THE IMPACT OF HERPESVIRUSES

ON

REPRODUCTIVE PERFORMANCE IN HORSES

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The impact of herpesviruses on reproductive performance in horses

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THE IMPACT OF HERPESVIRUSES ON REPRODUCTIVE PERFORMANCE IN HORSES

De invloed van herpesvirussen op
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CHAPTER 1
General Introduction

1.1 The impact of herpesviruses on reproductive performance in horses

Over millions of years, the equid herpesviruses (EHVs) have co-evolved with their hosts to the point where they are now ubiquitous in domestic and wild equid populations, but still responsible for frequent and serious disease episodes. The successful adaptation and high prevalence of these major pathogens poses a significant challenge to the health and welfare of horse populations worldwide, and consequently has considerable management and economic implications for the horse industry. Although there are currently nine EHVs described, two of the alphaherpesviruses, namely equid herpesvirus 1 (EHV-1) and equid herpesvirus 4 (EHV-4), are clinically, economically and epidemiologically by far the most important pathogens. These two viruses are associated with a range of disease manifestations of varying severity that, despite increasingly widespread vaccination, affect horses of all ages and breeds. The impact of EHV-1 and -4 includes the genetic and economic losses associated with abortion and fatal neonatal disease, the loss of days in work or training as a result of respiratory infections, and the potentially dramatic effects on health and welfare of the increasingly prevalent neurological form of disease. The economic effects associated with veterinary treatment, vaccination and preventative disease control strategies are considerable. The EHVs also exert a significant, albeit indirect, impact on the international horse industry as a consequence of the wastage associated with poor athletic performance or interference with local and international movement of horses for breeding and competition purposes.

Classically, both EHV-1 and -4 were regarded as primary respiratory pathogens; arguably, however, their most serious consequences are late term abortion, neonatal death and equine herpesvirus myeloencephalopathy (EHM), particularly when these manifest as epizootics. These significant sequelae occur more often following EHV-1 infection because it is more commonly associated with cell-associated viraemia and has a greater ability to infect a range of cell types, including the tissues of the uterus and nervous system. Equid herpesvirus 4 much less frequently results in significant viraemia or infects cells distant to the respiratory system and consequently EHV-4 abortions and neurological disease are both much less common and less likely to result in epizootic spread. One of the undoubted keys to the success of both EHV-1 and -4 in terms of their maintenance within a population are that, after the acute phase, they establish prolonged, possibly life-long, latent infections in asymptomatic horses which act, *via* periodic reactivation, as reservoirs of infection for new, susceptible horses. Given the key role of latency in the epidemiology of EHV-1 and -4 associated disease, additional data describing host and environmental risk factors that predispose to recrudescence, and in particular the

often postulated association with physiological stress, would be expected to contribute greatly to better understanding of the diseases and development of management strategies to mitigate the impact and risks of these important viral pathogens.

1.2 Personal perspective and research goals

From a personal perspective this thesis reflects my involvement in providing a clinical service at the University of Pretoria's Veterinary Faculty and in an advisory role to the South African Thoroughbred breeding industry. From these positions, I have been exposed to the challenges and practical realities of managing EHV-1 abortion epizootics and become increasingly convinced of the need to develop better strategies to enhance prevention or mitigate the severity of disease outbreaks in South African commercial horse breeding systems. The abortion epizootics have occurred in populations as diverse and geographically separated as Thoroughbred farms in northern California, the Western Cape and KwaZulu-Natal Provinces of South Africa, to Nooitgedacht ponies in Onderstepoort, Gauteng Province, South Africa.

The initial goal of this research endeavour was to develop a better understanding of the incidence and causes of pregnancy losses within the South African horse industry and to determine the contribution of pregnancy loss to overall reproductive performance. This was hitherto largely unreported in a South African context, with the exception of earlier studies by Van Niekerk and co-workers [1; 2] and Gilbert and Marlow [3] that looked primarily at endocrinological aspects of early pregnancy loss and, in particular, the possible relevance of stress.

Despite increased understanding and technological developments contributing to the marked improvements in pregnancy rates over recent decades, pregnancy attrition rates have remained relatively unchanged during the corresponding interval. Reportedly, approximately one fifth of all diagnosed pregnancies are lost. This pregnancy attrition, further categorised into early embryonic death and post-implantation abortion due to either infectious or non-infectious aetiologies, represents the most significant source of reproductive wastage. Bacteria are the most-frequently implicated infectious agents, however viruses, of which EHV-1 is indisputably the most important pathogen are an important contributor to late abortion in mares [4-6].

The focus of the current project shifted towards infectious abortion in response to requests for assistance in the management of significant herpesvirus abortion epizootics. It became

clear that there was a pressing need to better understand the epidemiology of herpesvirus abortion and provide more informed and locally-relevant prevention and intervention advice and strategies. It was also important for the various stakeholders to examine and quantify longer-term influences on reproductive outcomes and performance.

The sequence of the component chapters of this thesis parallels the steps taken in shifting from an initial focus on the impact of pregnancy loss on reproductive performance, to a narrower focus on the impact of infectious abortion, and in particular, herpesvirus-associated abortion and then moving onto the factors related to the recrudescence of herpesvirus infection as the likely starting point for an abortion epizootic. Each chapter was shaped and informed by the preceding one as successive research questions arose. The final chapter of the thesis synthesises the lessons learnt along the way, in an attempt to provide an informed approach to interventions that aim to reduce the impact of EHV on reproductive performance.

1.3 Scope of the thesis

That EHV-1 and -4 have a significant impact on the health and welfare of horses, and on trade and economics within the international horse industry is undisputed. Currently, EHV research efforts are biased towards untangling the complex immunological interactions between virus and host to facilitate vaccine development. In addition, the emergence of epizootic neurological disease as a growing threat has increasingly occupied researchers and animal health regulatory bodies. Recently, the introduction of molecular techniques has enhanced diagnostic speed and sensitivity and has helped to better define the prevalence and pathogenesis of these viruses. Despite general recognition of the association of these viruses with abortion and neonatal disease, their influence on reproductive performance in horse-breeding systems internationally remains largely undefined.

This thesis addresses the EHVs from the perspective of significant pathogens that affect the reproductive performance of horses. The primary aim was to define the effects of abortion epizootics on outcomes that determine reproductive performance in broodmares. A secondary aim was to examine an association that is frequently implicated in abortion episodes, namely the role of physiological stress in recrudescence of latent infection.

The populations included in this series of retrospective and prospective studies were all South African Thoroughbreds. The data were thus generally appropriate for comparison with those obtained from demographically-similar Thoroughbred breeding populations worldwide.

Chapter 2 is a review of selected literature reporting on the most important EHVs, namely EHV-1 and -4 from a veterinary and, in particular, a reproductive disease perspective. The review investigates how these ubiquitous viruses have evolved unique biological features over millennia to evade immunological recognition and thereby adapt to their equid hosts. In so doing, these features have shaped the pathogenesis and epidemiology and enabled the herpesviruses to continue to pose a significant threat to equine health and welfare. The review highlights epidemiological features and pathogenesis of the reproductive manifestations (i.e. late-term abortion and neonatal mortality) that are associated particularly with EHV-1. Other EHV syndromes including upper respiratory tract infections and neurological disease are reviewed in terms of their associations with abortion and neonatal disease, with the focus on epidemiological features influencing viral biology, in particular latency and recrudescence of infectious virus. These features are summarised in a table compiled from surveys and case series originating from several countries over a number of decades. Similarly, epidemiological risk factors associated with epizootic EHV-1 abortion are reviewed and summarised in table form. The importance of advances in laboratory-based diagnostics (particularly the advent of molecular methodologies) and interventional and preventative strategies, including vaccination, are also critically evaluated.

Chapter 3 presents a retrospective survey of reproductive performance in the South African Thoroughbred broodmare population during 1975-1999. The inclusion of this survey is to provide a context for subsequent assessment of the impact of EHV-1 abortion on pregnancy attrition and reproductive performance within subsets of the same population. The survey covered a transitional period during which novel reproductive technologies and therapies, and a compulsory reproductive health scheme were introduced. These advances contributed to significantly improved production outcomes, with the notable exception of late pregnancy loss which remained essentially unchanged despite concurrent improvements in initial pregnancy rates.

Chapter 4 describes a prospective cohort study using reproductive data obtained from broodmares following EHV-1 abortion epizootics. This was prompted by the need to objectively define the association of EHV-1 abortion with subsequent reproductive performance and additionally to differentiate the relative effects of early embryonic death (EED) and abortion (including abortion due to EHV-1) on subsequent reproductive performance. Uniquely, these data were used to develop predictive models to evaluate the relative influences and

interactions associated with EHV-1 abortion, compared with other causes of pregnancy attrition, on key reproductive performance outcomes in Thoroughbreds.

Chapter 5 is a retrospective study describing epidemiological and reproductive data from two geographically and temporally separated EHV-1 abortion epizootics affecting unvaccinated broodmares but with markedly contrasting morbidities. The study aimed to identify differences in epidemiological features and interventions that influenced the divergent outcomes of the two epizootics. The findings would be used to enhance prevention strategies and management advice for future EHV-1 abortion outbreaks.

Chapter 6 describes the development of a model for the minimally-invasive measurement of physiological stress via faecal glucocorticoid metabolites to evaluate its contribution to event-related recrudescence of latent EHV-1 and -4. Clinical monitoring and nasal swabbing for viral nucleic acids using a real time quantitative duplex PCR assay were applied to detect the presence of EHV-1 and -4 in adult horses. Although frequently cited as an important feature of EHV pathogenesis, few studies of stress-associated viral recrudescence in horses, and none with a concurrent measure of physiological stress, have been reported. The model was applied in a prospective study of pregnant broodmares consigned to a sales event during late-gestation. The model tested the association of viral nucleic acid excretion with previously-cited epidemiological risk factors including late gestation status, social disruption, road transport and exposure to a novel event.

Chapter 7 presents data obtained by monitoring a group of horses for evidence of EHV-1 and -4 virus shedding in nasal secretions, and clinical signs associated with viral infection during a period of residence at a bloodstock sales venue in South Africa. To enhance the probability of detecting viral nucleic acid, the study population included young horses (<2 years old) during the winter months, both factors previously associated with increased viral detection. This study also included a larger population monitored over a longer interval. In addition, serological assessment for EHV-1 and -4 in the horses at arrival was used to indicate their prior exposure before consignment to the sale. The study applied the model reported in Chapter 6 for non-invasive sampling to determine physiological stress by variation in FGM concentrations.

Chapter 8 summarizes and discusses the studies presented in the preceding chapters, in the context of the previously reported studies reviewed in Chapter 2. This chapter also addresses any possible shortcomings in achieving the aims of the current series of studies, and lists key areas that warrant future investigation. The chapter concludes with an update to

recommended interventions for both the control and prevention of EHV abortion epizootics on the basis of the data recorded in this series of studies.

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CHAPTER 2
The herpesviruses and their association with reproductive performance in horses

2.1 The equid herpesviruses in context

Taxonomically, the herpesviruses are currently classified within the Order *Herpesvirales* that accommodates three families: the *Alloherpesviridae,* the *Malacoherpesviridae* and the *Herpesviridae.* The *Herpesviridae* has three subfamilies: *Alpha, Beta* and *Gamma-herpesvirinae* and contains the mammal, bird and reptile adapted viruses including the herpesviruses identified in association with the *Equidae*. Herpesviruses infecting horses are named after the family of their primary natural host, the names ending in 'id', however the historically applied synonym in frequent usage is 'equine' herpesviruses. Within the sub-family *Alphaherpesvirinae,* the genus Varicellovirus contains six species associated with the *Equidae*: equid herpesvirus 1 (EHV-1) or the 'abortion virus', equid herpesvirus 3 (EHV-3) or coital exanthema virus, equid herpesvirus 4 (EHV-4) or rhinopneumonitis virus, equid herpesvirus 6 (EHV-6) or asinine herpesvirus 1, equid herpesvirus 8 (EHV-8) or asinine herpesvirus 3 and equid herpesvirus 9 (EHV-9) or gazelle herpesvirus 1. In addition, equid herpesviruses 2, 5 and 7 (EHV-2, EHV-5 and EHV-7) and recently, zebra herpesvirus 1, have been assigned to the sub-family *Gammaherpesvirinae* [1-6].

In vivo each herpesvirus has a primary association with a single species or, at most, a narrow range of hosts. They are nonetheless characterised by shared biological properties including the ability to establish latency following primary infection in their preferred host. Over the course of millions of years of coevolution, host species have developed a complex immune response and, in their turn, the viruses have responded with various survival strategies to evade this response. The viral adaptations include an ability to down-regulate MHC-1 protein expression on infected cells, mechanisms for avoiding cell-mediated lysis or for interfering with antibody-dependant and cell-mediated lysis, interactions with the host cytokine response to suppress lymphocyte activation and finally, latency [4; 6]. As with many successful pathogens, the resultant immunological balance is characterised by most infections being asymptomatic. The characteristic life-long persistence in the host as a result of failure to eradicate infection may however result in recrudescence of disease in both immunocompromised and immunocompetent hosts thereby establishing a permanent reservoir of infectious agent within a given population [4]. Herpesviruses cause pathology during primary infection or, alternatively, subsequent to viral reactivation following latency. Pathology is the result of the production of viral progeny leading to lysis of the host cell and, in certain herpesviruses, *via* their inherent oncogenic potential [4].

The horse is the natural host for EHV-1, -2, -3, -4, and -5, with EHV-1 and -4 being indisputably the most relevant from an equine health perspective [1; 6]. These two viruses (EHV-1 and -4) contain linear double-stranded type 'D' DNA genomes separated into a unique long (UL) region and unique short (US) segment, and share a high degree of genetic similarity. The larger EHV-1 genome is 150-kbp in length with 76 distinct genes and duplication of genes 64, 65, 66 and 67 resulting in 80 open reading frames (ORF's). Equine herpesvirus 4, at 146-kbp also has 76 genes but only three of these (64-66) are duplicated. Despite their close genetic and antigenic similarity, these two herpesviruses show markedly different pathogenicity [7]. Equine herpesvirus 4 is associated almost exclusively with upper respiratory tract (URT) infection, whereas EHV-1 typically has a systemic distribution and affects multiple organ systems with a consequently diverse range of diseases of varying severity [6]. Internationally, serological surveillance studies have generally demonstrated a far greater prevalence of EHV-4 than EHV-1 infection in various populations [1; 6; 8].

Equine herpesvirus 1 was first isolated in Kentucky in 1932 but was retrospectively associated with mare abortion since 1921; EHV-1 continues to be the source of significant genetic and economic losses to the international equine breeding industry, primarily because it is the most common infectious cause of abortion and a highly fatal neonatal disease but also because of its role in respiratory and neurological disease and chorioretinopathy [9-11]. The reported contribution of EHV-1 in recorded equine abortions in selected countries varies between 4.5% (UK), 8.9% (USA) and 15% (France) [11]. By contrast, EHV-4 is seldom detected in abortions, accounting for <1% (Kentucky) and <16% (UK) of viral abortions over ten and seven year-periods respectively [12]. On the other hand, both EHV-1 and, especially, EHV-4 are associated with acute upper respiratory tract (URT) infections that have a significant impact on performance horses, in particular racehorses, causing both economic losses accrued as a result of the need for veterinary interventions and wastage through poor performance and lost training time [13]. Herpesviruses have an important impact on both the local and international movement of horses for breeding and competition. The apparently-increasing incidence of EHV-1-associated neurological disease, or equine herpes myeloencephalopathy (EHM), has prompted both a growing awareness and stimulated research efforts to define the pathogenesis and epidemiology of this manifestation of EHV-1 associated disease [1; 14-18].

