry and increase the risk association with IURD in young horses. Future investigation into management practices to reduce the impact of physiological stress on the health and welfare of young Thoroughbreds consigned to sales, is essential. Determining the prevalence of infectious respiratory pathogens in the South African Thoroughbred racing population, as well as the role of lesser-characterised respiratory infections during sales consignment, will facilitate the design of appropriate risk mitigation and control strategies. EHV vaccination is one potential strategy of limiting viral shedding and transmission which warrants investigation.

Chapter 5

References

AHARONSON-RAZ, K., DAVIDSON, I., PORAT, Y., ALTORY, A., KLEMENT, E. and STEINMAN, A. (2014) Seroprevalence and rate of infection of equine influenza virus (H3N3 and H7N7) and equine herpes virus (1 and 4) in the horse population of Israel. *Journal of Equine Veterinary Science*, **34**, 828 - 832.

AKAIKE, H. (1974) A new look at the statistical model identification. *IEEE Transactions on Automatic Control*, **19**, 716 - 723.

ARNOLD, T.W. (2010) Uninformative parameters and model selection using Akaike's Information Criterion. *Journal of Wildlife Management*, **74**, 1175 - 1178.

BAILEY, C.J., ROSE, R.J., REID, S.W.J. and HODGSON, D.R. (1997) Wastage in the Australian thoroughbred racing industry: a survey of Sydney trainers. *Australian Veterinary Journal*, **75**, 64 - 66.

BELL, S.A., BALASURIYA, U.B.R., GARDNER, I.A., BARRY, P.A., WILSON, W.D., FERRARO, G.L. and MACLACHLAN, N.J. (2006) Temporal detection of equine herpesvirus infections of a cohort of mares and their foals. *Veterinary Microbiology*, **116**, 249 - 257.

BLUNDEN, A.S., SMITH, K.C., BINNS, M.M., ZHANG, L., GOWER, S.M. and MUMFORD, J.A. (1995) Replication of equid herpesvirus 4 in endothelial cells and synovia of a field case of viral pneumonia and synovitis of a foal. *Journal of Comparative Pathology*, **112**, 133 - 140.

BORCHERS, K., WOLFINGER, U., LAWRENZ, B., SCHELLENBACH, A. and LUDWIG, H. (1997) Equine herpesvirus 4 DNA in trigeminal ganglia of naturally infected horses detected by direct in situ PCR. *Journal of General Virology*, **78**, 1109 - 1114.

BURNHAM, K.P. and ANDERSON, D.R. (2002) *Model selection and multimodel inference: a practical information-theoretic approach*. 2nd edn., Springer, New York.

BUSTIN, S.A., BENES, V., NOLAN, T. and PFAFFL, M.W. (2005) Quantitative real-time RT-PCR – a perspective. *Journal of Molecular Endocrinology*, **34**, 597 - 601.

CARDWELL, J.M., SMITH, K.C., WOOD, J.L.N. and NEWTON, J.R. (2013) A longitudinal study of respiratory infections in British National Hunt racehorses. *Veterinary Record*, **172**, 637 - 639.

CARLSON, J.K., TRAUB-DARGATZ, J.L., LUNN, D.P., MORLEY, P.S., KOHLER, A., KASPER, K., LANDOLT, G.A., BARNETT, D.C. and LUNN, K.F. (2013) Equine viral respiratory pathogen surveillance at horse shows and sales. *Journal of Equine Veterinary Science*, **33**, 229 - 237.

CARR, E., SCHOTT, H. and PUSTERLA, N. (2011) Absence of equid herpesvirus-1 reactivation and viraemia in hospitalized critically ill horses. *Journal of Veterinary Internal Medicine*, **25**, 1190 - 1193.

CHOWDHURY, S.I., LUDWIG, H. and BUHK, H.J. (1988) Molecular biological characterization of equine herpesvirus type 1 (EHV-1) isolates from ruminant hosts. *Virus Research*, **11**, 127 - 139.

CRABB, B.S., MACPHERSON, C.M., REUBEL, G.H., BROWNING, G.F., STUDDERT, M.J. and DRUMMER, H.E. (1995) A type-specific serological test to distinguish antibodies to equine herpesviruses 4 and 1. *Archives of Virology*, **140**, 245 - 258.

CRABB, B.S. and STUDDERT, M.J. (1993) Epitopes of glycoprotein G of equine herpesvirus 4 and 1 located near the c-termini elicit type-specific antibody responses in the natural host. *Journal of Virology*, **67**, 6332 - 6338.

DAVISON, A.J., EBERLE, R., EHLERS, B., HAYWARD, G.S., McGEOCH, D.J., MINSON, A.C., PELLET, P.E., ROIZMAN, B., STUDDERT, M.J. and THIRY, E. (2009) The order herpesvirales. *Archives of Virology*, **154**, 171 - 177.

DIALLO, I.S., HEWITSON, G., WRIGHT, L.L., KELLY, M.A., RODWELL, B.J. and CORNEY, B.G. (2007) Multiplex real-time PCR for the detection and differentiation of equid herpesvirus 1 (EHV-1) and equid herpesvirus 4 (EHV-4). *Veterinary Microbiology*, **123**, 93 - 103.

DYNON, K., BLACK, W.D., FICORILLI, N., HARTLEY, C.A. and STUDDERT, M.J. (2007) Detection of viruses in nasal swab samples from horses with acute, febrile, respiratory disease using virus isolation, polymerase chain reaction and serology. *Australian Veterinary Journal*, **85**, 46 - 50.

EDINGTON, N. and BRIDGES, C.G. (1985) Experimental reactivation of equid herpesvirus 1 (EHV 1) following the administration of corticosteroids. *Equine Veterinary Journal*, **17**, 369 - 372.

ESPY, M.J., UHL, J.R., SLOAN, L.M., BUCKWALTER, S.P., JONES, M.F., VETTER, E.A., YAO, J.D.C., WENGENACK, N.L., ROSENBLATT, J.E., COCKERILL, F.R. and SMITH, T.F.

(2006) Real-time PCR in clinical microbiology: applications for routine laboratory testing. *Clinical Microbiology Reviews*, **19**, 165 - 256.