2.2 Reproductive performance and pregnancy failure in horse breeding systems

Arguably, the current yardstick for reproductive efficiency is represented by a live foal rate (FR) of around 80%, given the similar FR of 82.7%, 80.2%, 79.8% and 78.3% reported in well-managed Thoroughbred mares bred in the UK, New Zealand, Sweden and USA, respectively [19-22]. The corresponding end-of-season pregnancy rates (PR) in these studies exceeded 90%, representing a considerable improvement on previous decades. The difference between initial PR and FR remains a cause for concern however, because in addition to the increasingly challenging economic imperatives faced by horse breeders, pregnancy loss rates have remained relatively unchanged during the decades in which overall fertility improved markedly, such that they now represent the most significant source of reproductive wastage [23-25]. A multivariate analysis of the factors associated with the economics of breeding Thoroughbred horses showed that for a broodmare owner in Kentucky to generate a profit on their 'investment', the mare must produce a live foal in 6/7 successive years at stud [23]. Pregnancy rates are usually described as either per mated oestrous cycle or per season, with the former being the superior variable for describing reproductive efficiency [19; 20; 26; 27]. The FR is obviously what is left of the PR once pregnancy losses have been accounted for and, as a dependant variable, may be expressed as either the FR per cycle or per season. Pregnancy attrition is the sum of all pregnancy losses, and may be further categorised into early embryonic death (EED) and later, post-implantation loss. Post-implantation losses are additionally subdivided into abortion (fetal loss at <300 d gestation) and stillbirth (delivery of a dead foal at ≥300 d gestation), while neonatal mortality is defined as a foal death occurring ≤7 d after birth [28]. Mare-level factors including advanced age and barren reproductive status have been consistently shown to negatively influence reproductive outcome in broodmares including Thoroughbreds [19; 22; 29]. In recent surveys, approximately 20% of all pregnancies diagnosed by day 15 post-ovulation were reportedly lost, with 57% (USA) and 58% (UK) of these losses occurring during the post-implantation stage of gestation [21; 26]. More specifically, post-implantation loss rates of 12.9%, 8.9% and 8.7% were reported from Thoroughbred mares in Kentucky, the UK and Japan respectively [21; 26; 30]. These and other reports confirm that pregnancy failure is a major factor influencing reproductive efficiency in Thoroughbred and other horse breeding systems worldwide, which warrants further investigation to better understand the most common factors underlying these pregnancy losses [21; 26; 30] and help to develop strategies to manage or mitigate the losses.

Most reports describing Thoroughbred reproductive performance examine the effect of possible contributory factors at the mare level. These reports have generally used univariable analyses to examine the effect of a single explanatory variable or predictor such as age or reproductive status on an outcome, accepting conditions of independence. Many of these selected variables are, however, related. An example is the interrelatedness of mare age and reproductive status or parity, given that maiden mares are most likely to be located within both the youngest age and primiparous cohorts. Mare-level outcomes cannot be considered as independent events and, moreover, are subject to two sources of variation in the form of both individual mare differences and 'clustering' that further reduces the effective sample size and thus the power of the study and thereby increases the chances of a type 1 statistical error [27], i.e. a false positive effect. In addition, such analyses are unable to differentiate between the relative influences of the many explanatory variables on any particular outcome. There is a distinct risk of drawing a misleading conclusion when only considering the crude relationships between the predictors and the reproductive outcome of interest. Multivariable analyses are required to improve the likelihood of identifying true explanatory variables, because many effects are heterogeneous and subject to modification by other factors.

2.3 Equine herpesviruses and their role in infectious abortion

Fetal losses due to either infectious or non-infectious aetiologies are reported to range from 5-25% of all pregnancies [31-33]. The most common infectious agents implicated are bacteria, with ascending bacterial placentitis accounting for the majority of infectious abortion in late gestation mares [33]. Viruses are however an important contributor to infectious abortion, and are reportedly responsible for 4.1-9.6% of all diagnosed abortions and perinatal losses, and 13-18.2% of those confirmed to be of infectious aetiology [33]. Furthermore, EHV-1 is undisputedly the most important viral pathogen associated with equine abortion because of its prevalence, potential for epizootic spread (particularly within naïve populations [9; 31; 34]) and economic impact [9; 10; 12; 35; 36]. The epizootic manifestation (abortion 'storm') can involve approximately 1/3 of exposed mares, and abortion rates of up to 87% have been reported for unvaccinated mares [37; 38]. Vaccination, heightened awareness and more effective disease reporting appear to have reduced the frequency of EHV-1 abortion, and in particular the incidence of abortion storms, in many countries [18; 36]. Equine disease monitoring in Central Kentucky has indicated a marked decrease in the frequency of EHV-1 abortions (despite a

tripling in the broodmare population) with most recorded incidents during a 51 year reporting period up to 2008 being sporadic manifestations [39]. A review of data from 14 retrospective epidemiological surveys that include data for EHV-abortion and are derived from eight countries during the 42 year interval from 1969-2011 is summarised in Table 1. Where available, the table highlights the diagnostic rate and aetiological differentiation achieved from submissions of samples from abortions and neonatal deaths, with the aim of clarifying the relative importance of EHV-abortions and neonatal disease to the complete picture of reproductive attrition in horse breeding systems around the world. The inclusion of data describing confirmatory diagnostic methods helps illustrate the influence that technological development and introduction of novel tests, in particular polymerase chain reaction (PCR) assays have had in improving the speed, sensitivity and specificity of diagnosis. The improvements have included the sequential development of the ability to initially differentiate EHV-1 from -4, and thereafter of some specific neuropathogenic from non-neuropathogenic EHV-1 viruses accompanied by recognition of the importance of advising fetal membrane submissions to diagnose atypical (fetal-negative) EHV-1 abortion. Despite these advances, ongoing surveillance indicates that EHV-1 remains an important cause of both sporadic and epizootic abortions, where the countries in which epizootics continue to be common typically being those in which interventions, in particular vaccination, are less routinely applied. A 24-year retrospective survey of 1822 equine abortions in France implicated bacteria as causal agent in nearly 80% of all diagnosed abortions, with viruses accounting for almost 10% including >15.0% of infectious cases, with EHV-1 accounting for 14.5% of the latter [33]. By comparison, EHV-4 was implicated in a mere four cases and the only other virus implicated, equine arteritis virus (EAV) accounted for only a single case. The annual incidence of EHV-abortion was variable, depending mainly on the number and size of outbreaks, accompanied by sporadic manifestations in fully-vaccinated mare populations [33]. A similar survey over approximately 24 years in Poland reported EHV-1 to be responsible for 23% of abortion diagnoses and similarly reported a variable annual incidence and a seasonal distribution [40]. In Italy, 103 cases of abortion with a presumptive diagnosis rate of 64% were reported, and EHV-1 was the most frequently diagnosed cause [28].

2.4 The other equine herpesviruses: EHV-3 and the equine gammaherpesviruses

Equine herpesvirus 3 and the equine gammaherpesviruses (EHV-2 and -5) are also widely prevalent in horse populations worldwide and, although they are associated with disease, their manifestations are generally of lesser veterinary and economic importance [5; 6; 41]. Equine herpesvirus 3 is the aetiological agent of coital exanthema a classic, albeit self-limiting, venereal infection affecting the external genital mucosae of both mares and stallions. Transmission is primarily *via* coitus, although fomites have been suggested to play a secondary role in transmission. Recurrence *via* reactivation in successive breeding seasons is suspected but not proven [41]. There is currently no evidence for an association between EHV-3 and either infertility or abortion [1; 11; 42]. Although these gammaherpesviruses are frequently detected, their role in disease manifestations is somewhat complicated in that both EHV-2 and -5 have been detected in immunocompetent hosts without overt signs of disease whereas they have also been detected in horses with clinical signs ranging from mild respiratory disease, pneumonia, pharyngitis and lymphadenopathy, to keratoconjunctivitis, poor performance and equine multinodular pulmonary fibrosis (EMPF). To date, neither EHV-2 nor -5 have been reported in association with pregnancy loss or abortion in mares [1; 5; 43].

2.5 Epidemiology of EHV-1 and EHV-4

Both EHV-1 and -4 are regarded as ubiquitous pathogens of horses with a global distribution [1; 17; 34; 44]. Improved serological testing *via* type-specific ELISAs has improved differentiation between the two species, although there are complications associated with interpretation in vaccinated populations [1; 6]. The EHVs are characterised by a short life-cycle, early exposure of young horses and widespread establishment of (probably) life-long latent infections following primary infection of young horses. An important discovery was the identification of broodmares and their foals as important reservoirs of EHV-1 and most likely of EHV-4 too, with viral transmission occurring prior to weaning and as early as 30 d of age [13; 45]. Virus was found to circulate between mares and foals despite vaccination of the mares [46]. Thereafter, recovered horses act as reservoirs of infection for other in-contacts and previously unexposed, susceptible horses *via* periodic, generally asymptomatic ('silent') viral recrudescence with shedding [10]. Since >50% of horses are probably latently infected, with either or both EHV-1 and -4, the recrudescence of latent virus is thought to be the primary source of infective virus

[44; 47; 48]. Maintenance of endemic infection is associated with cycles of silent, subclinical infection among adults, between adults and foals and among foals [44]. The short environmental persistence of these labile, easily inactivated viruses is probably <7 d with a maximum of 35 d and environmental contamination is consequently unimportant for the maintenance of EHVs in the global population [44]. Environmental transmission is however central to outbreaks, which are typically associated with closely-confined horse populations [44].

Risk factors reportedly associated with the prevalence of EHV-1 and -4 latency include geographical location; host origin, age and breed; management practices; diagnostic method and sensitivity; and other undetermined factors [17; 48]. Sero-epidemiological surveys have further shown a consistently higher prevalence of EHV-4 than EHV-1 in Thoroughbreds and other equine populations in several countries [1; 6]. A seasonal influence has also been reported, with EHV-1 infections occurring predominantly in winter (compared to EHV-4 which is reported throughout the year) which in turn, coincides with the expected period of late gestation in this long-day seasonal-breeding species [1; 49]. These variations in sero-prevalence, although supporting epidemiological differences between the two viruses, may alternatively or additionally have been biased by EHV-1 vaccination or variations in assays and study designs [8; 48; 49].

Recent research has focussed increasingly on the neuropathogenic (alternatively termed neurotropic or neurovirulent) strains of EHV-1 viruses. These neuropathogenic and non-neuropathogenic strains were thought to be distinguishable *via* a single nucleotide polymorphism (SNP) in the open reading frame (ORF) 30 of the gene encoding viral DNA polymerase with a single nucleotide substitution of adenine (A) for guanine (G) at nucleotide 2254 (i.e. $A \rightarrow G_{2254}$) with a resultant change from asparagine (N) to aspartic acid (D) at amino acid position 752 (i.e. $N \rightarrow D_{752}$) [52]. This led to development of RT-PCR assays using allelic discrimination for the detection and differentiation of these strains [53; 54]. Neurological disease appeared to have a stronger association with the ORF30 G_{2254} genotype whereas the non-neurological biovariant (the ORF30 A_{2254} genotype) has been linked primarily with non-neuropathogenic disease [52]. The G_{2254}: A_{2254} ratio was also reported to vary geographically; for example, the Thoroughbred broodmare population in Kentucky, USA showed a larger proportion of latent ORF30 G_{2254} than described in other areas [6; 53], notably reflected by submissions of aborted foetuses assayed by allelic-distinction PCR [15]. Disease monitoring in Central Kentucky indicated a significantly increased incidence of EHV-1 neurological disease

since 2000 [15; 39; 53]. The prevalence of ORF30 A_{2254} is probably greater than neuropathogenic EHV-1 strains in most horse populations, supporting the observed low frequency of EHM. However, a significant proportion of neurological cases are reported in animals with the supposedly non-neuropathogenic genotype [48; 55].

2.5.1 Epidemiological role of young animals

Both EHV-1 and -4 are reported to circulate widely in both vaccinated and unvaccinated populations of mares and foals on stud farms, with EHV-4 more likely to be identified as a clinical entity whereas reports associating EHV-1 with acute respiratory disease in foals are relatively rare [56]. Sero-epidemiological evidence of EHV-1 antibodies in foals in unvaccinated populations in Australia demonstrated that lactating mares are a source of infection to their foals, which in turn infect other foals in close contact [13; 45; 57]. This constitutes a cycle of 'silent' infection without overt clinical signs both before and after weaning. A subsequent study in a vaccinated population detected EHV-1 and -4 by PCR assay of nasal swabs recovered from both vaccinated mares and their unvaccinated foals [46]. This suggested that even comprehensive vaccination of broodmares was ineffective in preventing spread of EHV-1 and sero-conversion of foals some of which, as adult broodmares, re-enter the breeding population as latent carriers and help perpetuate the disease. Although it clearly did not prevent infection, vaccination appeared to offer partial protection since it reduced the frequency of virus shedding, thereby potentially reducing both prevalence and risk of new infections in a herd [46].

2.5.2 Epidemiology of EHV-1 and EHV-4 respiratory disease

Both EHV-1 and -4 are important aetiological agents associated with viral respiratory disease and are enzootic in most horse populations, with EHV-4 reported by one survey to be the most common pathogen detected in horses showing signs of acute infectious upper respiratory tract disease (IURD) [58]. In addition, the two gammaherpesviruses EHV-2 and -5 have been detected worldwide, and are classified among the 'less well-characterised' group of respiratory viruses, and are associated with very early primary infection of foals, development of life-long latency and periodic recrudescence. They have been detected in respiratory secretions from

horses with signs of IURD, but also in immunocompetent animals without symptoms. Their role as primary pathogens, co-factors or incidental findings is currently undefined [5; 59].

A frequent anecdotal citing is that approximately half of the callouts attended to by racetrack veterinarians are for URT disease cases of an apparently viral origin, but frequently of unverified aetiology [44; 45; 59]. These infections are generally both self-limiting and rarely life-threatening, but have a considerable erosive impact accrued directly via costs associated with veterinary care and indirectly via interruptions in training and performance schedules. As previously described, type-specific serological testing on stud farms showed both that many foals are infected early in life, with a far greater prevalence of antibodies for EHV-4 although a significant proportion of foals were seropositive for EHV-1 prior to weaning. The probable primary sources are the dams of these foals or other asymptomatic adult mares with additional horizontal transmission between foals prior to or following weaning. Transmission is via aerosolised virus or contact with infected respiratory secretions or various fomites. This endemic infection is probably due to a combination of sub-clinical infection cycles and reactivation episodes within the mare population or individual mares respectively [13]. Interestingly, despite the high exposure rates, low rates of EHV-1 isolation compared to EHV-4 in young horses with URT disease signs are reported from various extensive sero-epidemiological surveys [42; 58]. Age, season, increased stocking density associated with indoor, sales and training facilities have been reported as risk factors for viral shedding [49; 58]. The highest frequency of EHV-4 was reported in horses <1 year of age [49; 58].

2.5.3 Epidemiology of EHV-1 and EHV-4 abortion and fatal neonatal infection

Abortion due to EHVs can be in the form of either sporadic occurrences or, less frequently, epizootics or outbreaks [16-18; 37]. The reported incubation period preceding abortion varies widely and has been reported to range between 9-121 d [38]. Under natural conditions, EHV-1 abortion affects mares of any age although it has been reported to occur mainly in first-foal mares [36; 60]. Abortions occur more frequently in late gestation, with 95% in the last trimester, 74.9% between 8-10 months and almost none before 4 months [9; 33; 60]. Mares are thought to very rarely abort due to EHV-1 in successive pregnancies [61].

Retrospective surveys over the past quarter of a century have documented the prevalence of EHV-associated abortion and fatal neonatal infection and are summarised in Table 1. The reported data must be assumed to be biased by factors including the absence until quite

recently of molecular techniques enhancing the sensitivity and specificity of diagnosis. The relatively recent recognition of 'atypical' abortion of virus-negative fetuses, emphasized that testing fetal membranes in addition to conventionally-tested fetal target organs, has probably further increased diagnostic sensitivity [62]. The association of EHV-1 with abortion and neonatal death was quoted from numerous sources by Szeredi *et al* (2003) to occur with a frequency of 3-25% in Europe and 3-9% in the USA [63]. In Germany, EHV-1 was reported to be responsible for 10% of all recorded abortions (or 0.65% of all confirmed pregnancies) among the 6.5% of pregnant Thoroughbred mares that aborted during 1968-1992 [64]. In a 1988-1997 UK survey, 82/1252 (6.5%) abortions were diagnosed as being due to EHV-1 (n=78; 95%) and EHV-4 (n=4; 5%), and EHV-1 accounted for 12.2% of neonatal deaths based on viral isolation and immunohistochemical confirmation [65]. In the Netherlands during 1983-1995, isolates obtained from abortions and neonatal deaths and analysed by type specific ELISA indicated that 244/254 (96%) incidents were due to EHV-1, with 10 (4%) ascribed to EHV-4 [66]. Szeredi *et al* (2003) reported EHV-1 positive diagnoses in Hungary during 1998-2000 despite regular use of EHV-1 / -4 vaccination, from 15 (14.9%) and 13 (12.9%) of 101 cases using immuno-histochemical and virological methods, respectively [63]. A survey from Poland (1977-2010) similarly used viral isolation and immunohistochemistry to demonstrate that EHV-1 was responsible for 106/452 (23.5%) abortions and neonatal deaths [40]. Infrequent vaccination in Poland was suggested to explain the greater prevalence in the latter report. Two surveys of abortions and neonatal deaths were recently reported from France. The first spanning 2002-2005 reported data from 407 cases analysed *via* nested consensus PCR, and showed 67 (15%) incidents to be associated with EHVs, of which 59 (14.5%) were due to EHV-1 [11]. The data from 1822 cases examined during 1986-2010 in Normandy were reported; during the course of the survey, testing by viral isolation and immunohistochemistry was replaced (in 2002) by a consensual EHV-PCR and discriminatory EHV-1 / -4 PCR assays. Final figures, indicated 15.1% of abortions to be infectious in origin with a viral aetiology in 9.6% cases, of which 126 (6.9%) were due to EHV-1 and only four to EHV-4 [33].

2.5.4 Neuropathogenic EHV-1, the other EHVs and abortion

In Kentucky, 426 EHV-1 isolates archived from fetal tissues collected during 1951-2006 were analysed by RT qPCR to examine the temporal variation in abortion frequency associated with neuro- versus non-neuropathogenic genotypes. The prevalence of the ORF30 G_{2254} genotype

(shown to be present in the 1950s) was found to have increased during the 2000s [15]. A German survey from 1987-2009 included data from 66 EHV-1 cases, including 32 derived from six epizootics and 34 from sporadic cases and differentiated 7 (10.6%) ORF30 G_{2254} from 59 (89.4%) ORF30 A_{2254} genotypes [67]. In Italy during 2004-2011, a diagnostic rate of 58.7% for 116 abortion and neonatal mortality cases was achieved using virus isolation and a type-specific nested EHV-PCR that identified 27 EHV cases including EHV-1, -2 and -5 with 22 (81.5%) positive for EHV-1 of which 16 (72.7%) were neuropathogenic and 6 (27.3%) were non-neuropathogenic strains [68].

Prevalence of neurological disease associated with abortion

Outbreaks of neurological disease with concurrent abortion have been reported and include a 1983 Austrian EHV-1 epizootic at a Lipizzaner stud where 22 abortions and neonatal deaths occurred and 10/17 (58.8%) mares with neurological signs died [69]. Other combined outbreaks of neurological disease and abortion include those in a 23-year retrospective study from France, an Australian report and the two recent reports from Croatia and Germany respectively [16; 17; 56; 69]. The ORF30 G_{2254} genotype strains are reported at low incidence in abortion events not associated with neurological disease cases [15; 55; 67]. A recent abortion outbreak in Germany associated with the ORF30 G_{2254} genotype was notably, however, unaccompanied by neurological disease, a finding hypothesised to be due to affected pregnant mares' solid vaccination history and immune status [18]. Both pregnancy and lactation with a foal at foot have been proposed as risk factors for neurological disease [56].

2.5.5 Epidemiology of epizootic abortion

A review of the epidemiological data reported in association with EHV-1 epizootics during 1983-2012 from six countries with different management and vaccination practices is summarised in Table 2. The prevalence of EHV-associated abortion, and deaths among the neonatal foals, respectively, for pregnant mares in eight case series were:

- 22/30 (73%) abortions in Austrian Lipizzaners [69];
- 33/38 (87%) abortions and one neonatal death in Australian Thoroughbreds [37];
- 7/12 (58.3%) and one in trotting horses in the Netherlands [64];
- 50/173 (29%) and five in polo ponies in Argentina [70];
- 11/18 (61%) and none in Welsh ponies in the UK [71];

- 4/40 (10%) and one, in Quarter horses and Arabians (site 1) and 17/32 (53.1%) and three in Lipizzaners (site 2) in Croatia [16];
- 6/7 (86%) and none in sport horses in Germany [17]; and
- 16/25 (64%) and two in Standardbred horses in Germany [18].

Epidemiological risk factors for epizootic abortion

A number of epidemiological risk factors have been identified for EHV-1 epizootic abortion, and a review of ten selected case series and retrospective surveys describing these risks is summarised in Table 3. The commonly identified risk factors are believed to either increase the risk of novel introduction of infection into a naïve population or, perhaps more commonly given the reported prevalence of latency, to promote physiological stress-induced recrudescence of infectious virus from within a population. Seven (70%) of the reports included unvaccinated or incompletely vaccinated populations, and a history including recent local (30%) or international (20%) transport was also a common feature. Management practices and, in particular, introductions of novel mares into established social groups (20%) often without appropriate quarantine (20%), large groups of late-gestation broodmares (20%), high-stocking densities (30%), and co-mingling of different epidemiological populations (30%) were other common risk factors. Several of the reports suggested that the outcome of an outbreak was biased by intervention strategies, including the time elapsed prior to establishing an aetiological diagnosis and the availability of isolation facilities (40%).

2.6 Pathogenesis and disease syndromes

2.6.1 Transmission

Polymerase chain reaction-based assays demonstrated the transmission of EHV-1 and -4 in clinical samples *via* direct contact and aerosolisation of nasal secretions, with significantly larger virus loads in the fluids and tissues associated with aborted foetuses and foetal membranes. Less commonly, transmission occurs *via* fomites including water sources, food and equipment [9; 36; 77; 78]**.** Horizontal transmission of EHV-1 and -4 in semen *via* the venereal route has been suggested but its significance is undefined, with no evidence of infectious virus transmission despite presence of EHV-1 in semen [79; 80].

2.6.2 Virus entry

Equine herpesvirus 1 has a greater cellular and host spectrum than EHV-4, and is able to infect three cell types in three different organ systems including epithelial cells, mononuclear cells of the lymphoid tissue and peripheral circulation and the endothelial cells of internal organs [7]. Equine herpesvirus 4 is however generally limited *in vivo* to the epithelial cells of the URT. Equine herpesvirus 1 binds various cellular receptors and enters host cells *via* a range of pathways, which serves to maximise its host range. By contrast, EHV-4 binds a limited range of receptors with a consequent reduction in host range [6]. Upon cellular entry, the virus enters either a lytic cycle, releasing new virus particles from the infected cell, or a latent infection cycle where, following entry of viral DNA into the nucleus, further transcription is blocked with the exception of a 'latency-associated transcript' (LAT) [44; 81]. These two cycles may occur independently or concurrently [44]. Subsequent to nasal entry, the virus replicates in URT epithelial cells with consequent shedding and typical herpetic lesions affecting the mucosae. Both EHV-1 and -4 infections may result in respiratory disease with pyrexia, anorexia and ocular and nasal discharges [1; 7].

2.6.3 Viraemia and secondary replication

Following the respiratory infection phase, EHV-1 invades the *lamina propria* assisted by migrating mononuclear cells and then, by courtesy of a leukocyte-associated viraemia, becomes widely distributed throughout the body. The secondary sites of virus replication include the vascular endothelium of the gravid uterus, the central nervous system (CNS) and the eye [7]. The widespread endothelial infection and associated vasculitis, thrombosis and ischaemic damage, together with the host's response, are eventually responsible for the ensuing manifestations of abortion, neurological disease or chorioretinopathy [12]. Unlike the associations of pathogenicity with viraemia and endothelial cell infection for EHV-1, EHV-4 infections are generally limited to the URT and a leukocyte-associated viraemia appears to be a rare occurrence. As a consequence, abortion and neurological disease associated with EHV-4 are much less common [1; 7]. Acute EHV-4 infection is associated with pyrexia, anorexia and nasal discharge with ocular discharge being rarer and milder than reported in association with EHV-1 infection.

2.6.4 Latency and reactivation

Latently infected animals are not infectious and are clinically normal. The prevalence of latent infection with EHV-1 and, or -4 was estimated at >50% of the horse population, with a range between 54-88% reported for EHV-1 [36; 44; 53]. Latency is established in a similar manner and at comparable anatomical locations to the other alphaherpesviruses [1]. The predominant site of latency is variously reported to be lymphoid tissue and lymphocytes, typically CD8+ lymphocytes and neuronal cells of the trigeminal ganglia [6; 81-85]. The latently-infected cells do not express viral antigen and consequently evade immunological detection. The numbers and dynamics of latently-infected neuronal cells are unknown, but circulating latently-infected lymphocytes are rare and decline over time, unless supplemented by reactivation or *de novo* infection [44]. Molecular detection of latent EHV-1 and -4 has been reported in several studies *via* post-mortem sampling of neuronal and lymphoid tissues, specifically the trigeminal ganglia and submandibular and bronchial lymph nodes [8]. A prevalence of 53.8% EHV-1 (mostly non-neurotropic strains) was reported from samples derived from 132 Thoroughbred broodmares of various ages from Kentucky [53]. Latent EHV-1 was detected in 15% of 132 horses undergoing routine necropsy in California and 32.7% of 52 horses at slaughter in New Zealand [8; 85]. Similar to the sero-prevalence reports, a greater prevalence of EHV-4 (82.8%) than EHV-1 (25.7%) with frequent dual infection (17.1%) of sampled horses was described for 70 young Thoroughbred racehorses in California, supporting the dogma of frequent early exposure to EHV-4. In addition, non-neurotropic strains were more prevalent than neurotropic strains in this last study, with the trigeminal ganglion as the commonest site of latency [48].

Recrudescence of latent virus is a crucial feature in the epidemiology of EHVs and which may or may not be accompanied by clinical disease [85]. The reactivation of virus in latently-infected EHV-1 horses by a switch from a latent to a lytic cycle with viraemia or shedding is poorly understood but is thought to occur under conditions associated with either physiological stress or immunosuppression, possibly by administration of pharmacological agents including corticosteroids or immunomodulators [9; 48; 86; 87]. Equine herpesvirus 4 and other EHVs including EHV-2 and -3 have been shown to reactivate, albeit without development of clinical signs, after experimental administration of high dose corticosteroids [5; 87]. Additional risk-factors reported for EHV viral recrudescence include management and environmental stressors such as transport, disease, hospitalisation, inclement weather and social disruption [18; 70]. Upon reactivation of the latent genome, the un-enveloped virus capsids are assembled for translocation to the respiratory epithelium where they become enveloped to make infectious

virus particles. Thereafter, the virions will either be neutralised by local mucosal immunity or contribute to a new cycle of respiratory infection with viral shedding into respiratory secretions or viraemia [44; 87]. Most reactivation episodes terminate with respiratory mucosal neutralisation without consequent nasal shedding or viraemia. Clinically, reactivation is only detectable if nasal shedding or viraemia follows, such that the actual frequency is unknown, furthermore, the differentiation between reactivation and reinfection by exogenous virus is rarely possible [87].

2.6.5 Neonatal disease

Although highly fatal, EHV associated neonatal disease is relatively uncommon and is predominantly associated with EHV-1, and far more rarely with EHV-4 infection [44; 88]. A rare field report of an epizootic, primarily characterised by fatal neonatal disease mostly in full-term foals and without associated abortions and stillbirths, was observed with 29/43 (67.4%) foal deaths that were virologically diagnosed as being due to EHV [60].

Infection may be aquired either *in utero* or shortly post-partum from a shedding dam or other in-contact animal. The affected neonates are generally weak and diseased at birth. Signs of respiratory disease associated with a primary pneumonitis are the most common manifestation, characterised by rapid deterioration with respiratory distress, dyspnoea with hypoxia and an invariably fatal outcome. More rarely, the affected foals are born apparently normal but develop signs within a few days, often due to a complicating secondary bacterial bronchopneumonia. The risk association for viral shedding postpartum by subclinical neonates is undefined [78].

2.6.6 Stallions

In pigs, cattle and men, certain herpesviruses are shed in the ejaculate and have been associated with infertility. Reports describing the risk of stallions shedding EHV-1 and -4 into their semen are however limited [79; 80; 89; 90]. Although scrotal oedema, reduced libido and sperm quality are described in viraemic stallions, the reported leukocyte-associated shedding of EHV-1 into the ejaculate is of greater potential interest. One study detected EHV-1 (but not EHV-4) DNA in semen sampled from 51/390 (13%) stallions without concurrent signs of disease nor apparent effect on their fertility [79].

2.6.7 Abortion pathogenesis

The pathogenesis of EHV-associated abortion is not fully defined, with two methods of viral transmission from the pregnant endometrium to the placenta having been described that may occur concurrently [44; 76; 91; 92]. The key stage, namely endometrial endothelial cell infection, follows the viraemia (an essential pathogenic prerequisite) associated with the initial respiratory lytic infection or reactivation. The EHV-1-infected leukocytes reach the endometrium where they bind to endothelial cell surfaces, particularly in the smaller arterioles supplying the endometrial glands at the base of the microcotyledons. A subsequent sequence of vasculitis, thrombosis, microcotyledonary infarction with perivascular cuffing and transplacental spread at the vascular lesion sites ensues [65; 78]. The transfer of infected leukocytes may occur in >50% of cases during EHV-1 abortion outbreaks [44]. This may take the form of direct transmission via endometrial infarcts as endothelial cells lyse, freeing large amounts of free virus that enters the fetal trophoblasts, propagating widespread endometrial infarction and rapid detachment of fetal membranes with expulsion before the fetus is infected [93]. This may explain the phenomenon of viral-negative fetuses in 'atypical' cases where viral antigen and nucleic acids are found only in association with the fetal membranes. Alternatively or additionally, transplacental cell-to-cell migration of the infected maternal leukocytes may occur, with infected monocytes transiting the chorion's epithelial layers, and small amounts of virus being slowly transferred from the endometrium via the fetal membranes. This latter scenario is probably seen in abortion cases where infarction results in milder lesions and with virus reaching the fetus prior to expulsion, generating the viral-positive foetuses reported most-commonly. These cases with fetal infection are additionally associated with viral loads in fetal tissues far greater than in the fetal membranes [78]. If this transplacental infection occurs close to term, it is possible for an infected foal to be born alive; however, these neonates almost invariably succumb to interstitial pneumonia within a few days of birth [93].

The uterine endothelial cells are reportedly more susceptible to EHV-1 infection in late than early gestation [36]. Susceptibility during the early embryonic phase of gestation and any association with early embryonic death is currently unknown [44]. Endothelial and leukocyte cell surface adhesion molecules, the expression of which may be regulated by hormonal factors associated with late gestation, have been proposed to facilitate infection of the endothelium [94; 95]. The viral factors associated with the occurrence of abortion include strain variation; for example, there is a purported strong association of the N752 strain variant of the non-neuropathogenic ORF30 A_{2254} in natural abortions [52]. Nevertheless, while abortogenic

potential reportedly varies between strains of EHV-1, most strains are presumed to be capable of inducing abortion, in contrast to the situation for neurological disease which is associated with only a few strains. Both the N752 and the D752 strains of the neuropathogenic ORF30 G_{2254} EHV-1 genotype have been associated with abortion, although neurotropic strains are apparently less commonly associated with abortion in the absence of neurological disease [11; 18; 52; 78]. The 'less-virulent' EHV-1 strains are reported to have a reduced tropism for endothelial cells, nevertheless associated abortion is still a consequence of the vascular lesion and thrombo-ischaemia due to viral replication in the endothelial cells as would be the case in the rare incidents of EHV-4 associated abortion which result when EHV-4 is able to replicate in the endometrial and trophoblastic endothelial cells [44]. Vasculitis is a feature described at between five to nine months of gestation, suggesting that gestational age or additional host factors may be predictors of abortion as an outcome [10]. In short, a combination of both viral and host factors probably influence the outcome of EHV-1 infection of the gravid uterus, given that pregnant mares can become infected without abortion being an inevitable sequel [10; 47; 78].