FOOTE, C.E., LOVE, D.N., GILKERSON, J.R., WELLINGTON, J.E. and WHALLEY, J.M. (2006) EHV-1 and EHV-4 infection in vaccinated mares and their foals. *Veterinary Immunology and Immunopathology*, **111**, 41 - 46.

FOOTE, C.E., LOVE, D.N., GILKERSON, J.R. and WHALLEY, J.M. (2004) Detection of EHV-1 and EHV-4 DNA in unweaned Thoroughbred foals from vaccinated mares on a large stud farm. *Equine Veterinary Journal*, **36**, 341 - 345.

FRIDAY, P.A., SCARRATT, W.K., ELVINGER, F., TIMONEY, P.J. and BONDA, A. (2000) Ataxia and paresis with equine herpesvirus type 1 infection in a herd of riding school horses. *Journal of Veterinary Internal Medicine*, **14**, 197 - 201.

GANSWINDT, A., HEISTERMANN, M., BORRAGAN, S. and HODGES, J.K. (2002) Assessment of testicular endocrine function in captive African elephants by measurement of urinary and fecal androgens. *Zoo Biology*, **21**, 27 - 36.

GANSWINDT, A., MUENSCHER, S., HENLEY, M., PALME, R., THOMPSON, P. and BERTSCHINGER, H. (2010) Concentrations of faecal glucocorticoid metabolites in physically injured free-ranging African elephants (*Loxodonta africana*). *Wildlife Biology*, **16**, 323 - 332.

GELMAN, A. (2008) Scaling regression inputs by dividing by two standard deviations. *Statistics in Medicine*, **27**, 2865 - 2873.

GILKERSON, J.R., JORM, L.R., LOVE, D.N., LAWRENCE, G.L. and WHALLEY, J.M. (1994) Epidemiological investigation of equid herpesvirus-4 (EHV-4) excretion assessed by nasal swabs taken from thoroughbred foals. *Veterinary Microbiology*, **39**, 275 - 283.

GILKERSON, J.R., TEAGUE, N., WHALLEY, J.M. and LOVE, D.N. (1999a) A prospective cohort study of upper respiratory tract disease in one and two year old racehorses. Serological evaluation of the role of equine herpesviruses 1 and 4 (EHV-1 and EHV-4) in respiratory disease. *Australian Equine Veterinarian*, **17**, 76 - 81.

GILKERSON, J.R., WHALLEY, J.M., DRUMMER, H.E., STUDDERT, M.J. and LOVE, D.N. (1999b) Epidemiology of EHV-1 and EHV-4 in the mare and foal populations of a Hunter Valley stud farm: are mares the source of EHV-1 for unweaned foals. *Veterinary Microbiology*, **68**, 27 - 34.

GILKERSON, J.R., WHALLEY, J.M., DRUMMER, H.E., STUDDERT, M.J. and LOVE, D.N. (1999c) Epidemiological studies of equine herpesvirus 1 (EHV-1) in Thoroughbred foals: a review of studies conducted in the Hunter Valley of New South Wales between 1995 and 1997. *Veterinary Microbiology,* **68**, 15 - 25.

HARLESS, W. and PUSTERLA, N. (2006) Equine herpesvirus 1 and 4 respiratory disease in the horse. *Clinical Techniques in Equine Practice*, **5**, 197 - 202.

HEBIA-FELLAH, I., LEAUTE, A., FIENI, F., ZIENTARA, S., IMBERT-MARCILLE, B.M., BESSE, B., FORTIER, G., PRONOST, S., MISZCZAK, F., FERRY, B., THORIN, C., PELLERIN, J.L. and BRUYAS, J.F. (2009) Evaluation of the presence of equine viral herpesvirus 1 (EHV-1) and equine viral herpesvirus 4 (EHV-4) DNA in stallion semen using polymerase chain reaction (PCR). *Theriogenology*, **71**, 1381 - 1389.

HEISTERMANN, M., PALME, R. and GANSWINDT, A. (2006) Comparison of different enzymeimmunoassays for assessment of adrenocortical activity in primates based on fecal analysis. *American Journal of Primatology*, **68**, 257 - 273.

HENNINGER, R.W., REED, S.M., SAVILLE, W.J., ALLEN, G.P., HASS, G.F., KOHN, C.W. and SOFALY, C. (2007) Outbreak of neurologic disease caused by equine herpesvirus-1 at a university equestrian centre. *Journal of Veterinary Internal Medicine*, **21**, 157 - 165.

IRVINE, C.H.G. and ALEXANDER, S.L. (1994) Factors affecting the circadian rhythm in plasma cortisol concentrations in the horse. *Domestic Animal Endocrinology*, **11**, 227 - 238.

KLEIN, D. (2002) Quantification using real-time PCR technology: applications and limitations. *Trends in molecular medicine*, **8**, 257 - 260.

KO, S., KANG, J., YEH, J., MOON, J., CHOI, G., WON, S. and CHAE, J. (2013) First report on molecular detection of equine upper respiratory infectious viruses in republic of Korea. *Journal of Equine Veterinary Science*, **33**, 628 - 636.

KYDD, J.H., TOWNSEND, H.G.G. and HANNANT, D. (2006) The equine immune response to equine herpesvirus-1: the virus and its vaccines. *Veterinary Immunology and Immunopathology*, **111**, 15 - 30.

LUNN, D.P., DAVIS-POYNTER, N., FLAMINIO, M.J.B.F., HOROHOV, D.W., OSTERRIEDER, K., PUSTERLA, N. and TOWNSEND, H.G.G. (2009) Equine herpesvirus-1 consensus statement. *Journal of Veterinary Internal Medicine*, **23**, 450 - 461.

MA, G., AZAB, W. and OSTERRIEDER, N. (2013) Equine herpesvirus type 1 (EHV-1) and 4 (EHV-4) – masters of co-evolution and a constant threat to equids and beyond. *Veterinary Microbiology*, **167**, 123 - 134.

MACKAY, I.M., ARDEN, K.E. and NITSCHE, A. (2002) Real-time PCR in virology. *Nucleic Acids Research*, **30**, 1292 - 1305.