2.6.8 Complications associated with abortion

A mare that has suffered an EHV-associated abortion is not immune to recurrence, but this is apparently rare [12]. Following abortion, virus is cleared rapidly from the genital tract, and future breeding capacity is not impaired unless damage to the uterus or caudal reproductive tract has occurred due to dystocia [12; 17; 18]. Dystocia and other obstetrical complications are rarely reported as complications of EHV-1 abortion [17; 64]. Postpartum deaths are also only occasional complications associated with uterine prolapse, dystocia or to concurrent EHV-1 neurological disease [16]. Interestingly, the clinical manifestation of premature placental separation (PPS), also known as 'red-bag delivery', has been reported on several occasions in association with EHV-1 abortion, including a recent report of a statistically significant association with EHV nucleic acid [17; 62; 65; 68; 71]. Retained fetal membranes (RFM), including one reported incidence of 66.7%, and uterine prolapse with subsequent fatal outcomes have additionally, albeit infrequently been reported following EHV-1 abortion [13; 64].

2.7 Diagnosis of EHV

2.7.1 General diagnostics

Diagnosis of viral infections can be achieved directly by detection of virus in clinical samples or indirectly by detection of an increase in virus-specific antibody in paired sera obtained at an interval of 7-14 d. Classically, the direct detection by isolation of EHV-1 or -4 virus in a cell culture system was considered the gold standard for laboratory diagnosis. This method provides sensitive evidence of the presence of infectious virus by demonstrating a characteristic cytopathic effect (CPE) within 5-7 d of inoculating cell cultures with supernatant from clinical samples [44]. Histopathology is important for confirming EHV infection in both aborted tissues and post mortem-derived samples from EHM cases [44]. Histopathology and immunohistochemistry (IHC) on formalin-fixed tissue sections to observe characteristic lesions and the presence of viral antigen associated with the vasculitis in infected epithelial and endothelial cells are used to confirm EHV in fetal, placental and CNS tissues [44]. Direct immunofluorescence (IF) tests are used as rapid front-line diagnostic tests, although their utilisation in clinical samples is limited by their requirement for live virus to detect viral antigen.

The EHV-1 and -4 genomes were first sequenced by Telford *et al* in 1992 and 1998 respectively, greatly facilitating both the improved detection and understanding of the pathogenesis of these viruses [10]. Currently, PCR assays, and, in particular, type-specific assays are considered the routine diagnostic test of choice for EHV-1. They have a high degree of sensitivity and specificity even, in the case of RT-qPCR, in the presence of low viral loads [36; 77; 96-99]. Moreover, fresh, frozen and fixed-tissue samples can all be submitted for PCR assays [44]. Various conventional PCR protocols (single or nested PCR) targeting specific genes within EHV-1 and -4 have been reported for the molecular detection of these viruses, although interpretation is somewhat complicated by a lack of protocol standardisation. Molecular strain analyses have identified the single-point mutation in ORF30 in the case of 86% of neuropathogenic EHV-1 strains [52; 97; 99]. Polymerase chain reaction assays also facilitate studies of viral kinetics by quantifying viral loads from respiratory secretions collected *via* nasal (more-sensitive) or nasopharyngeal swabs to document shedding, and in un-coagulated blood to demonstrate and monitor viraemia [100]. Quantitative RT-PCR assays have potential application in the identification of the elusive latent status by measuring the discrepancy in magnitude of virus loads between viraemic leukocytes (far lower magnitude) and lytic-infected cells shed in nasal secretions [101]. This can assist in defining the severity of infection and

monitoring disease progression and enables estimation of the risks associated with transmission. However, a recent EHV-1 consensus statement warned of assay limitations with respect to sensitivity and specificity that affect their ability to distinguish between the presence of high levels of infectious virus and low or undetectable levels during latent infection [36]. Furthermore their application for random testing or surveillance studies in healthy populations was not supported, and it was recommended that these tests be reserved for cases of clinically-suspected EHV-1 infection alone. The statement concluded by recommending the following steps to document active EHV-1 infection:

'*Un-coagulated blood and nasal swab for PCR analysis (preferentially by quantitative real-time PCR assay).*

Some laboratories prefer EDTA as an anticoagulant, because heparin can interfere with PCR reactions.

Un-coagulated blood and nasal swab for EHV-1 virus isolation when clinical signs and PCR results are suggestive of infection.

Paired-serum samples collected 15-21 d apart for serology (VN assay and ELISA for specific virus antigen) when available.

In the absence of clinical signs consistent with EHV-1 infection, use of current diagnostic methods, including real-time PCR, as screening tests are not recommended.'

Indirect or presumptive evidence of infection is possible by serological detection of a >4-fold increase in EHV-1 or -4 antibody titres in paired serum samples collected 7-21 d apart. Although serology has limited utility for confirming a diagnosis of EHV infection in an individual horse, testing paired serum samples from in-contact horses can be useful, because a proportion of both affected and unaffected in-contact animals will seroconvert, providing indirect evidence of virus presence. Serological surveillance for the two closely-related viruses is however complicated by both their antigenic cross-reactivity and the scarcity of type-specific antibodies. Serological testing has developed from tests for virus-neutralizing or complement-fixing antibodies to an EHV-1 / -4 type-specific ELISA based on recombinant antigens from the variable region at the carboxy-terminus of the glycoprotein G (gG) of EHV-1 and -4 that elicits type-specific humoral antibodies [6]. The latter test has, *via* paired sera, been reported to reliably identify horses infected with either EHV-1 or -4 [36; 50].

2.7.2 Abortion diagnosis

Herpesvirus abortion diagnosis is performed on tissue samples obtained from the fetus, fetal membranes or preferably both [35]. The diagnostic approach to late-gestation mare abortion has conventionally included gross post-mortem examination of the aborted fetus and fetal membranes, and supplemental histopathology for macroscopic and microscopic lesions characteristic of EHV-1 abortion. The importance of including fetal membrane samples in diagnostic submissions following abortion and stillbirth is emphasized by instances when either the fetal membranes alone are available and, more specifically, by the existence of 'atypical' abortions [35; 62]. In the latter, EHV-1 virus is present solely in fetal membranes and is absent in the fetus, possibly due to virus-induced uteroplacental infarction triggering fetal membrane detachment and abortion before the passage of virus into the fetus. The more commonly encountered abortion phenotype involves milder placental lesions, which allow virus to be transferred transplacentally and to replicate within fetal organs [11; 33; 62; 65; 71; 76; 92; 93]. The inclusion of fetal membrane samples for investigation in the diagnostic protocol is further emphasized by the fact that ascending placentitis (bacterial and fungal) is still the most commonly diagnosed cause of late-term abortion [33; 65; 74].

Given the potential for epizootic manifestation of herpesvirus abortion, the appropriate diagnostic approach to mare abortion should include assays with rapid turnaround times but still offering optimal sensitivity and specificity. The more rapid serological and molecular methods that permit both biological and molecular identification of virus are particularly useful when managing disease outbreaks. The increase in the reported prevalence of EHV-1 abortion in recent years may be due simply to improved molecular diagnostics and heightened awareness of the need to include fetal membrane samples in diagnostic submissions [11].

An array of diagnostic tests including virus isolation, histopathology, immunostaining and molecular assays have been applied to target organs from aborted fetuses and fetal membranes. These direct detection methods have evolved from classical viral isolation in cell culture, *via* histopathology to antigen detection methods and, most recently, rapid and type-specific PCRs to detect viral DNA [8; 77; 96-99]. Immunohistological methods include *in-situ* hybridisation (ISH) using labelled probes for selected EHV-1 and -4 genomic strains [92] and immunoperoxidase (IP) staining using polyclonal antisera cross-reactive with EHV-1 and -4 [35]. PCR assays and enzyme immunostaining have proven to be useful in the diagnosis of EHV-1 abortion [36; 76; 78]. For example, when allantochorion samples from 49 known EHV abortions were analysed by PCR in parallel with *in situ* hybridisation (ISH) and immunoperoxidase staining

(IP), the PCR detected 41 (EHV-1) and 5 (EHV-4) cases, compared to 21 and 4 cases respectively by ISH and 15 by IP [35]. The main advantages of a PCR based approach include rapid turnaround times and the ability to detect even minute quantities of viral nucleic acids in a wide variety of sample types. In the case of suspected EHV abortion, important tissues to sample for PCR assay include the fetal lung, liver, thymus, adrenal and spleen together with multiple sites on the allantochorion [35]. As previously described, an added advantage of PCR is the ability to not only detect genetic material, but simultaneously provide an accurate measure of the amount of virus present, which can be valuable when monitoring viral shedding to help improve infection control strategies [102].

The indirect serological diagnosis of EHV infection from maternal and fetal serology alone is unreliable despite availability of virus neutralisation (VN), complement fixation (CF) and type-specific enzyme linked immunosorbent assays (ELISAs) for EHV-1 / -4 antibody detection [8; 15]. Extensive vaccination and maternal antibody presence are important confounders of serological investigation, including during an abortion outbreak [44].

2.8 Immunology and EHV abortion

Much of the current immunological data is derived from experimental infections, while information from field infections or outbreaks is limited. Both natural infection and experimental challenge with EHV-1 virus induce a solid, albeit transient protective immunity for approximately 3-6 months [44]. Pregnant mares exposed to infection in early gestation are protected from aborting during the late, susceptible stages of gestation. Equine herpesvirus 1 abortions have been reported to occur mainly in first-foal mares and, although individual mares may suffer more than one EHV abortion, mares aborting in a particular year rarely abort in the subsequent year [60; 92]. The systemic humoral responses to infection have been comprehensively defined and are characterised by both a transient (<3 m) complement fixing (CF) and a longer (>12 m) virus neutralising (VN) antibody response. The principal antibody isotypes reported are IgM and IgG (a, b and c) with minimal IgA detected in the circulation. The VN antibody titres are poorly correlated with protection from infection, and high titres are reflected by reduced nasal shedding of virus rather than any block to viraemia. In contrast, the mucosal IgA response to primary and subsequent EHV-1 infections does correlate with protection. This suggests that although short-lived, IgA has an important role as a first line of defence against infection [10]. With regard to the cellular response to EHV-1 infection,

viraemia elicits a leukocyte response mainly from CD8+ and possibly CD4+ cells. The cytotoxic T lymphocyte (CTL) response which was refined recently using tests to measure the frequencies of CTL precursors (CTLp), has shown a correlation of high CTLp numbers with protection from infection and, significantly, from abortion post-EHV-1 challenge [10; 103].

2.8.1 Vaccination to protect against EHV abortion

Vaccination currently constitutes the mainstay of protection against EHV-1 abortion, despite uncertainty regarding its efficacy [36]. Studies examining the efficacy of vaccines for reducing the incidence of abortion are somewhat conflicting and confounded by methodological differences and limitations in experimental design [36; 103]. Nevertheless, the widespread application of vaccination, probably in conjunction with other management measures, and improvements in diagnostic methodologies and disease reporting appear to have reduced the incidence of EHV-1 abortions in several populations [36]. Vaccination protocols to prevent EHV-1 abortion aim to stimulate immune responses that incorporate both neutralising antibody and CTL cells that will lyse virus-infected cells and thus reduce or eliminate the cell-associated viremia responsible for uterine infection [103; 104]. However, the increased serum antibody titres resulting from vaccination were reportedly unable to reduce the duration of viraemia or prevent abortion during an experimental challenge with EHV-1 [103]. On the other hand, examination of the protective immune responses demonstrated that vaccination will increase secretion of respiratory tract mucosal antibodies, albeit transiently. These antibodies prevent infection of the respiratory tract and reduce respiratory shedding in vaccinated horses; indeed, inactivated vaccines are able to induce high VN antibody frequencies which in turn limit spread of virus during an outbreak [105; 106]. Currently, the vaccines that claim to offer protection against EHV-1 abortion are inactivated whole-virus single-component (EHV-1) vaccines e.g. Pneumabort K-1B (Fort Dodge) and Prodigy (Intervet), or combinations (EHV-1 and -4) such as Duvaxyn (Fort Dodge) that are administered to mares at 5, 7 and 9 months of gestation [107].

Immunological protection against EHV-1 is associated with high neutralising antibody titres and EHV-1-specific CTLp levels in the peripheral circulation [103]. Modified live vaccines (MLV) would thus be expected to offer at least partial protection against the development of EHV disease, an assumption that helped define goals for improved future vaccine developments [108; 109]. It is proposed that vaccine formulations should induce both systemic and mucosal immune responses, as reflected by high frequencies of both CTLp and mucosal VN antibody

[10]. A recent study in a large group of pregnant mares comparing the inactivated combination vaccine with a commercial EHV-1 monovalent MLV vaccine, found no difference in abortion rates or EHV-1 specific antibody titres, albeit with minor enhancement in neutralising antibody titres at delivery and for the first three months of life in foals whose dams received the MLV vaccine [108]. A challenge trial that similarly compared the MLV and inactivated bivalent vaccines in a group of young ponies reported both suppressed clinical disease and reduced nasal shedding, while inactivated vaccine reduced the duration of viraemia [110].

2.9 Control and prevention of EHV-1 disease outbreaks

Any notion of eliminating of EHV-1 from horse populations is unrealistic because their ability to maintain an endemic status within the global population appears assured. This is mainly because of the early establishment of life-long latency, despite the presence of maternal antibody and the ability of the virus to modulate or suppress host immune systems [111]. The additional silent cycles of sub-clinical or mild respiratory disease, during which infectious virus is shed, facilitate transmission and re-infection with sequelae such as abortion in closed-herds without recent, prior exogenous viral exposure, further complicate any plans for control. Currently-available vaccines are unable to reliably prevent initial infection, viraemia or latency. Controlling clinical disease syndromes is consequently dependent on reduction in both the magnitude and duration of nasopharyngeal viral shedding and cell-associated viraemia to secondary replication sites.

2.9.1 Outbreak intervention and preventative strategies for EHV-1 abortion

Consideration of risk factors to minimise viral transmission

Recommendations for control generally target minimisation of exposure to the three principal sources of infectious EHV-1, namely; respiratory shedding by actively-infected horses, environmental contamination and transfer *via* fomites of the large amounts of virus associated with the fetus, membranes and lochia from the reproductive tract of aborting mares, and recrudescence of endogenous virus in a latently-infected carrier [112]. Infected neonatal foals are an additional potential source of infectious virus that warrant consideration in the event of epizootics. Transmission generally requires close contact facilitating infection via the URT mucosa. In addition, fomitic transmission *via* contaminated hands and clothing of personnel,

feed, water, equipment and vehicles is possible. Minimising exposure on a breeding farm is achievable *via* interventions including vaccination, subdivision of pregnant mares into smaller groups, physical separation of groups, isolation of newly acquired horses and reduction of physiological stress, because of the association with endogenous recrudescence of latent virus [36; 112].

Epidemiological risk factors for EHV-1 abortion epizootics associated with management practices (summarised in Table 3) include; large groups of susceptible, heavily-pregnant mares; the introduction of horses, in particular pregnant mares, into established social groups; absent or incomplete vaccination, physiological stressors such as international or local road transport, competition events and high-stocking densities particularly with co-mingling of epidemiologically distinct populations and exacerbated by winter-housing [16-18; 37; 40; 65; 70; 71]. A negative bias on the outcomes has been associated with absent or inadequate isolation facilities or quarantine protocols and prolonged intervals from a first abortion to establishing an aetiological diagnosis [37; 40; 64; 70; 71].

Intervention strategies during EHV-1 abortion outbreaks

The recommended priorities during suspected epizootic EHV-1 disease are firstly to make an early diagnosis, and thereafter to prevent further spread of infectious virus both within and beyond the index group, and thirdly to manage any clinical cases [36; 112]. Key principles for controlling the spread of virus are: subdivision into small, epidemiologically-isolated subgroups; minimise introduction of exogenous and endogenous (stress-induced recrudescence) virus; and vaccinate to maximise herd immunity [36; 112]. The management of clinical cases is possibly of less relevance in the case of an aborting mare than in cases of neonatal disease and EHM, as there are few reports of clinical complications or mare mortality other than isolated incidences of obstetrical complications including uterine prolapse and RFM (Tables 1 and 2).

A review of case reports describing abortion epizootics over the past decades has highlighted the following interventions:

- movement restrictions onto and within an affected property [17; 18; 64; 71];
- separate aborting mares to an isolated area [18; 37; 71];
- separate clothing and equipment for use on isolated groups [17; 18; 71];

- sampling of resident horses by nasal swabbing, and blood (serum and EDTA); clinical monitoring for signs of disease, e.g. rectal temperatures for pyrexia [17; 18];
- subdivision of susceptible mares into smaller groups [71];
- treatment of aborting mares with antimicrobials and topical antiseptics [37; 71];
- secure removal of abortus and membranes for disposal by incineration [17];
- disinfection of the contaminated environment [37; 64; 71] and
- minimise physiological stress [18].

Diagnostic measures to enhance intervention strategies

After any late-term abortion, measures to limit spread of possible infectious virus should be implemented without delay and maintained until a diagnosis that excludes EHV is established. Achieving a rapid laboratory-based aetiological diagnosis enables the implementation of specific intervention strategies that may more effectively minimise or contain further spread of infection. Several reports detail both the deleterious effects of delayed diagnosis and, in contrast, the benefits of rapid diagnosis on eventual outcome (Table 2). An early and accurate diagnosis is currently best achieved from abortions by qPCR assay which is more practical, robust and has both superior sensitivity and specificity and, moreover, a potentially rapid turn-around time compared to histological and viral isolation methods [36; 96]. An appropriate diagnostic action plan and readily-available sampling materials, including suitable swabs and containers, are a prerequisite to rapid diagnosis [36].

Sample submissions from abortions should include both fetal and placental tissues, and ideally the entire fetus and fetal membranes for post-mortem investigation backed up by histopathology and virology (PCR and virus isolation). Practical constraints imposed by geographical location and distance from a diagnostic centre, costs and the presence of scavengers may limit the efficacy of the diagnostic plan. This may be addressed by submitting appropriate samples in duplicate in both buffered 10% formalin and unfixed in sealed, UV-protected containers for molecular diagnostics. Submission of whole blood and nasal swabs from suspicious neonatal foals, and nasal and caudal reproductive tract (lochia) swabs from aborting or in-contact broodmares and other suspect or in-contact horses is also advised.

Biosecurity measures to limit exposure of susceptible horses

Various EHV or EHV-1 abortion specific outbreak management protocols have been produced [36; 91; 112]. Prompt separation into isolated groups of post-abortion mares likely to be shedding high viral loads *via* the infected lochia, together with the secure disposal of aborted material and efforts to decontaminate the environment will significantly assist in reducing spread of infectious virus. Attempts to contain infectious virus within the initially-affected group in an outbreak scenario includes the application of a 'DISH' protocol, i.e.: **disinfection** of areas potentially contaminated by the large amounts of infectious virus associated with the aborted fetus, fetal membranes and lochia; **isolation** of affected horses; **submission** of clinical samples to a diagnostic laboratory; and **hygienic** procedures to prevent spread of infection (biosecurity).

Valuable guidelines include:

- AAEP guidelines for Infectious Disease Outbreak Control (http://www.aaep.org/info/infectious-disease-control);
- Equine Herpesvirus -1 and -4 Related Diseases (http://www.aaep.org/custdocs/EquineHerpesvirusFinal030513.pdf); and
- The UK's Horserace Betting Levy Board (HBLB) Codes of Practice (http://codes.hblb.org.uk/).

The post-abortion mare and any horse exhibiting clinical signs (e.g. pyrexia, nasal discharge or neurological deficit) are kept physically isolated until the infection risk has passed. This may be achieved by measures including geographical removal with intervening physical and sanitary barriers, assisted by chemical disinfection. Personnel caring for isolated animals must use protective clothing, hand washes and disinfectant footbaths and, ideally, are restricted from contact with other horses. Feed and water sources are separated and appropriate bedding and waste disposal measures implemented.

In-contact animals include mares in the same paddock as the aborting mare or those in adjacent areas in direct contact *via* fence-lines and considered to be at high risk of potential exposure to infectious virus. Relocation or co-mingling of in-contacts during an outbreak poses a significant potential risk for further dispersal of virus. These animals require movement-restricted quarantine and daily monitoring for signs suggestive of EHV-1 infection. This may include twice-daily measurement of rectal temperature and observation of nasal discharge, in addition to monitoring for abortion. The addition of nasal swabbing and blood sampling to detect viraemia and virus shedding may be useful adjuncts. The in-contact group often includes the most susceptible population i.e. pregnant mares in advanced gestation. This is why the

immediate separation of this cohort of pregnant mares (preferably with subdivision into small groups) is advised to reduce abortion rates within the population [13; 36; 112]. These mares should also be subject to isolation measures until they themselves abort or deliver either a diseased or a live-healthy foal. An important additional consideration is the potential physiological stress resulting from social disruption following separation of an established resident group [36].

Isolation and quarantine intervals

The recommended intervals for isolation and quarantine in the face of EHV-1 disease outbreaks are somewhat conflicting. A 21 d interval from last recorded disease incident was previously advocated as reasonable, based on three times the shedding interval for EHV-1 (7 d) by mature horses [112]. This was then extended to 28 d in the AAEP guidelines published in 2006, chiefly because of the observation of more protracted shedding intervals associated with EHM [36; 112]. Alternatives that have been suggested, primarily to expedite reduction of quarantine to 14 d, are supplementary nasal swabbing for qPCR analysis of all resident horses for two to four consecutive days. This may be augmented by twice daily monitoring of rectal temperatures, aiming at 14 consecutive pyrexia-free days followed by qPCR assay for virus presence [36]. The HBLB 'Codes of practice' are more prescriptive in defining the recommended response to EHV-1 abortion and neonatal disease, as compared to the response applicable to respiratory and neurological disease outbreaks (http://codes.hblb.org.uk/). The following is an excerpt from the most recent code (2015), which recommends a 28 d isolation and movement restriction protocol for EHV-1 abortion:

'Barren mares, maiden mares and mares with healthy foals at foot on the affected premises can be moved 28 d after the last EHV abortion, providing they can be placed in quarantine for 14 d following arrival at their new premises. Serological monitoring at a 10-14 d interval to look for signs of seroconversion during the period is advised. It may be possible, under the direction of the attending veterinary surgeon and in consultation with stud owners/managers of where they may move, to move non-pregnant mares earlier than 28 days'.

Additional points to note concerning advised management of broodmares on an affected farm include withholding aborting mares from breeding until their second post-partum oestrus or, alternatively until 30 d post-partum [112]. Most recommendations agree that in-contact

pregnant broodmares must remain resident on the affected farm until they either abort or foal normally at term (http://codes.hblb.org.uk/).

Vaccination during the course of an outbreak

If confronted with an EHV-abortion epizootic it is probably useful to vaccinate horses that are at increased risk of exposure. In previously-vaccinated horses, a booster vaccination may provide a rapid anamnestic response that will facilitate attempts to reduce spread of infectious virus. The possibility that vaccination in the face of an outbreak exacerbates the risk of neurological disease is poorly substantiated and controversial [36].

2.9.2 Strategies for preventing abortion epizootics

It is noteworthy that multiple abortions due to EHV-1 rarely, if ever, occur on fully vaccinated studs and have predominantly been reported in unvaccinated and densely stocked broodmare populations [39]. On the other hand, broodmare management reliant solely on vaccination without effective biosecurity and infection control measures will also be unlikely to prevent abortion episodes [15; 61]. The aim should be to apply management strategies that minimise or prevent exposure to infectious virus together with concurrent application of an effective immunisation strategy [39]. Key preventative interventions in addition to appropriate immunisation are maintaining smaller, isolated groups of broodmares of a similar gestation stage. Avoiding movement of horses, in particular avoiding the introduction of late-gestation pregnant mares to an established group simply because they have a similar foaling date, as is commonplace in Thoroughbred breeding worldwide, is a highlighted recommendation [36; 70; 112].

Recent reports recommend the following preventative strategies based on observations during outbreaks:

- ensure appropriate vaccination of introduced horses [17];
- restrict contact of pregnant broodmares with other populations [16; 17];
- effective quarantining or isolation of new arrivals [16];
- subdivision into groups based on age, sex and management [16]; and
- separation of all resident and transient animals [16].

Vaccination for prevention of EHV-1 abortion

As described above, vaccination with either a modified live or an inactivated virus vaccine with additional sanitary measures provides partial clinical and virological protection against EHV-1- and -4-induced diseases, including a reported decline in epizootic abortion episodes with widespread vaccination [108; 110; 113]. The prevention of EHV-1 abortion based entirely on vaccination is currently unrealistic, primarily because of the biology and evasion strategies evolved by herpesviruses over millennia. In addition, and largely because of these adaptive features, obtaining a 'herd-protection' by vaccination against the EHVs, unlike for other viruses, requires an extremely high vaccination rate. A recent paper reported that sustaining a vaccination rate of 79.3-85.3% for an entire population of three year-old Japanese racehorses resident in training facilities was required for the prevention of epizootic pyrexia due to EHV-1 [49]. By extrapolation, if a similar vaccination rate is required to prevent abortion, it is unlikely to be achieved on breeding farms where geography, demographics and environmental factors influencing susceptibility to EHV-1 epizootics are very different.

Stress reduction to prevent recrudescence of infectious virus

The physiological stress response in horses has been described in terms of various metabolic, immunological and neuro-endocrinological mechanisms, including hormones that reflect the central roles of the hypothalamic-pituitary-adrenocortical axis and the sympatho-adreno-medullary system [114-118]. Increases in cortisol levels after long-distance journeys confirm that cortisol is a useful indicator of stress in horses [116-118]. Prolonged exposure to stress is presumed to cause immunosuppression and increased susceptibility to disease in horses [114; 117]. However, while EHV recrudescence with nasal shedding of infective virus has been initiated experimentally by administration of immunosuppressive agents including corticosteroids, in-the-field proof of an association between potentially stressful events and recrudescence is lacking [87], and the risk factors for reactivation (including stressors) are poorly understood. Few reported studies have investigated the relationship between EHV shedding and specific stress factors, and one such study describing long-distance transport of horses documented only a low incidence of viral reactivation and shedding [85]. The reports that have been produced are additionally limited by the absence of data to prove the existence of or to quantify extent of physiological stress. A list of procedures or events that have been suggested to act as stressors include weaning, castration, long-distance transport, strenuous

exercise and concurrent disease [86]. In addition, disruption of social hierarchies via introductions to and removals from broodmare groups are considered to be important stressors. The consignment of pregnant broodmares for sale has anecdotally been associated with subsequent EHV-abortion, assumed to be associated with exposure to one, or a combination of, potential social, environmental and management-associated stressors. The exposure to stress and reactivation of EHV infection in these mares at a vulnerable (late) stage of pregnancy apparently fulfils many of the criteria currently assumed to contribute to the pathogenesis of EHV-abortion.

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Table 1 Summary of epidemiological factors associated with EHV-1 and -4 abortion and diagnostic methods from 14 selected retrospective surveys in eight countries during 1969-2011

Incidence & diagnostic rates of abortion, stillbirth & neonatal death	EHV-1 & -4 prevalence	Diagnostic method to confirm EHV	Breed	Country	Additional epidemiological data	Interval	Ref.
166 abortions	EHV=5	VI; HP	TB	UK	EHV>8.75 m gestation	1969-71	[72]
393 cases a. 1977: 5 sites b. 1981: 3 sites abortion; stillborn; neonate†	a. EHV=46 b. EHV=14	VI; HP; VN	All	Aus (NSW)	respiratory disease & abortion; sero-survey of yearlings: most = both sub-types 1 & 2	1977-82	[73]
1211 cases abortion; stillborn; undiagnosed = 16.9%	EHV-1=41 (3.3%)	VI; IF	TB=75%; SB=10.2%	USA (KY)	vaccinated; no epizootics; non-infectious : infectious = 2 : 1; placentitis = 24.7%	1988-89	[74]
254 EHV isolates abortion; neonate†	EHV-1=244 (96%); EHV-4=10 (4%)	VI; HP (IP); ELISA	All	NL	vaccination status undefined	1983-95	[66]
290 cases abortion; stillborn	EHV-1=26 (8.9%)	VI; HP	22 breeds: SB=30%; TB=14.1%	USA (MI)	EHV-1 in SB=2x other breeds; EHV-1 in TB: MI% ≈ 3x KY%; placentitis = 12.7%	1985-96	[75]
1252 cases abortion; neonate†	EHV=6.5%; EHV-1=95%; EHV-4=5%	VI; HP (IP)	All	UK	epizootics in unvaccinated mares; epizootics in high stocking density; placentitis = 9.8%	1988-97	[65]
241 abortions fetus & fm submissions	EHV-1=15 (6.2%); a. typical=9 (60%) b. atypical=6 (40%)	VI; HP (IP); PCR	All	UK	atypical case series (b): TB=5, Welsh = 1; vaccinated; dystocia = 2	2001-03	[62]
101 cases a. fetus = 93; neonate†=8 b. fm=76	a. EHV-1=15 (14.9%) neonate†=1 b. EHV1=11 (14.5%)	VI; IF	All	Hun	vaccination status undefined; EHV-1: RFM=1 (9%)	1988-2000	[63; 76]

Incidence & diagnostic rates of abortion, stillbirth & neonatal death	EHV-1 & -4 prevalence	Diagnostic method to confirm EHV	Breed	Country	Additional epidemiological data	Interval	Ref.

407 cases
fetus = 279;
stillborn = 49;
premature =77
	EHV-1=59/407 (15%)	VI; HP; qPCR	TB=45%;
			SB=39%;
			SFr=11%;
			All=5%
				Fr	EHV-2=3;
					EHV-5=1;
					EHV-3=0;
					EHV-4=0
						2002-05	[11]

426 EHV-1 isolates all = sporadic abortions	1960's=3.3%; 1990's=14.4%; 2000-06=19.4%	qPCR (ORF30)	TB	USA (KY)	between 1960's-2006: increased prevalence of ORF30 G_{2254}	1951-2006	[15]
1822 cases diagnosis = 74.9%; infectious abortions >47.7%; fm lesions ≈ 50%; bacterial = 79.9%; viral = 15.1%	EHV-1=6.9%; EHV-4=0.2%	VI; HP; qPCR	All; TB=49.6%	Fr	vaccinated mares: fewer EHV-1 cases; 91.2% abortion; >6 m gestation; 79.4% EHV-1 abortions = 8-10 m; seasonal abortions; placentitis = 9.9%	1986-2009	[33]
66 EHV-1 isolates outbreaks (n=6)=32; sporadic cases = 34	EHV-1=66; ORF30 G_{2254}=7 (10.6%); ORF30 A_{2254}=59 (89.4%)	VI; qPCR (ORF 30)	All	Ger	vaccination status undefined; 1 outbreak: neuro signs pre-abortion; ORF30 G_{2254} shed in semen	1987-2009	[67]

116 cases
diagnosis = 68 (58.7%);
fm submissions = 79%;
abortion = 67;
stillborn = 22;
neonate† = 14;
placental disease = 3
	EHV-1=22 (81.5%);
	ORF30 G_{2254}=16 (72.7%);
	ORF30 A_{2254}=6 (27.3%)
		VI; HP; qPCR (ORF 30)	All	It	vaccination in most mares;
					EHV-1 = main cause abortion;
					EHV-1 co-infected with EHV-2 &-5;
					EHV associated with co-infection;
					EHV associated with PPS
						2004-11	[68]

Key: All: multiple or undefined breeds; Aust: Australia; CF: complement fixation assay; EHM: equine herpes myeloencephalopathy; EHV-1, -2, -3, -4, -5: equine herpesvirus types -1, -2, -3, -4, -5; ELISA: enzyme-linked immunoassay; Fr: France; fm: fetal membrane; Ger: Germany; HP: histopathology; Hun: Hungary; IF: immunofluorescence; IP: immunoperoxidase staining; It: Italy; KY: Kentucky; ORF30 G_{2254}: neuropathogenic genotype; ORF30 A_{2254}: non-neuropathogenic genotype; neonate†: neonatal death; NL: Netherlands; NSW: New South Wales; MI: Michigan; other: other breeds' ORF 30: ORF 30 A/G_{2254} allele discrimination qPCR assay; PCR: polymerase chain reaction assay; PPS: premature placental separation; qPCR: quantitative (real-time) polymerase chain reaction assay; QH: Quarterhorse; RFM: retained fetal membranes; SFr: Selle Francais; SB: Standardbred; UK: United Kingdom; VI: virus isolation; VN: virus neutralisation test.