MATSUMARA, T., SUGIURA, T., IMAGAWA, H., FUKUNAGA, Y. and KAMADA, M. (1992) Epizootiological aspects of type 1 and type 4 equine herpesvirus infections among horse populations. *Journal of Veterinary Medical Science*, **54**, 207 - 211.

MERL, S., SCHERZER, S., PALME, R. and MÖSTL, E. (2000) Pain causes increased concentrations of glucocorticoid metabolites in horse feces. *Journal of Equine Veterinary Science*, **20**, 586 - 590.

MORLEY, P.S., TOWNSEND, H.G.G., BOGDAN, J.R. and HAINES, D.M. (2000) Risk factors for disease associated with influenza virus infections during three epidemics in horses. *Journal of the American Veterinary Medical Association*, **216**, 545 - 550.

MÖSTL, E. and PALME, R. (2002) Hormones as indicators of stress. *Domestic Animal Endocrinology*, **23**, 67 - 74.

MUMFORD, J.A., ROSSDALE, P.D., JESSETT, D.M., GANN, S.J., OUSEY, J. and COOK, R.F. (1987) Serological and virological investigation of an equid herpesvirus 1 (EHV-1) abortion storm on a stud farm in 1985. *Journal of Reproduction and Fertility*, **35**, 509 - 518.

O'KEEFE, J.F., ALLEY, M.R., JONES, D. and WILKS, C.R. (1995) Neonatal mortality due to equid herpesvirus 4 (EHV-4) in a foal. *Australian Veterinary Journal*, **72**, 353 - 354.

OSTERRIEDER, N. and VAN DE WALLE, G.R. (2010) Pathogenic potential of equine alphaherpesviruses: the importance of the mononuclear cell compartment in disease outcome. *Veterinary Microbiology*, **143**, 21 - 28.

PALME, R., FISCHER, P., SCHILDORFER, H. and ISMAIL, M.N. (1996) Excretion of infused 14C-steroid hormones via faeces and urine in domestic livestock. *Animal Reproduction Science*, **43**, 43 - 63.

PALME, R. and MÖSTL, E. (1997) Measurement of cortisol metabolites in faeces of sheep as a parameter of cortisol concentration in blood. *International Journal of Mammalian Biology*, **62**, 192 - 197.

PATEL, J.R. and HELDENS, J. (2005) Equine herpesviruses 1 (EHV-1) and 4 (EHV-4) – epidemiology, disease and immunoprophylaxis: a brief review. *The Veterinary Journal*, **170**, 14 - 23.

PUSTERLA, N., HUSSEY, S.B., MAPES, S., JOHNSON, C., COLLIER, J.R., HILL, J., LUNN, D.P. and WILSON, W.D. (2010) Molecular investigation of the viral kinetics of equine herpesvirus-1 in blood and nasal secretions of horses after corticosteroid-induced recrudescence of latent infection. *Journal of Veterinary Internal Medicine*, **24**, 1153 - 1157.

PUSTERLA, N., HUSSEY, S.B., MAPES, S., LEUTENEGGER, C.M., MADIGAN, J.E., FERRARO, G.L., WILSON, W.D. and LUNN, D.P. (2009a) Comparison of four methods to quantify equid herpesvirus 1 load by real-time polymerase chain reaction in nasal secretions of experimentally and naturally infected horses. *Journal of Veterinary Diagnostic Investigation*, **21**, 836 - 840.

PUSTERLA, N., KASS, P.H., MAPES, S., JOHNSON, C., BARNETT, D.C., VAALA, W., GUTIERREZ, C., McDANIEL, R., WHITEHEAD, B. and MANNING, J. (2011) Surveillance programme for important equine infectious respiratory pathogens in the USA. *Veterinary Record*, **169**, 12.

PUSTERLA, N., LEUTENEGGER, C.M., WILSON, W.D., WATSON, J.L., FERRARO, G.L. and MADIGAN, J.E. (2005) Equine herpesvirus-4 kinetics in peripheral blood leukocytes and nasopharyngeal secretions in foals using quantitative real-time TaqMan PCR. *Journal of Veterinary Diagnostic Investigation*, **17**, 578 - 581.

PUSTERLA, N., MADIGAN, J.E. and LEUTENEGGER, C.M. (2006) Real-time polymerase chain reaction: a novel molecular diagnostic tool for equine infectious diseases. *Journal of Veterinary Internal Medicine*, **20**, 3 - 12.

PUSTERLA, N., MAPES, S., MADIGAN, J.E., MACLACHLAN, N.J., FERRARO, G.L., WATSON, J.L., SPIER, S.J. and WILSON, W.D. (2009b) Prevalence of EHV-1 in adult horses transported over long distances. *Veterinary Record*, **165**, 473 - 475.

PUSTERLA, N., MAPES, S., WADEMAN, C., WHITE, A. and HODZIC, E. (2013) Investigation of the role of lesser characterised respiratory viruses associated with upper respiratory tract infections in horses. *Veterinary Record*, **172**, 315 - 317.

PUSTERLA, N., WILSON, W.D., MADIGAN, J.E. and FERRARO, G.L. (2009c) Equine herpesvirus-1 myeloencephalopathy: a review of recent developments. *The Veterinary Journal*, **180**, 279 - 289.

R CORE TEAM (2012) R: A language and environment for statistical computing. [http://www.R-project.org/]. Vienna: R Foundation for Statistical Computing.

REBHUN, W.C., JENKINS, D.H., RIIS, R.C., DILL, S.G., DUBOVI, E.J. and TORRES, A. (1988) An epizootic of blindness and encephalitis associated with a herpesvirus indistinguishable from equine herpesvirus 1 in a herd of alpacas and llamas. *Journal of the American Veterinary Medical Association*, **192**, 953 - 956.

SABINE, M. (1980) Differentiation of equine herpesviruses of type 1. *Australian Veterinary Journal*, **56**, 44 - 45.

SCHATZ, S. and PALME, R. (2001) Measurement of faecal cortisol metabolites in cats and dogs: a non-invasive method for evaluating adrenocortical function. *Veterinary Research Communications*, **25**, 271 - 287.

SCHMIDT, A., BIAU, S., MÖSTL, E., BECKER-BIRCK, M., MORILLON, B., AURICH, J., FAURE, J.-M. and AURICH, C. (2010a) Changes in cortisol release and heart rate variability in sport horses during long-distance road transport. *Domestic Animal Endocrinology*, **38**, 179 - 189.

SCHMIDT, A., MÖSTL, E., WEHNERT, C., AURICH, J., MÜLLER, J. and AURICH, C. (2010b) Cortisol release and heart rate variability in horses during road transport. *Hormones and Behaviour*, **57**, 209 - 215.

SCHULMAN, M., BECKER, A., GANSWINDT, S., GUTHRIE, A., STOUT, T. and GANSWINDT, A. (2014) The effect of consignment to broodmare sales on physiological stress measured by faecal glucocorticoid metabolites in pregnant Thoroughbred mares. *BMC Veterinary Research*, **10**, 25.

SLATER, J. (2007) Equine herpesviruses. In: *Equine infectious diseases*, 1st edn., Eds: D.C. Sellon and M.T. Long, Saunders Elsevier, St Louis. pp 134 - 153.

SLATER, J.D., BORCHERS, K., THACKRAY, A.M. and FIELD, H.J. (1994) The trigeminal ganglion is a location for equine herpesvirus 1 latency and reactivation in the horse. *Journal of General Virology*, **75**, 2007 - 2016.

SONIS, J.M. and GOEHRING, L.S. (2013) Nasal shedding of equid herpesvirus type 1 and type 4 in hospitalized, febrile horses. *Journal of Equine Veterinary Science*, **33**, 756 - 759.

STUDDERT, M.J., SIMPSON, T. and ROIZMAN, B. (1981) Differentiation of respiratory and abortigenic isolates of equine herpesvirus 1 by restriction endonucleases. *Science*, **214**, 262 - 264.

THE THOROUGHBRED BREEDERS' ASSOCIATION OF SOUTH AFRICA. Website, accessed 10 May 2014 [http://www.tba.co.za/bsa/price.php?url=20132yo].

TOUMA, C. and PALME, R. (2005) Measuring fecal glucocorticoid metabolites in mammals and birds: the importance of validation. *Annals of the New York Academy of Science*, **1046**, 54 - 74.

VALASEK, M.A. and REPA, J.J. (2005) The power of real-time PCR. *Advances in Physiology Education*, **29**, 151 - 159.

VANDEKERCKHOVE, A.P., GLORIEUX, S., GRYSPEERDT, A.C., STEUKERS, L., VAN DOORSSELAERE, J., OSTERRIEDER, N., VAN DE WALLE, G.R. and NAUWYNCK, H.J. (2011) Equine alphaherpesviruses (EHV-1 and EHV-4) differ in their efficiency to infect

mononuclear cells during early steps of infection in nasal mucosal explants. *Veterinary Microbiology*, **152**, 21 - 28.

VAN MAANEN, C. (2002) Equine herpesvirus 1 and 4: an update. *Veterinary Quarterly*, **24**, 57 - 78.

WALTER, J., BALZER, H.J., SEEH, C., FEY, K., BLEUL, U. and OSTERRIEDER, N. (2012) Venereal shedding of equid herpesvirus-1 (EHV-1) in naturally infected stallions. *Journal of Veterinary Internal Medicine*, **26**, 1500 - 1504.

WALTER, J., SEEH, C., FEY, K., BLEUL, U., OSTERRIEDER, N. (2013) Clinical observations and management of a severe equine herpesvirus type 1 outbreak with abortion and encephalomyelitis. *Acta Veterinaria Scandinavica*, **55**, 19.

WANG, L., RAIDAL, S.L., PIZZIRANI, A. and WILCOX, G.E. (2007) Detection of respiratory herpesviruses in foals and adult horses determined by nested multiplex PCR. *Veterinary Microbiology*, **121**, 18 - 28.

WELCH, H.M., BRIDGES, C.G., LYON, A.M., GRIFFITHS, L. and EDINGTON, N. (1992) Latent equid herpesviruses 1 and 4: detection and distinction using the polymerase chain reaction and co-cultivation from lymphoid tissues. *Journal of General Virology*, **73**, 261 - 268.

WOHLSEIN, P., LEHMBECKER, A., SPITZBARTH, I., ALGERMISSEN, D., BAUMGARTNER, W., BOER, M., KUMMROW, M., HAAS, L. and GRUMMER, B. (2011) Fatal epizootic equine herpesvirus 1 infections in new and unnatural hosts. *Veterinary Microbiology*, **149**, 456 - 460.

WORLD ORGANISATION FOR ANIMAL HEALTH. Website, accessed 11 April 2014 [http://www.oie.int/wahis_2/public/wahid.php/Countryinformation/Animalsituation].

WORLD WEATHER ONLINE. Website, accessed 21 February 2014 [www.worldweatheronline.com/].

XU, R. (2003) Measuring explained variation in linear mixed effects models. *Statistics in Medicine*, **22**, 3527 - 3541.

APPENDIX 1

Standard operating procedure: Multiplex qPCR for EHV-1 and -4

A. Sample preparation

Agitate nasal swabs for 5 s in 0.5 ml of 0.1 M phosphate buffered saline (pH 7.4) in a 1.5 ml Eppendorf tube.

B. Nucleic acid extraction using the MagMax™ Pathogen DNA/RNA kit (Life Technologies) and Kingfisher 96 Magnetic Particle Processor (Thermo Fisher Scientific Inc.)

Plate 1- Sample plate: add 100 µl sample to each well. Add 20 µl of beads mix (10 µl RNA binding beads, 10 µl Lysis Binding Enhancer) to sample, centrifuge for short period, then shake plate for 1 min at max setting on an IKA TTS3D shaker (Merck). Add 400 µl of Lysis Solution Mix (200 µl Lysis Binding Solution, 200 µl Isopropanol) to each sample.

Plates 2 and 3- Wash Solution 1 (300 µl/well).

Plates 4 and 5- Wash Solution 2 (450 µl/well).

Plate 6- Elution Buffer (90 µl/well).

Add two positive and one negative control samples to the sample plate with Lysis Solution.