Table 2 Summary of the epidemiological data and diagnostic methods in eight selected case reports describing EHV-1 abortion epizootics in seven countries during 1983-2012

Case presentation	EHV-1 & -4 prevalence	Diagnostic method to confirm EHV	Breed	Country	Epidemiological data including vaccination status	Year	Ref.
30 cases abort = 22 neonate†=8 (?)	EHV-1=30 (100%)	VI; DNA REA	Lip	Austria	no vaccination; 17 mares showed EHM & 10 died (8† either peri-partal or post-abortion)	1983	[69]
44 pregnant mares abort & neonate†=33 (75%)	33 submissions EHV-1=32 (97%)	VI; HP	TB	Aus	no vaccination; all mares in 1 camp; no premonitory signs; no introductions; no complications	1986	[37]
12 pregnant mares abort = 7 (58.3%); neonate†=1 (8.3%)	EHV-1=8 (66.7%)	VI; IF; PCR; serology	Trot	NL	post-transport; introduction into resident groups; 1† due to uterine prolapse post-abortion	1991	[64]
173 pregnant mares abort = 50 (28.9%)	EHV-1=50 (28.9%)	VI; VN	Polo	Arg	vaccinated; mares in 4 groups on 3 sites; assisted reproduction centre; post-transport; introduction into resident groups; large groups pregnant mares; no isolation protocols; delayed diagnosis	1998	[70]
21 mares (18 pregnant) abort = 11/18 (61%)	4 submissions EHV-1=4 (100%) atypical=1 (25%)	HP (IP); PCR; CF	W/A	UK	unvaccinated; large groups pregnant mares; atypical; PPS in all submissions; delayed diagnosis; serology unreliable	2004	[71]

Case presentation	EHV-1 & -4 prevalence	Diagnostic method to confirm EHV	Breed	Country	Epidemiological data including vaccination status	Year	Ref.

Site 1: 40 pregnant mares
abort = 4 (10%);
neonate†=1 (2.5%);
EHM=6 cases (4†)
Site 2: 32 pregnant mares
abort = 17 (53.1%);
neonate†=3 (9.4%);
EHM=1 case (mild)
	Sites 1 & 2:
	EHV-1=5 (12.5%)
	ORF30 G_{2254}
		VI; PCR; serology	Site1: QH
			Site 2: Lip
				Croatia	no vaccination;
					international transport;
					introduction between Sites 1 & 2;
					breed-dependant abortion rate: Lip>QH
						2009	[16]

Population = 79 horses **pregnant mares =7** abort & neonate†=6 (85.7%); EHM=8; pyrexia = 55	EHV-1=6 (85.7%) ORF30 G_{2254}	VI; HP; qPCR; VN	SpH	Ger	vaccination status variable; high stocking density; mixing epidemiological groups; no quarantine protocol **aborting mares:** EHM preceding abortion = 3 (50%); PPS=1 (16.7%); RFM=4 (66.6%); dystocia = 1 (16.7%)	2009	[17]
25 pregnant mares abort = 16 (64%); neonate†=2 (8%)	EHV-1=18 (72%) ORF30 G_{2254}	VI; qPCR; VN; ELISA	SB	Ger	vaccinated; international transport; introduction into resident groups; no quarantine protocol; no EHM	2012	[18]

Key: All: various breeds; Arg: Argentina; Aust: Australia; CF: complement fixation assay; EHM: equine herpes myeloencephalopathy; EHV-1, -4: equine herpesvirus types 1 and 4; ELISA: enzyme-linked immunoassay; Ger: Germany; HP: histopathology; IF: immunofluorescence; IP: immunoperoxidase staining; ORF30 G_{2254}: neuropathogenic genotype; Lip: Lipizzaner; neonate†: neonatal death; NL: Netherlands; PCR: polymerase chain reaction assay; qPCR: quantitative (real-time) polymerase chain reaction assay; Polo: Polo pony; PPS: premature placental separation; QH: Quarterhorse; REA: restriction enzyme analysis; RFM: retained fetal membranes; SB: Standardbred; SpH: Sports horse; TB: Thoroughbred; Trot: Trotters; UK: United Kingdom; VI: virus isolation; VN: virus neutralisation test; W/A: Welsh Section A pony

Table 3 Epidemiological risk factors identified in EHV-1 abortion epizootics in selected case series and retrospective surveys reported in eight countries during 1991-2014

Reported risk factor	Country	Reference
International movement of asymptomatic animals	Croatia	[16]
	Germany	[18]
Immunosuppression associated with disease or hospitalisation	Germany	[18]
Absent, irregular or incomplete vaccination	Australia	[37]
	Argentina	[70]
	UK	[65; 71]
	Poland	[40]
	Croatia	[16]
	Germany	[17]
Breed	Croatia	[16]
Recent transport	Netherlands	[64]
	Argentina	[70]
	Germany	[18]
Intermingling of different epidemiological groups	Netherlands	[64]
	Argentina	[70]
	Germany	[17]
Overcrowding and high stocking density	Netherlands	[64]
	Argentina	[70]
	Germany	[17; 18]
Disruption of social groups by introductions	Argentina	[70]
	Germany	[18]
Lack of isolation protocols or facilities	Australia	[37]
	Argentina	[70]
Large management groups of susceptible mares	Australia	[37]
	UK	[71]
Seasonal occurrence	France	[33]
	Poland	[40]
Financial constraints or other delays affecting diagnostic submissions	Australia	[37]
	Netherlands	[64]
	UK	[71]
	Poland	[40]

CHAPTER 3
A survey of reproductive success in South African Thoroughbred breeding from 1977 to 1999

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Abstract

The data and an analysis of the statistical summaries from the 'Return of Mares' of the *General Stud Book* of The Jockey Club of Southern Africa from 1975-1999 are presented. The total number of mares covered per season ranged from 7393 (1992) to 5180 (1995). The total living produce in the period surveyed was 95317 foals. The categories of data examined include: the total number of mares covered per season; the total numbers and percentage of their living produce; the total numbers and percentage dead produce, 'slips' and foals born dead, barren and 'no return' mares; and the total number of live twins reported. The percentage live foals per season increased from 52 to 62% and the percentage barren and 'no return' mares declined from 35.50 to 28.40% over the period surveyed. The number of live twins reported showed a dramatic reduction from 156 to 5. These apparent improvements are ascribed to a combination of factors including advances in veterinary knowledge and technology. The findings are similar to those reported by similar surveys of national Thoroughbred populations from North America and Germany. There is an indication to broaden this annual survey by recording additional parameters more accurately reflecting reproductive efficiency rather than a cumulative annual total of live foals.

Keywords: horse, reproduction, Thoroughbred

Introduction

Reproductive success as an assessment of reproductive performance

Whether a specific mare produces a live foal or not will define her reproductive success. Reproductive success as an assessment of reproductive performance of broodmares is most commonly documented by recording foaling rates by either one of two methods. First, per season, and second, per cycle foaling rates. An increase in reproductive success for an individual mare, farm or population is reflected by an increase in either or both of these[4].

The per season rate is independent of the number of coverings (and cycles) it took to result in a live foal. There is also little indication of the role of pregnancy attrition prior to the point of foaling. It fails to adequately evaluate the contribution of the stallion and other aspects central to reproductive management. The preferred parameter is thus the per cycle rate as it takes into account the number of oestrous cycles a mare is bred in order to

produce a foal. Recording this parameter allows analysis of reproductive efficiency in terms of not only the cumulative seasonal success, but also the management effort and veterinary input.

Average ('normal') reproductive performance of broodmares

Internationally, the largest record repositories for broodmare reproductive performance are those for Thoroughbreds provided by the various national jockey clubs. Generally these are annual records of the live returns to registered mares comprising a national population on a per season live foaling rate. The jockey clubs are currently unable to monitor and record each cycle on which a particular mare is bred. No per cycle population success rates are thus available. There are, however, data in several instances providing per cycle live foaling rates for particular farms.

Records of reproductive performance in Thoroughbred horse populations

Surveys of pregnancy rates per cycle reportedly vary from 43-56% in Thoroughbreds, Standardbred and Quarter Horse mares[7]. These authors also report cumulative end-of-season pregnancy rates and foaling rates of 70-80% and 50-70%, respectively, in a number of studies. Surveying Thoroughbred population data over a 5-year period in North America showed a yield of 241958 foals (a 5-year average of 59%) for 40512 stallions bred to 408275 mares[4].

Reproductive performance of the Thoroughbred as a breed has been and continues to be influenced by several additional factors. These primarily include a traditionally imposed official breeding season related to age-imposed horse racing. This is partially at odds with the normal physiological breeding season. The mare is seasonally polyoestrous with multiple oestrus (and ovulatory) cycles confined to a 'breeding season' determined by long day-length[4]. The artificially imposed Thoroughbred breeding season with the official birth date of all registered Thoroughbred horses being 1 August in South Africa obviously influences the opportunities for mating and establishment of pregnancy (reproductive success).

There have additionally been no selection criteria in the breed other than for pedigree and athletic performance. There is furthermore a complete restriction on any form of assisted or artificial reproduction technology.

Factors contributing to reproductive performance

A number of factors thought to contribute to reproductive performance have been reported[5-8]. Only a few of these factors have been assessed through controlled studies. The following list includes some of the reported factors thought to influence overall reproductive performance:

- booksize[5,7];
- mare availability[5];
- mare age[6,7];
- mare status[7,8];
- economic factors[6];
- Stud Health programmes[6];
- stallion factors[5,6,7,8]; and
- month of mating[5,7].

Table 1 Summary of reproductive data of registered Thoroughbred mares in southern Africa for breeding season intervals from 1975-1999

Year of birth	Mares covered	Living produce		Dead produce		Slips and stillborn		Barren & no return		Twins
	(n)	(n)	(%)	(n)	(%)	(n)	(%)	(n)	(%)	(n)
1975-79	30386	15887	52.3	930	3.1	2866	9.4	10679	35.1	192
1980-84	3 066	19165	51.2	1348	3.6	3189	8.6	10025	27	165
1985-89	34371	20364	59.2	1327	3.9	2976	8.7	9888	28.8	138
1990-94	33410	19892	59.5	1272	3.8	2590	7.8	9767	29.2	25
1995-99	27535	16917	61.4	682	2.5	2076	7.5	7967	29	5

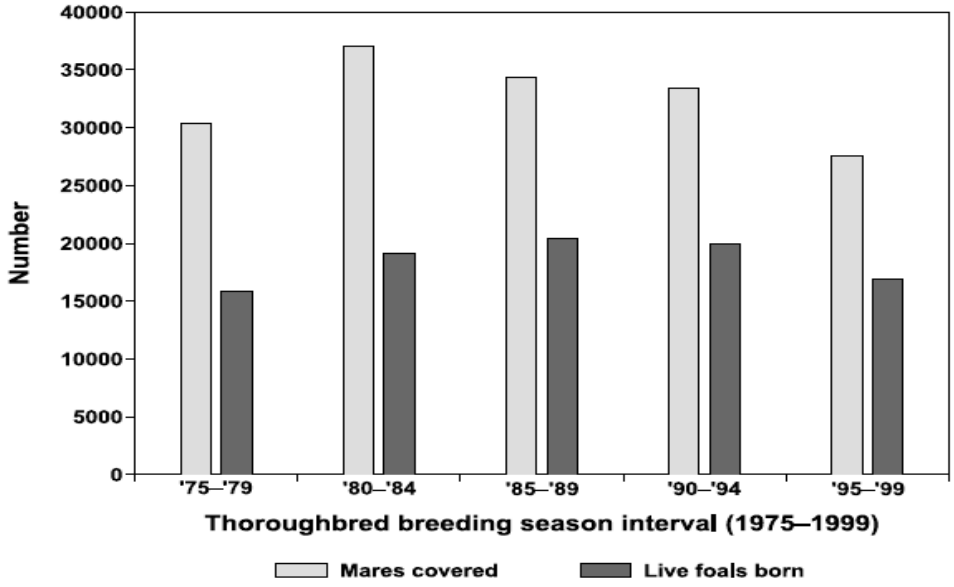

Figure 1 Number of registered Thoroughbred mares covered and live foals produced during each of the 5-year intervals of the study (1975-1999)

The Jockey Club of Southern Africa initiated statistical analysis of reproductive performance in 1974 by recording 'living produce'. Until the 1970s, all reports seem to indicate only that the live foal percentage was below 50%, with a mare population of approximately 6500. There was reportedly an 'overall pregnancy rate' of 72% for the years 1960-1962[1].

Materials and methods

This retrospective study analysed the data available from the statistical summaries published annually from 1976 by the Jockey Club of Southern Africa. The publications appeared as the *General Stud Book of South Africa,* Supplements to Volumes 21-25 (Return of Mares 1975-76 to 1990-91) and *The General Stud Book of Southern Africa,* Supplements to Volumes 25-27 (Return of Mares 1991-92 to 1999-2000)[2,3]. These summaries are derived from the information supplied to the Jockey Club on an annual basis for purposes of registration of mares at stud and their living produce. The reported data were subdivided into several categories selected for this study as being the most accurate indicators of average reproductive performance of Thoroughbred broodmares. The categories were the following: total number of mares recorded; total number of mares covered; total number of living produce (including the total number of

colts and fillies, respectively); and total number of live twins reported. The percentage of live foals born by month was also derived from the data.

The categories of 'slips and stillborn' and 'barren and no return' as reported were also examined. The 'no return' component included in the category of 'barren and no return' mares is difficult to define and report on with accuracy. The category includes barren mares, mares that have aborted singleton foetuses or twins, mares that produce weak or dead singleton foals or twins at term, foals dying *post partum* and foals that are not registered. There is a probable overlap of this category with that of 'slips and stillborn'. Both categories probably reflect other components of pregnancy wastage and incomplete reporting, including unreported barren mares, dead or stillborn foals, 'slipped foals' and twins.

Data analysis

All categories were examined within five data cohorts, each comprising five successive breeding seasons. The first cohort shows data reported for the years of birth 1975-79 and represents the produce for mares covered in the breeding seasons of the years 1974-78. The last cohort similarly represented the data for the years of birth 1995-99, and of mares covered during the 1994-98 breeding seasons. The means of the categories were then calculated for each 5-season cohort. The first and last cohorts were then compared to establish any trend between the inception of data reporting and the most recent breeding seasons.

Results

Table 1 summarises the data surveyed between 1975 and 1999. All categories examined are shown. The largest number of mares covered in a particular season (data not shown) was in 1992 (7393). The lowest number of mares covered was 5180 in 1995 (data not shown). Between 1975 and 1999, a total of 95317 live foals was born.

Between 1975 and 1979, a mean annual live foal production rate of 52.3% was recorded. The final data cohort (1995-99) shows 61.4% mares covered produced live foals (Figure 1). The last season surveyed (1999), shows a live foal percentage of 62.0% (data not shown).

The reported percentages of both dead produce and 'slips and stillborn' in the study period has stayed relatively consistent with a mean cohort range of 2.5-3.9% and 7.5-9.4%, respectively (Table 1).

The 'barren and no return' category however has shown a positive trend with a decline from 35.1% in the 1975-79 breeding seasons to 29.0% in the final cohort until 1999 (Table 1).

The reported incidence of living twins was radically reduced with a mean of 127.75 live twins per season between 1975 and 1990 dropping to a mean of seven reported live twins per season between 1991 and 1999 (data not shown).

Figure 2 shows the percentage of live foals born per month for the 1975-99 breeding seasons.

Discussion

The South African survey presents data similar to those reflected in other studies undertaken in recent years covering a similar period and categories of Thoroughbred horse populations in the North America and Germany[4-6]. These reported on the much larger and smaller North American and German Thoroughbred populations, respectively, as well as data from four well-managed stud farms in the State of Kentucky[4-6].

The number of mares being covered per season is similar at both the beginning of this survey and the present day. This period also marks some significant fluctuations in the economic scenario in South Africa. By extrapolation to data from other countries it can be assumed that this is reflected in the number of mares covered per season and hence the number of live produce, if not the percentage on a per season basis.

The percentage of living produce over the period from 1980 was consistent around the 60.0% mark. This reflects a considerable improvement on the mean of 52.4% in the initial period surveyed from 1975-1979. During this latter period of the survey there were several significant innovations in breeding technology and advances in the field of equine reproduction applied in South Africa. From this period onwards there was increased utilisation of PgF2-alpha and other pharmacological agents for manipulation of the mare's cycle and induction of ovulation. The widespread utilisation of diagnostic ultrasound from the mid-1980s and the Stud Health Scheme in 1980 has undeniably also played a role.