C. Duplex real-time PCR preparation

Master Mix (17 µl/sample):

Kapa Probe Fast ABI Prism® 2X PCR Master Mix (Kapa Biosystems)	10 µl
EHV-1 primer/probe mix	1 µl
EHV-4 primer/probe mix	1 µl
Nuclease-free water	5 µl

D. Plate setup

Use a 0.1 ml PCR plate:

Add 17 µl of the Master Mix per sample.

Add 3 µl of each extracted sample to the wells individually.

E. Duplex real-time PCR

Place the plate on the real-time PCR machine and close the machine.

Select the 'Advance setup' option.

Create run name.

Select 'Fast run 40 min' (step 1 - 95ºC for 20 sec; step 2 - 95ºC for 1 sec, 60ºC for 20 sec, step 2 repeated for 40 cycles).

Click on 'Run method' and change volume to 20 µl.

Click on 'Plate setup'.

Import sample sheet by clicking on file, then import, then browse and then select file.

Select targets: TaqMan Probe EHV-1 and EHV-4.

Click on 'Assign targets and samples'.

Add targets to each sample.

Save run.

Ensure machine is closed and that samples are in correct rows.

Select 'Start run'.

Run lasts approximately 45 min.

APPENDIX 2

Standard operating procedure: EHV-1 and EHV-4 ELISA

- Get fresh aliquot of antigens from -70ºC. These last one to two weeks at 4ºC. Briefly spin down antigen in Eppendorf tube and coat Nunc MaxiSorp® flat-bottom 96 well plates with 100 µl of GST*, Peg1var* and Peg4var* antigens at 0.25 µg/ml in coating buffer (tap plates to ensure even coverage) overnight at 4ºC. Several plates can be coated that will last for at least one week or more at 4ºC. Make up in Macartney bottles ~ 4 ml per plate (4 ml coating buffer, 2 µl antigen). Wrap in cling wrap and put in fridge overnight. * - *Dilute these antigens in coating buffer, not water.*

  Coating buffer (bicarbonate / carbonate buffer):

  - 0.1 M Na_2CO_3 (store at 4ºC to prevent growth)
  - 0.1 M $NaHCO_3$ (store at 4ºC to prevent growth)
  - Mix 16 ml and 34 ml of each, respectively (total 50 ml) and check that the pH = 9.6. Can make ~ 200 ml coating buffer at a time and store at 4 ºC. Discard once growth is detected.

- Aspirate antigens with plate washer. Wash 2x with PBST and dry plates. Block unoccupied sites with Block 100 µl BSA_{10}PBS + 5% normal sheep serum for 2 hours at 37ºC (CO_2 incubator).

  - **PBST** (1x) – 0.05% Tween 20 (Sigma) in PBS

- **10x PBS** – 1 Litre
  - NaCl 80 g
  - KCl 2 g
  - Na_2HPO_4 11.5 g
  - KH_2PO_4 2 g
  - in dH_2O
- for 10x PBST + 5 ml Tween 20

BSA_{10}PBS + 5% normal sheep serum (Block):

- 10 mg/ml BSA Fraction V (Boehringer)
- 5% normal sheep serum
- in 1x PBS

- Aspirate block and wash plates 2x with PBST and dry plates.
- Spin down and then dilute primary antibody 1/100 in BSA_5PBST + 2.5% normal sheep serum and add 100 µl to each well (tap plates). Place plates on shaker for 2 hours at room temperature.

BSA_5PBST + 2.5% normal sheep serum (diluent):

- 5 mg/ml BSA Fraction V
- 2.5% normal sheep serum
- 0.05% Tween 20
- in PBS

- Aspirate antibody and wash plates 4x with PBST and dry plates.
- Add 100 µl of HRPO conjugated Goat anti Horse IgG (KPL – Kirkegaard and Perry Laboratories) diluted 1/1000 in BSA_5PBST + 2.5% normal sheep serum to each well (tap plates). Place on shaker for 45 minutes to 1 hour.

- Aspirate conjugate and wash plates 4x with PBST and dry plates.
- Add 100 µl of TMB substrate to each well.

TMB substrate – per 10 ml

- 1 TMB tablet (Sigma)
- 10 ml developing buffer pH = 5.0
- 2 µl 30% H_2O_2
- Add TMB tablet before aspirating conjugate and it will be dissolved by the time you have finished.
- Add H_2O_2 immediately before use.

Developing buffer – (0.1 M phosphate-citrate buffer stock)

- 0.2 M dibasic sodium phosphate
- 0.1 M citric acid
- Mix 25.7 ml and 24.3 ml (50 ml) of each, respectively, and check pH = 5.0
- **Working concentration use at 0.05 M:**
- Add 50 ml ddH_2O (total 100 ml)

- Develop plates for 6 minutes. Add 50 µl of 1 M HCl to each well to stop the reaction and read the plates at 450 nm.

APPENDIX 3

Standard operating procedure: Faecal steroid extraction

1) Receiving Samples:

i) Please check for the following:

- faecal samples have to be frozen (if samples are received dry, continue with step 3).

ii) Store faecal samples at -20°C until freeze-drying.

2) Freeze-Drying:

i) Ensure that samples are thoroughly frozen prior to lyophilisation.

ii) Check freeze dryer for working condition.

iii) Start pump and freezer.

iv) Open containers, remove caps/stoppers, place containers on the trays.

v) Close the freeze-dryer (dry samples according to manufacturer's instructions).

vi) Clean and dry caps/stoppers.

vii) Check for pressure and temperature regularly, enter into log-file provided.

viii) Remove samples from freeze-dryer after 48 or 72 hours (depending on number and volume of samples), check samples for dryness and close containers.

ix) Store dry faecal samples at room temperature.

3) Pulverisation:

You will need: 80% ethanol in a spray bottle, paper to place underneath, sieves, tweezers (maybe pestle and mortar), waste bags, paper towel roll, and the dry faecal samples.

Please wear laboratory coat, gloves, and surgical mask.

i) Clean all surfaces with 80% ethanol prior to pulverisation and between the samples, and wipe dry.

ii) Clean sieve and tweezers (and pestle and mortar if needed) prior to pulverisation and between the samples with 80% ethanol, and wipe dry.

iii) Work on a piece of paper, empty contents of the container into the sieve (or mortar, if samples are very hard/compact) placed on the paper.

iv) Work faecal powder through the sieve with tweezers by moving sample around in the sieve, or by crushing it with the pestle, if samples are very hard/compact.

v) Fill faecal powder back into the container, remove coarse material that remained in the sieve into the prepared waste bag, and discard paper.

vi) Clean and dry the surface and tools before starting with the next sample.

vii) Take a new piece of paper, and continue with pulverising the next sample.

viii) Continue for all samples.

ix) Store faecal powder in labelled box at room temperature.

x) Have the waste incinerated.

4) Weighing:

You will need scales (at least accurate to the mg), spatula, 80% ethanol, tissue paper, rack with properly labelled tubes (5 ml) and lids for all samples, sample list containing an empty column for writing down the sample weight, pen.

i) Clean surfaces, scales, spatula prior to weighing and clean spatula between samples.

ii) Put empty labelled tube (cap removed) on the scale, set weight to zero.

iii) Weigh 0.100 g to 0.110 g of faecal powder of the respective sample (unless stated otherwise, e.g. for projects with small sample amounts only 0.05 g - 0.060 g are used)

iv) Write down the exact weight of faecal powder in the sample list.

v) Close tube and put the tube back into the sample rack.

vi) Clean spatula with 80% ethanol, wipe dry and continue with the next tube and sample.

ix) Have the waste incinerated.

x) Enter sample weights into prepared Excel sample list.

5) Steroid Extraction:

You will need a multitube vortex (alternatively an over-head shaker), a centrifuge with a respective rotor fitting for the used tubes, lab timer, multi-stepper pipette, 50 ml syringe, 80% ethanol (freshly prepared), properly labelled micro centrifuge tubes (3ml) for all extracts

i) Remove caps from the tubes and put them in order to ensure individual caps will be replaced on the correct tubes.

ii) Add 3 ml 80% ethanol (unless stated otherwise) per sample, preferably using a multistepper pipette.

iii) Vortex on a multitube-vortex for 15 min or alternatively shake over-head for 15 min; please ensure that all of the faecal powder is in suspension before starting.

iv) Centrifuge the tubes for 10 min at 1500 x G.

v) Double-check matching labels on tubes and micro-centrifuge-tubes, and decant supernatant into the respective micro-centrifuge-tubes, taking care not to stir up the pellet; alternatively use pipette to transfer the supernatant.

vi) Discard the remaining excess supernatant unless discussed otherwise.

vii) Let the pellets dry in the tubes, and discard afterwards unless discussed otherwise (e.g. if needed for determination of organic weight; see separate protocol).

viii) Store steroid extracts in labelled box at -20°C until used for EIA analysis.

Standard operating procedure: EIA

1) EIA day 1:

i) Defrost, sort and dilute the faecal extracts according to the assay sample list.

ii) Defrost respective standard, quality controls (QCs), antibody, steroid label, and coated microtiter plate.

iii) Prepare standard curve serial dilutions with assay buffer according to the protocol sheet.

iv) Add assay buffer to antibody and labelled steroid according to the protocol sheet.

v) Wash the coated plate 4 times with 300 µl washing solution per well in the washer, pat dry. Label plate on the side using permanent marker (assay type, project name, date).

vi) Pipette 100 µl assay buffer into wells A1 + A2. Pipette 50 µl assay buffer, standard, QCs and diluted extracts into the respective wells.

vii) Pipette 50 µl labelled steroid into every well using a multipette.

viii) Pipette 50 µl antibody into every well (**EXCEPT** into the BLANK A1 + A2) using a multipette.

ix) Cover the plate with cling wrap, mix contents carefully, and place the plate into fridge overnight.

2) EIA day 2:

i) Defrost streptavidin-POD aliquot (20 µl), add to 16 ml of cool assay buffer, rinse tube 2x.

ii) Discard the contents of the plate into the BSA waste, wash the plate 4 times with 300 µl cool washing solution per well, pat dry.

iii) Pipette 150 µl streptavidin-POD solution into every well using a multipette.

iv) Incubate the plate in the fridge on the plate shaker for 45 min.

v) Discard the contents of the plate into the BSA waste, wash the plate 4 times with 300 µl cool washing solution per well, pat dry.

vi) Mix 250 µl TMB with 17 ml cool substrate using solution directly before use.

vii) Pipette 150 µl substrate solution into every well using a multipette.

viii) Incubate plate in the fridge on plate shaker for 20-90 min (ideally 30-60 min), until OD of the zero wells is about 1.0 (check for colour change after 5 min for the first time).

ix) Start computer and printer, open respective protocol in the Gen5 software, start the reader only briefly before use.

x) Stop the enzyme reaction by carefully adding 50 µl H_2SO_4 (2 M) per well using a multipette.

xi) In Gen5, enter the plate information into the experiment window, carefully place the plate onto the reader carrier and read the plate.

xii) Take the plate out of the reader and let it dry at RT on lab bench.

xiii) Print out the results, export results into excel file, transfer the excel file data into pre-prepared file on your computer and analyse the results (see below).

3) Analysis of results:

i) Determine linear range for the plate on the print-out.

ii) Determine duplicate outliers and linear-range-outliers for re-measurement.

iii) Transfer results to be used to the prepared Excel results table and calculate concentrations.

iv) Transfer results of the QCs together with the added information into the quality control list.

v) Mark the samples for re-measurement in the results table and determine new dilution factors for the linear-range-outliers

APPENDIX 4

Farm owner questionnaire

- **Name of farm of origin:**

- **Location of farm of origin** (town; district; province; GPS coordinates)**:**

- **Resident horse population on farm of origin:**

Total no. of horses on farm:		Stallions:	
Mares:		Colts (1 – 3 years):	
Fillies (1 - 3 years):		Colts (< 1 year)	
Fillies (< 1 year):		Geldings:	

- **Quarantine procedures on farm of origin:**

Open or closed herd?	Open	Closed
Were any new horses introduced to the farm during the past 3 months?	Yes	No
If yes, please describe any isolation or quarantine procedures followed on farm for new arrivals:		

- **Departure of consigned horses from farm of origin:**

Date (DD/MM/YY):	……../……../……..	Time:	

- **Arrival of consigned horses at sales complex:**

Date (DD/MM/YY):	……../……../……..	Time:	

- **Stop-overs en-route to sales complex:**

Date (DD/MM/YY):	……../……../……..	Location:	
Date (DD/MM/YY):	……../……../……..	Location:	

- **Number of horses in farm's draft consigned to sale:**

Colts:		Fillies:	

- **Sales preparation:**

Were horses kept in groups during preparation?	Yes	No
If yes, number horses per group:		
Were horses housed/ stabled during preparation?	Yes	No
If yes, number days housed pre-travel:		
Roughage type fed pre-sale:		
Concentrate type fed pre-sale:		
Were sales horses dewormed pre-sale:	Yes	No
If yes, number days dewormed pre-travel:		

APPENDIX 5

EHV-1 and -4 ELISA results for all arrival and departure serum samples.