The categories of percentages of dead produce and of 'slips and stillborn' mares appears relatively unchanged. The percentage of 'barren and no return' mares as a proportion of the overall population is, however, declining and the incidence of twins being born is almost negligible.

The distribution by month of birth, of all live foals born between 1975 and 1999 has remained consistent throughout the survey with most foals being born in September and October each year.

Similar studies suggest that improved overall management and Health Schemes do have a positive impact on reproductive success. The positive effects on pregnancy rates of both increased mare availability and increased booksize in North American studies reflect this. These undoubtedly result from improved management, *e.g.* ensuring earlier ovulatory cycles and optimal stallion utilisation. Other important factors appear to be the application of infectious disease surveys, the average age of the mares in the breeding populations and the overriding dictates of economics.

The improvements in veterinary knowledge and technology contributing to a decline in the proportion of barren mares and pregnancy wastage due to factors such as twinning should arguably have yielded more dramatic improvements to the live foal yield. It is currently impossible to reliably comment on the impact of these innovations on reproductive efficiency as reflected by parameters such as per cycle foal rate due to the non-availability of these data. If these were available it will permit the analysis of the efficiency of reproductive management. Ultimately this may answer the question of whether or not real progress is being made.

There is an indication to broaden this annual survey by recording additional parameters more accurately reflecting reproductive efficiency rather than a cumulative annual (per season) total of live foals. This will require the recording of per cycle reproductive data such as a live foal percentage per cycle. Other useful parameters to evaluate would be recording the mean age of broodmares and the individual farm average booksize for their stallions as well as individual stallion booksize and their effect on both the per cycle and per season foal percentages. These data will be more readily accessed with the introduction of computerisation, and a standardised reporting system based on individual microchip identification of mares and their produce. This is readily integrated with the current computerised system of recordings obtained from all registered Thoroughbred broodmares, stallions and foals.

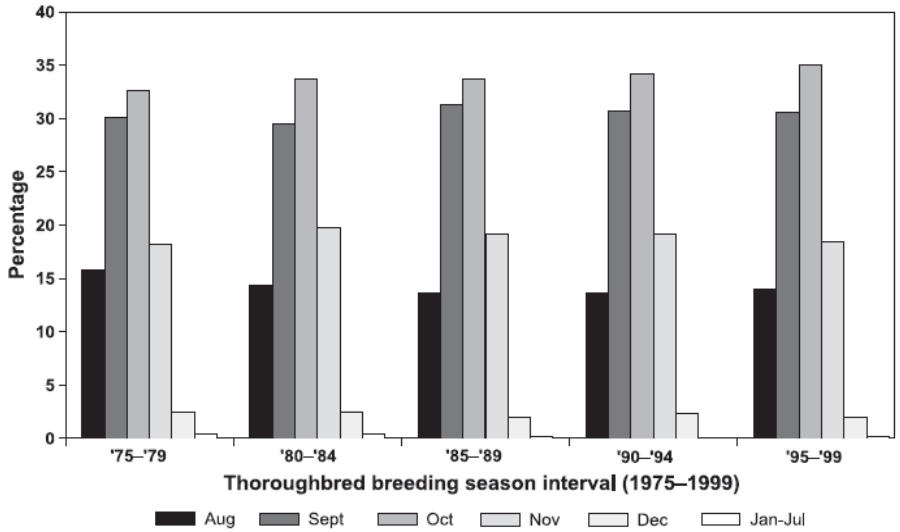

Figure 2 Percentage live foals born per month of each of the South African breeding season intervals in the study (1975-1999)

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CHAPTER 4
A predictive model for reproductive performance following abortion in Thoroughbred mares

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Abstract

Pregnancy losses include early embryonic death (E.E.D.) and later (post-implantation) abortion. Abortions, particularly equid herpesvirus (EHV-1) abortion epizootics cause severe economic and production losses. The long-term effects of EHV-1 and other abortions on subsequent reproductive performance in broodmare populations however remain undefined. This study described the relationships of E.E.D. and abortion with the following reproductive outcomes in Thoroughbred systems: breeding efficiency, month of last breeding, subsequent pregnancy and live foal rates. A prospective cohort study in broodmare populations following EHV-1 epizootics on two South African farms was used to develop predictive models of the relative influences and interactions of reproductive variables associated with EHV-1 and other abortion causes on reproductive performance. Early embryonic death predicted all the reproductive outcomes. Abortion predicted increased effort and month of breeding to establish pregnancy, but not becoming pregnant or foaling. Increasing age predicted decreased reproductive efficiency, and pregnancy and foaling probabilities. Mare reproductive status predicted breeding efficiency and the last month of breeding, but not establishing pregnancy. The last month of breeding predicted efficiency, pregnancy and foaling. Interestingly, breeding in the first month of the season was associated with an improved probability of pregnancy among barren mares.

Introduction

Various aspects of reproductive performance have been reported in Thoroughbred breeding systems. Reproductive variables include pregnancy rates (P.R.) per oestrous cycle or season (cumulative) and live foal rate (F.R.) (Bruck and others 1993, Morris and Allen 2002, Schulman and others 2003, Hemberg and others 2004, Allen and others 2007, Bosh and others 2009). Pregnancy losses include early embryonic death (E.E.D.) following confirmed pregnancy within six weeks of conception and later (post-implantation) loss or abortion. Mare age and reproductive status category are associated with reproductive performance, including pregnancy loss (which rises with age and barren status), and must be considered to understand reproductive outcomes in Thoroughbred populations (Bain 1969, Morris and Allen 2002, Hemberg and others, 2004, Allen and others 2007, Yang and Cho 2007).

Abortion causes are subdivided into infectious or non-infectious origin (Acland 1995, Jonker 2004, Laugier and others 2011) with equid herpesvirus (EHV-1) as the most important viral

cause of infectious abortion (Allen and Bryans 1986, Smith 1997, Gilkerson and others 1999, Gerst and others 2003, Smith and others 2003, Slater and others 2006, Brown and others 2007, Lunn and others 2009, Laugier and others 2011). Following abortion, EHV-1 virus rapidly clears from the genital tract, and future breeding capacity is unimpaired unless uterine damage has occurred from dystocia (Smith 1997).

This study aimed to describe the relationship between abortions and other reproductive variables with subsequent key outcomes in Thoroughbred pregnancy, including associated reproductive efficiency, and successful foaling. This study also aimed to enhance understanding of poorly supported assumptions regarding effects of EHV-1 and other abortion causes on reproductive performance.

Materials and methods

Design

A retrospective analysis of reproductive events affecting pregnant broodmares during unrelated abortion epizootics from confirmed EHV-1 infection during 2007 (Farm 1) and 2009 (Farm 2), respectively was conducted on two Thoroughbred stud farms in geographically-separated areas of South Africa (Schulman and others in submission). This prospective cohort study was undertaken in the same broodmare populations to assess the association of EHV-1 and other causes of abortion with subsequent reproductive performance.

Background

Farm 1 is located in the Western Cape Province of South Africa. Nine of 30 (30%) pregnant resident broodmares aborted between May and September, 2007. All abortions were confirmed as caused by EHV-1 through submission of fetal tissue and membranes for histopathology. One mare died following abortion, the remaining 21 foaled normally. During the subsequent breeding season (September 1, 2007 to January 31, 2008) all resident broodmares (n=45) were bred including 21 that foaled, eight surviving EHV-1 abortion mares (allocated as barren mares), and an additional 16 (4 foaling, 6 maiden and 6 barren) mares.

Farm 2, in KwaZulu-Natal Province, experienced an EHV-1 abortion epizootic between May and September, 2009. Eighteen (5.7%) EHV-1 abortions occurred amongst the resident pregnant mare population (n= 316). EHV-1 was confirmed by submission in all cases of fetal tissue and membrane samples using both qPCR assay and histopathology (Smith and others 1992, Gerst and others 2003). Forty three (13.6%) abortions were recorded during 2009, including 25 from other causes. A cause was diagnosed in 10 (40.0%) of the non-EHV abortions; six (24.0%) were associated with ascendant placentitis. During the subsequent breeding season (September 1, 2009 to January 31, 2010) 375 resident broodmares were bred, including 18 EHV-1 abortion mares and 21 non-EHV abortion mares, all re-allocated as barren mares.

Resident broodmare populations

Resident broodmares on both farms were divided into the following populations:

- non-abortion mares (foaling normally in the same season or not previously bred);
- abortion mares (aborting in the preceding season from any cause);
- EHV-1 abortion mares (aborting from EHV-1 in the preceding season);
- non-EHV abortion mares (aborting from non-EHV-related causes in the preceding season).

Mares were grouped into three age categories associated with Thoroughbred breeding systems: young (≤6 years), middle-aged (7-11 years), and old (≥12 years) mares.

Mares were further grouped by reproductive status: foaling (successful foaling in the current season); maiden (never previously bred); and barren (bred at least once previously but did not foal successfully in the current season, including the abortion mares).

Data

Sources of data were the two farms' broodmare and foaling records maintained by each respective farm's manager and veterinarian. The observation period commenced in the post-epizootic breeding (covering) season (September 1 to January 31) and ended with the associated foaling season from approximately August 1, one year later.

The variables selected for describing reproductive performance were: i) number of oestrous cycles per pregnancy or P.R. per cycle (by trans-rectal ultrasound at 14-16 d post-ovulation); ii) number of breeding attempts per pregnancy (from mare records); iii) incidence of E.E.D. observed ≤40 d post-ovulation (by trans-rectal ultrasound subsequent to initial positive

diagnosis); iv) cumulative pregnancy rate (P.R. cumulative) at the end of the breeding season by trans-rectal ultrasound and palpation; and v) successful (live) foaling (F.R.) in the subsequent foaling season.

The number of breedings was usually equivalent to the number of oestrous cycles. A maximum of two oestrus periods per month were available with an inter-ovulatory interval of 21-22 d (Aurich 2011). The farms commenced breeding in September (Month 1), continuing in October (Month 2) with most breeding attempts in November (Month 3) and declining through December (Month 4), with a negligible number of mares being bred in January (Month 5). The month of last breeding was an indirect indication of the month pregnancy was established. Early diagnosed pregnancy loss resulted in the mare being re-bred during a subsequent oestrus.

Data analysis

Statistical models were created for four defined outcomes to explain the relative influence and interactions of the different reproductive variables in the different broodmare populations in the subsequent breeding seasons. The data from both farms were pooled for analysis because 'farm' was neither a confounder nor effect modifier when examining main and interaction effects. Statistical significance was defined as $P<0.05$

Four outcomes were defined for these models:

- Pregnant at end of breeding season, analyzed with logistic regression, evaluated separately for all mares and only barren mares.
- Successful foaling in the subsequent season, analyzed with logistic regression.
- Number of breeding attempts to establish pregnancy between September 1 and January 31, analyzed as contingency table data using either an exact Kruskal-Wallis test for singly-ordered data (E.E.D., aborting mares, EHV and non-EHV-induced abortion, mare status) or an exact Jonckheere-Terpstra test for doubly-ordered data (age categories, month of last breeding), and as continuous data (age) using linear regression and Pearson correlation.
- Month of last breeding attempt between September 1 and January 31, analyzed as singly-ordered contingency table data (mare status, aborting mares) using an exact Kruskal-Wallis test.

Predictor variables evaluated by logistic regression analysis were:

- age
- mare reproductive status (barren, maiden or foaling)

- E.E.D. incident
- number of breeding attempts
- month of last breeding
- abortion due to any cause
- abortion due to EHV-1
- abortion due to non-EHV causes

Results of logistic regression analyses are presented as odds ratios (OR), 95% confidence intervals (95% CI), and P-values testing the null hypothesis that the OR=1. For continuous variables, the assumption of linearity in the log odds was verified in the analyses. Variables with significant odds ratios were then included in a multivariable logistic regression model, followed by using likelihood ratio tests to remove variables not significantly improving model fit.

Two logistic regression models were developed to predict probabilities of establishing pregnancy in the interval September 1 to 31 January 31 and for successful foaling in the associated season. Multivariable models were applied to hypothetical characteristic Thoroughbred broodmares typical of the internationally-expected demographic to illustrate the relative influence and interactions of the selected reproductive variables on the probabilities of two key outcomes.

Results

Reproductive performance of the different resident broodmare populations during the breeding seasons subsequent to the EHV-1 epizootics is summarized in Table 1 (Farm 1) and Table 2 (Farm 2). On Farm 1, although EHV-1 abortion mares required more cycles to become pregnant, all became pregnant compared with 89.2% of non-abortion mares. Twelve (26.7%) mares (including two EHV-1 abortion mares) subsequently aborted. The submission of samples for diagnostic screening universally failed to show EHV-1 or any other potentially epizootic-associated cause. The associated F.R. was similar in all mare populations and also to that reported in the previous, EHV-1 epizootic-associated season. On Farm 2, a similar P.R. per cycle and E.E.D. incidence was seen in all broodmares, P.R. cumulative and F.R. in the abortion populations was similar, but higher than that for the overall broodmare population.

Table 1 Reproductive performance of the resident broodmare populations on Farm 1 between September 1, 2007 to January 31, 2008

Reported risk factor	Mare populations		
	All mares (n=45)	All mares (n=45)	All mares (n=45)
Per cycle pregnancy rate (mean, range)	1.4 (1-5)	1.2 (1-4)	1.8 (1-5)
Early embryonic deaths	6 (13.3%)	5 (13.5%)	1 (12.5%)
Cumulative pregnancies	41 (91.1%)*	33 (89.2%)	8 (100%)
Not pregnant (January 31, 2008)	4 (8.9%)	4 (10.8%)	0 (0%)
Live foals (foaled in 2008)	27 (60.0%)	22 (59.4%)	5 (62.5%)

* two pregnant mares subsequently died

Table 2 Reproductive performance of the resident broodmare populations on Farm 2 between September 1, 2009 to January 31, 2010

Reported risk factor	Mare populations			
	All mares (n=368)	All abortion mares (n=43)	EHV-1 abortion mares (n=18)	Non-EHV abortion mares (n=25)
Per cycle pregnancy rate (mean, range)	1.7 (1-6)	1.9 (1-4)	1.9 (1-4)	1.8 (1-4)
Early embryonic deaths	31 (8.4%)	4 (9.3%)	2 (11.1%)	2 (8.0%)
Cumulative pregnancies	319 (86.7%)*	36 (83.7%)	16 (88.9%)	20 (80.0%)
Not pregnant (January 31, 2010)	49 (13.3%)	7 (16.3%)	2 (11.1%)	5 (20.0%)
Live foals (foaled in 2010)	279 (75.8%)	34 (79.1%)	16 (88.9%)	18 (72.0%)

* Five pregnant mares subsequently died; EHV-1 equine herpesvirus 1

The number of breeding attempts required to establish pregnancy

Age had a significant association with the number of breeding attempts, whether regarded as continuous (r=0.12, P=0.016) or as ordinal categories (P=0.040) (Table 3). Findings showed 68.5% young mares were bred once, compared with 60.6% middle-aged and 55.5% old mares, respectively. The trend with multiple breeding attempts was similar across all three age categories, two breeding attempts for 23.3%, 26.7% and 26.4% of young, middle-aged and old mares, respectively. Mare status had a significant association with the number of breedings (P<0.0001), with 73.3% maidens requiring a single breeding, compared with 63.8% foaling and 46.2% barren mares. A similar percentage (27.9% and 25.3%) of foaling and barren mares required a second attempt to establish pregnancy. E.E.D. had a significant association with the number of breedings (P<0.0001), associated with 46.0% second, and 21.6% third breeding attempts, with only three (8.1%) and one (2.7%) incidents of E.E.D. associated with a fourth and

fifth breeding, respectively. There was a significant association between the number of breedings and the last month of breeding a mare (P<0.0001). A single breeding and its association with pregnancy showed a monotonic decline with each successive month. From the first to the fourth month, 71 (85.5%), 67 (64.4%), 76 (57.1%), and 33 (37.9%) of mares, respectively became pregnant by a single breeding. The association with multiple (≥2) breedings and establishment of pregnancy showed that two breedings were required in only 12 (14.5%) of the mares bred in the first month. In the second, third and fourth months, a similar proportion of mares: 31 (29.8), 36 (27.1%), and 26 (29.9%), respectively required two breedings. Similarly, a third breeding was recorded from the second to the fourth month in five (4.8%), 18 (13.5%), and 12 (13.8%) mares, respectively.