Antibody positive Indeterminate Antibody negative * EHV-4 DNA detected

Farm	Province of origin	Horse identity	Sex	Arrival date	Departure date	Absorbance values			
						EHV-1		EHV-4	
						Arrival	Departure	Arrival	Departure
1	Western Cape	1-A	F	09.08.13	15.08.13	-0.00135	0.0003	1.8279	1.02955
1	Western Cape	1-B	C	09.08.13	15.08.13	0.0011	-0.0049	0.7503	0.70095
1	Western Cape	1-C	C	09.08.13	15.08.13	0.0019	-0.0076	1.30635	0.8938
1	Western Cape	1-D	F	09.08.13	15.08.13	-0.0028	0.00505	1.71815	1.07865
1	Western Cape	1-E*	C	09.08.13	15.08.13	0.00255	-0.00285	1.1248	0.8266
1	Western Cape	1-F	F	09.08.13	15.08.13	-0.0009	0.0034	0.42895	0.41345
1	Western Cape	1-G	C	09.08.13	15.08.13	-0.0017	-0.001	0.9365	0.75195
1	Western Cape	1-H	F	09.08.13	16.08.13	-0.00335	-0.00125	1.98445	1.6016
1	Western Cape	1-I	C	09.08.13	16.08.13	-0.00295	0.00405	1.3284	1.1236
1	Western Cape	1-J	F	09.08.13	16.08.13	0.0034	0.00265	0.21135	0.18705
1	Western Cape	1-K	C	09.08.13	16.08.13	0.00035	-0.00145	1.41525	1.13935
1	Western Cape	1-L	C	09.08.13	16.08.13	-0.00465	-0.0025	1.29785	1.23775
1	Western Cape	1-M	F	09.08.13	15.08.13	-0.0025	-0.00285	1.2542	0.78285
2	Western Cape	2-A	C	09.08.13	15.08.13	0.0005	-0.00165	1.7011	2.0487
2	Western Cape	2-B	C	09.08.13	15.08.13	-0.00365	-0.00055	1.2457	2.21405
2	Western Cape	2-C	C	09.08.13	15.08.13	0.0027	0.00625	0.7516	0.7467
2	Western Cape	2-D	C	09.08.13	15.08.13	-0.00225	-5E-05	0.72985	0.89655
2	Western Cape	2-E	C	09.08.13	16.08.13	-0.0027	0.00265	1.1863	0.88865
2	Western Cape	2-F	C	09.08.13	16.08.13	-0.03605	-0.011	1.03105	0.5496
2	Western Cape	2-G*	C	09.08.13	16.08.13	0.0008	-0.0009	0.52745	0.5005
2	Western Cape	2-H*	C	09.08.13	16.08.13	0.0043	0.0138	0.299	0.2645
2	Western Cape	2-I*	C	09.08.13	16.08.13	-0.003	-0.01485	0.7629	1.1064
3	Western Cape	3-A*	C	08.08.13	15.08.13	-0.0016	-0.0554	0.04075	0.0094
3	Western Cape	3-B	C	08.08.13	15.08.13	-0.00515	0.00985	0.75375	0.86995
3	Western Cape	3-C	C	08.08.13	15.08.13	0.0016	-0.00555	0.3533	0.44155
3	Western Cape	3-D*	F	08.08.13	16.08.13	-0.00135	-0.0024	1.11205	1.1826
3	Western Cape	3-E	F	08.08.13	16.08.13	0.01025	0.0099	0.6902	0.8215
3	Western Cape	3-F	F	08.08.13	16.08.13	-0.00395	0.00075	1.4111	1.3382
3	Western Cape	3-G	F	08.08.13	16.08.13	-0.00255	0.001	1.2633	1.7893
3	Western Cape	3-H	C	08.08.13	16.08.13	-0.0004	-0.00155	0.5031	0.7284
4	Eastern Cape	4-A	F	10.08.13	15.08.13	-0.0023	-0.0042	0.67165	0.966
4	Eastern Cape	4-B*	C	10.08.13	15.08.13	0.0096	0.00265	1.26555	1.5367
4	Eastern Cape	4-C	F	10.08.13	15.08.13	0.00495	0.005	0.98855	1.2896
4	Eastern Cape	4-D	F	10.08.13	15.08.13	-0.00695	0.008	0.58995	0.94775
4	Eastern Cape	4-E*	F	10.08.13	15.08.13	-0.00235	0.003	0.79535	0.88885
4	Eastern Cape	4-F	C	10.08.13	15.08.13	-0.0003	0.00255	0.7154	0.6478
4	Eastern Cape	4-G	F	10.08.13	15.08.13	-0.0073	-0.00365	1.14325	1.32945
4	Eastern Cape	4-H	C	10.08.13	16.08.13	0.0074	0.01435	1.23435	1.44015
4	Eastern Cape	4-I	C	10.08.13	16.08.13	-0.0009	0.0016	1.50175	1.89595
4	Eastern Cape	4-J*	F	10.08.13	15.08.13	0.0074	0.0002	1.37735	1.5319
4	Eastern Cape	4-K	F	10.08.13	15.08.13	-0.0373	-0.05085	0.4782	0.5157
4	Eastern Cape	4-L	F	10.08.13	16.08.13	0.01695	0.02705	0.37125	0.57245
4	Eastern Cape	4-M	C	10.08.13	16.08.13	No sample	0.0007	No sample	0.0366
5	Eastern Cape	5-A	C	10.08.13	15.08.13	0.00225	-0.0039	0.7548	1.10265
5	Eastern Cape	5-B*	F	10.08.13	15.08.13	0.0005	-0.00495	0.52555	0.2906
5	Eastern Cape	5-C	C	10.08.13	15.08.13	-0.00235	0.0005	0.0063	0.0017
5	Eastern Cape	5-D*	F	10.08.13	15.08.13	0.00295	-0.0037	0.8521	1.04245
5	Eastern Cape	5-E	C	10.08.13	16.08.13	0.00065	-0.00145	1.01375	1.189

6	KwaZulu-Natal	6-A	F	11.08.13	15.08.13	-0.00125	-0.0038	0.2773	0.254
6	KwaZulu-Natal	6-B	F	11.08.13	15.08.13	-0.0245	-0.0148	0.47075	0.27995
6	KwaZulu-Natal	6-C	F	11.08.13	15.08.13	0.06865	0.0729	0.39975	0.2959
6	KwaZulu-Natal	6-D	F	11.08.13	15.08.13	0.0057	0.0104	1.7498	1.39365
6	KwaZulu-Natal	6-E	C	11.08.13	15.08.13	0.17725	0.122	1.68245	1.0964
6	KwaZulu-Natal	6-F	C	11.08.13	16.08.13	0.0043	-0.00045	1.43985	0.6862
6	KwaZulu-Natal	6-G	F	11.08.13	16.08.13	-0.0077	-0.00055	1.0053	0.66035
6	KwaZulu-Natal	6-H	C	11.08.13	15.08.13	-0.00475	0.00075	2.6856	1.91745
6	KwaZulu-Natal	6-I*	F	11.08.13	15.08.13	-0.00135	0.0031	0.8036	0.8254
6	KwaZulu-Natal	6-J	F	11.08.13	16.08.13	0	0.0013	0.68705	0.6609
7	KwaZulu-Natal	7-A	C	09.08.13	15.08.13	-0.02355	-0.01625	0.25215	0.2226
7	KwaZulu-Natal	7-B	F	09.08.13	15.08.13	0.00385	0.01465	0.38735	0.42075
7	KwaZulu-Natal	7-C	C	09.08.13	15.08.13	-0.0059	0.00095	0.62505	0.45645
7	KwaZulu-Natal	7-D	F	09.08.13	15.08.13	0.0024	0.00015	0.9282	0.5448
7	KwaZulu-Natal	7-E	C	09.08.13	15.08.13	-0.0048	0.0009	1.43745	1.27465
7	KwaZulu-Natal	7-F	C	09.08.13	15.08.13	-0.00315	-0.01415	0.22725	0.17
7	KwaZulu-Natal	7-G	C	09.08.13	15.08.13	0.00575	0.0105	0.4097	0.4872
7	KwaZulu-Natal	7-H	F	09.08.13	15.08.13	0.0007	0.0029	0.9117	0.8227
7	KwaZulu-Natal	7-I	C	09.08.13	15.08.13	0.0035	0.0064	0.1768	0.41665
7	KwaZulu-Natal	7-J	F	09.08.13	15.08.13	-0.0034	-0.00375	0.9477	0.5937
7	KwaZulu-Natal	7-K*	C	09.08.13	15.08.13	-0.001	-0.0019	0.1552	1.0839
7	KwaZulu-Natal	7-L	C	09.08.13	15.08.13	0.00325	-0.0082	0.40855	0.2924
7	KwaZulu-Natal	7-M	C	09.08.13	15.08.13	0.00225	0.00355	0.3587	0.4754
7	KwaZulu-Natal	7-N	C	09.08.13	15.08.13	-0.00515	No sample	0.55485	No sample
7	KwaZulu-Natal	7-O	F	09.08.13	15.08.13	-0.00035	0.00075	0.59535	0.34735
7	KwaZulu-Natal	7-P	C	09.08.13	15.08.13	0.00575	No sample	0.05655	No sample
7	KwaZulu-Natal	7-Q	F	09.08.13	15.08.13	-0.00225	-0.10385	1.0942	0.71415
7	KwaZulu-Natal	7-R	F	09.08.13	16.08.13	-0.00695	-0.0035	1.3609	1.14765
7	KwaZulu-Natal	7-S	C	09.08.13	16.08.13	-0.0003	-0.00435	0.1343	0.19165
7	KwaZulu-Natal	7-T	F	09.08.13	16.08.13	0.0035	0.0076	0.2698	0.3846
7	KwaZulu-Natal	7-U	C	09.08.13	16.08.13	-0.00495	-0.0402	0.34635	0.2616
7	KwaZulu-Natal	7-V	C	09.08.13	16.08.13	0.00685	0.0018	1.1411	0.7208
7	KwaZulu-Natal	7-W	C	09.08.13	16.08.13	0.0063	0.0012	1.87615	1.82475
7	KwaZulu-Natal	7-X	C	09.08.13	16.08.13	0.00555	0.003	1.93785	1.7489
7	KwaZulu-Natal	7-Y	F	09.08.13	16.08.13	-0.0021	-0.00925	0.6567	0.35035
7	KwaZulu-Natal	7-Z	F	09.08.13	16.08.13	0.0007	-0.00075	0.61245	0.81095
8	KwaZulu-Natal	8-A	F	13.08.13	15.08.13	-0.0025	0.00705	1.8191	0.3782
8	KwaZulu-Natal	8-B	C	13.08.13	15.08.13	-0.00225	-0.00765	1.05995	0.50785
8	KwaZulu-Natal	8-C	C	13.08.13	15.08.13	0.30305	0.0019	1.05465	0.0255
8	KwaZulu-Natal	8-D	C	13.08.13	16.08.13	-0.0019	-0.0102	1.933	1.37295
8	KwaZulu-Natal	8-E	F	13.08.13	16.08.13	-0.0027	-0.0068	2.1333	1.74525
8	KwaZulu-Natal	8-F	C	13.08.13	15.08.13	0.00145	-0.00225	2.61425	1.3233