A mare having aborted due to any cause was associated with the number of breedings (P=0.021). A single breeding was sufficient in 62.4% of non-abortion mares, whereas less than half (46.8%) of abortion mares became pregnant from only one breeding. The requirement for a second breeding (25.4% and 29.8%) was however similar between the two mare populations.

The month of the last breeding attempt in the interval September 1 until January 31

A significant relationship was seen with mare status (P<0.0001) (Table 3). In the first month, September, 46.2% barren, 26.7% maiden and 9.7% foaling mares were bred for a last time. In the second month, October, a similar proportion of barren (23.1%) and foaling (24.4%) mares and 33.3% maidens were bred a last time. The largest proportion of mares was bred in the third month, November, with 16.5% barren, 25.0% maiden and 39.9% foaling mares. In December, a similar proportion of the barren and maiden (14.3% and 15.0%, respectively) and 25.2% of foaling mares were bred for the last time. A negligible proportion of mares were January. Abortion due to all causes (P=0.0001), EHV-1 abortion (P=0.0014) and non-EHV-1 abortion (P=0.0023) were all associated with the last month of breeding. Approximately half (48.9%) of abortion mares were bred for the last time in the first month, with a monotonic decline from this point (23.4%, 17.0% and 10.6%) from October to December. A significant association was seen with E.E.D. (P<0.0001). A low incidence of E.E.D. of only one (2.8%) and three (8.3%) was recorded from mares bred in the first and second months, respectively. A high E.E.D. incidence (47.2% and 41.7%) was seen in the third and fourth months, respectively. A significant association was also seen with the number of breedings required (P<0.0001), as described above.

Pregnancy at the end of the breeding season

Univariate analyses of potential determinants of pregnancy at the end of the breeding season are shown in Tables 3 and 4. Significant variables include age, history of E.E.D., number of breeding attempts, and month of last cover. Multivariable analysis of the significant predictors showed a significant main effect of age (P=0.0042), and significant interactions between E.E.D. and number of breedings (P=0.0023) and between number of breeding attempts and month of last breeding (P=0.014). For each additional year of age, the odds of establishing pregnancy declined approximately 10% (OR=0.89, 95% CI=0.82-0.96). The predictive probability of establishing pregnancy with consideration of the interactions between the significant predictors is shown by the logistic regression model in Table 5. Three hypothetical broodmares, representative of the demographic in Thoroughbred systems are included. These include a five-year old maiden and a 15-year old barren mare that are bred commencing in the first month, September, continuing until the end of the season. In addition a 10-year old mare that foaled in the middle of the second month and was subsequently bred, commencing in November and continuing until January 31 was included.

Foaling successfully in subsequent season

Univariate analyses of potential determinants of pregnancy at the end of the subsequent breeding season are shown in Tables 3 and 6. Significant variables include age, history of E.E.D., number of breeding attempts, and month of last cover. Multivariable analysis included significant interactions between the potential determinants (results not shown). The predictive probability of foaling in a mare with consideration of the interactions between the significant predictors is shown by the logistic regression model in Table 7. Hypothetical broodmares representative of the demographic in Thoroughbred systems are included in this model. These include a five-year old maiden mare, a 10-year old foaling or barren mare, and a 15-year old foaling or barren mare.

Table 3 A summary of outcomes-based analyses of reproductive performance of pregnant broodmares resident on both farms

Outcome	Variable							
	Age	Status	Early embryonic death	Number of breedings	Month of last breeding	Abortion: all causes	Abortion: EHV-1	Abortion: non-EHV
Cumulative pregnancy rate	P=0.0005	P=0.77* P=0.24†	P<0.0001	P<0.0001	P<0.0001	P=0.31	P=0.75	P=0.51
Cumulative pregnancy rate: barren mares	-	-	-	-	-	P=0.38	P=0.43	P=0.28
Foaling rate	P<0.0001	P=0.055†	P<0.0001	P<0.0001	P<0.0001	P=0.19	P=0.49	P=0.44
Number of breedings	P=0.016‡ P=0.040§	P<0.0001	P<0.0001	-	P<0.0001	P=0.021	P=0.086	P=0.16
Month of last breeding	P=0.19‡ P=0.28§	P<0.0001	P<0.0001	P<0.0001	-	P<0.0001	P=0.0014	P=0.0023

* comparing foaling to barren mares; † comparing maiden to barren mares; ‡ data as continuous; § data as ordinal categories

Table 4 Univariate logistic regression models of potential determinants for establishing pregnancy in two populations of resident broodmares

Determinants	Pregnant (n)	Not pregnant (n)	Odds ratio	95% confidence interval	P-value
4.1 Age (years)			0.90	0.84-0.95	0.0005
4.2 Early embryonic death			0.073	0.030-0.16	<0.0001
4.3 Number of breeding attempts			0.39	0.28-0.53	<0.0001
4.4 Monthly trend of last cover			0.24	0.15-0.38	<0.0001
4.5 Month of last cover					
September	81	2	1		
October	103	1	2.53	0.13-151.5	0.83
November	119	14	0.21	0.023-0.96	0.042
December	56	31	0.045	0.0051-0.19	<0.0001
4.6 Abortion (any cause)					
No	316	46	1		
Yes	44	3	2.13	0.64-11.17	0.31

Table 5 The predictive probability of establishing a pregnancy between September 1 until January 31 in three hypothetical broodmares according to number of breedings and the month of last breeding and either with or without an incident of early embryonic death in the logistic regression model

Month of last breeding	No early embryonic death incident Number of breedings					Early embryonic death incident Number of breedings			
	1	2	3	4	5	2	3	4	5
5.1 A five year-old maiden mare									
1	0.99	1.00	-	-	-	0.98	-	-	-
2	0.99	0.99	1.00	1.00	-	0.92	0.99	-	-
3	0.98	0.97	0.96	0.95	0.95	0.70	0.88	0.96	-
4	0.95	0.87	0.71	0.47	0.24	0.33	0.40	0.49	0.04
5	0.90	0.59	-	-	-	0.10	-	-	-
5.2 A 10 year-old mare foaling in the middle of the second month									
3	0.99	0.99	-	-	-	0.97	-	-	-
4	0.98	0.99	0.99	0.99	-	0.86	0.98	-	-
5	0.96	0.95	0.94	0.94	0.91	0.57	0.80	0.93	-
5.3 A 15 year-old barren mare									
1	0.98	1.00	-	-	-	0.94	-	-	-
2	0.96	0.98	0.91	0.99	-	0.77	0.96	-	-
3	0.92	0.91	0.89	0.87	0.84	0.42	0.69	0.87	-
4	0.85	0.68	0.43	0.21	0.09	0.13	0.17	0.22	0.28
5	0.74	0.31	-	-	-	0.03	-	-	-

Table 6 Univariate logistic regression models of potential determinants of successful foaling in two populations of resident broodmares in a subsequent season

Determinants	Foaled (n)	Did not foal (n)	Odds ratio	95% confidence interval	P-value
6.1 Age (years)			0.89	0.85-0.94	<0.0001
6.2 Early embryonic death			0.092	0.038-0.21	<0.0001
6.3 Number of breeding attempts			0.54	0.41-0.70	<0.0001
6.4 Monthly trend of last cover			0.38	0.28-0.50	<0.0001
6.5 Month of last cover					
September	74	8	1		
October	92	9	1.10	0.35-3.40	1.00
November	100	31	0.35	0.13-0.84	0.015
December	47	38	0.089	0.033-0.21	<0.0001
6.6 Abortion (any cause)					
No	266	89	1		
Yes	39	7	2.13	0.64-11.17	0.31
6.7 Mare status					
Barren	65	25	1		
Foaling	188	63	1.15	0.64-2.03	0.71
Maiden	52	8	2.49	0.98-6.92	0.055

Table 7 The predicted probability of successful foaling in hypothetical broodmares of different ages and status categories according to the month of their last breeding and with or without an incident of early embryonic death in the previous season in the logistic regression model

Month of last breeding	No early embryonic death incident		Early embryonic death incident	
7.1 A five year-old maiden mare				
1	0.98		0.84	
2	0.94		0.65	
3	0.86		0.40	
4	0.69		0.20	
5	0.44		0.08	
	Barren	**Foaling**	**Barren**	**Foaling**
7.2 A 10 year-old barren or foaling mare				
1	0.91	0.97	0.54	0.8
2	0.79	0.93	0.30	0.59
3	0.58	0.82	0.14	0.34
4	0.33	0.62	0.05	0.16
5	0.15	0.38	0.02	0.06
7.3 A 15 year-old barren or foaling mare				
1	0.91	0.94	0.53	0.64
2	0.78	0.85	0.29	0.39
3	0.57	0.67	0.13	0.19
4	0.32	0.42	0.05	0.08
5	0.15	0.21	0.02	0.03

Discussion

The reproductive efficiency, E.E.D. rates, and PR cumulative were similar in all populations. The P.R. per cycle in the various broodmare populations was similar to previous reports for Thoroughbred mares of all ages (Allen and others 2007 and Hemberg and others 2004). After establishing pregnancy, horses' abortion rates were similar to those during the previous, epizootic-associated season on both farms. The F.R on Farm 1 although similar to an earlier report in South African Thoroughbreds (Schulman and others 2003), was appreciably lower than that on Farm 2, which in turn was similar to reported ranges for other populations of a similar age distribution (Allen and others 2007, Hemberg and others 2004). In the breeding season following the epizootic, a better foaling outcome (in particular on Farm 2) was observed for mares with a recent abortion history than with non-abortion mare populations.

These observations are difficult to explain, but imply an advantageous relationship between a history of abortion and subsequent reproductive performance. The influence of epizootic-

associated versus endemic (from sporadic causes) abortion on reproductive outcomes in a particular season is also unclear from these data.

Results indicate that solely focusing and reporting on the crude relationships between predictors and subsequent reproductive performance can lead to misleading conclusions, because such effects are heterogeneous and consistent with modification by other factors, as evidenced in the multivariate model. The development of a predictive model for reproductive outcomes with inclusion of effect modifiers such as age and mare reproductive status was used to enhance understanding of the association and relative influence of the various variables at key points in the Thoroughbred reproduction process.

Increased age reduced the probability of a successful outcome in establishing pregnancy and foaling. Age also strongly predicted of the number of breedings: approximately one-third ≤6 years required a second attempt. The trend with multiple breeding attempts was conspicuously similar across all three age categories.

Mare status showed no predictive association with the establishing pregnancy, although barren mares have been reported in association with reduced fertility (Hemberg and others 2004, Allen and others 2007, Yang and Cho 2007). Foaling status conferred an age-dependent predictive advantage on successful foaling compared to maiden and barren statuses. As age increased, the advantage conferred by foaling status, however, declined. Status was predictive of the number of breedings, with a single breeding sufficient in the majority of maidens (73.3%) compared with foaling mares (63.8%) and less than half of the barren mares (46.2%). A similar percentage of foaling and barren mares required a second attempt to establish pregnancy. Mare status was predictive of her last month of breeding, as maidens and barrens were 'available' from the onset of the season in most cases, unlike the foaling mares foaling until December in some cases. Approximately half the barrens became pregnant in the modal first month. This may perhaps mitigate some of the effects of the reported inherent subfertility of barren mares, providing a potentially increased number of cycles available to rebreed them. Maidens showed a wider distribution by month of last breeding, as the modal month for becoming pregnant occurred in the second month. This may imply that in these populations, barrens are better prepared by management than maidens to resume cyclic activity, or perhaps barren versus maiden status confers fewer disadvantages at the onset of the breeding season. November, with the most breedings, resulted in pregnancy in nearly 40% of the foaling mares. A considerable number of mares in all categories were still being bred in December.

The number of breedings predicted establishment of pregnancy and foaling and was significantly associated with age and status, last month of breeding, and E.E.D. More maidens became pregnant with a single breeding than foaling or barren mares. Interestingly, a similar percentage of foaling and barren mares required a second attempt to establish pregnancy. There was an enhanced probability of establishing pregnancy early in the season, as breeding in September was surprisingly strongly predictive that a single breeding would result in pregnancy. The relative efficiency of breeding effort and implied result (i.e. pregnancy) monotonically declined. The 85.5% of mares becoming pregnant at the expected onset of physiological cyclicity in September contrasts with 37.9% in December, coincident with the physiologically optimal peak in ovulation rates. This may imply that any potential physiological advantage of a relatively late breeding is overcome by other negatively-associated factors related to breeding at a later stage. The number of breeding attempts significantly predicted F.R., with each additional breeding halving the probability of foaling. Month of last breeding was also a significant predictor with a monotonic decline in the probability of successful foaling between September and December.

E.E.D. was strongly predictive of multiple breeding attempts and pregnancy. Of interest and difficult to explain is E.E.D. associated with reducing the probability of foaling by almost 90%. In the predictive model of an incident of E.E.D. in a relatively young mare, the probabilities of her becoming pregnant and foaling are reduced compared with a substantially older mare with no E.E.D. The sudden and marked increase in E.E.D. in the third and fourth months coincided with commencing breeding the majority of the foaling mares. Many are bred within a short interval post-foaling, reportedly associated with the highest E.E.D. (Yang and Cho 2007).

Abortion from any cause (EHV-1 or non-EHV-1 causes) did not predict subsequent pregnancy or foaling. Abortion was predictive of the number of breedings to establish pregnancy: slightly fewer than half the abortion mares became pregnant from a single breeding, compared with nearly two-thirds of non-abortion mares. An abortion history, including EHV-1, predicted the last month of breeding: approximately half these mares became pregnant due to breeding in the first month, with a monotonic decline through December.

In conclusion, development of a predictive model for important outcomes enhanced understanding of complex interactions and relative influences of various reproductive variables. Outcomes such as pregnancy associated with the customary predictor variables by bivariate analysis appeared to be either unaffected, or confusingly, enhanced, in mares subsequent to abortion. Abortion was not predictive of becoming pregnant or foaling in contrast to previous

reports (Bain 1969, Hemberg and others 2004), but was associated with increased effort to establish pregnancy and last month of breeding. Increased age, supporting previous reports, was predictive of increased breeding efforts and decreased the probabilities of establishing pregnancy (Allen and others 2007, Yang and Cho 2007) and foaling (Hemberg and others 2004, Allen and others 2007). Reproductive status was not predictive of establishing pregnancy, contrasting with Allen and others (2007) and Hemberg and others (2004), but being predictive of the number of breedings and the last month of breeding was similar to other reports (Allen and others 2007 and Hemberg and others 2004). The increased probability of foaling success in foaling mares declined with age. In all mares, E.E.D. was strongly predictive of all outcomes: breeding attempts, the last month of breeding, pregnancy, and foaling. The last month of breeding was predictive of the effort and success of establishing pregnancy (in contrast to Hemberg and others 2004) and foaling. Interestingly, the combination of barren status and breeding in the first month of the season improved the probability of establishing pregnancy, as was seen in approximately half of the abortion mares.

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CHAPTER 5
Epidemiology and reproductive outcomes of EHV-1 abortion epizootics in unvaccinated Thoroughbred mares in South Africa

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Summary

Reasons for performing study*:* EHV-1 is one of the most common causes of infectious abortion in mares. Analyzing the demography of outbreaks and detailing subsequent reproductive performance of affected mares will assist in the management of future (threatened) epizootics.

Objectives: To examine the epidemiology and reproductive outcomes of two EHV-1 abortion epizootics with very different patterns of morbidity.

Study design: Epidemiological and reproductive data were analyzed retrospectively following abortion epizootics associated with EHV-1, but initiated via different routes, among unvaccinated mares on two Thoroughbred farms in South Africa.

Methods: Aborting mares were assigned to either the EHV-1 abortion cohort via positive immunostaining (farm 1 and 2) or qPCR (farm 2) on tissue samples, or to the non-EHV abortion cohort.

Results: During their respective epizootics, EHV-1 abortions affected 9/30 (30.0%) and 18/316 (5.7%) of the pregnant mares on farms 1 and 2 respectively; there were also 25 (7.9%) non-EHV abortions on farm 2. Epizootic differences included: durations (farm 1=135 d; farm 2=34 d), intervals between first and subsequent abortions (farm 1=39 d; farm 2=2 d), and intervals to confirmation of EHV-1 (farm 1=40 d; farm 2=2 d). The median age of EHV-1 abortion mares (8.0; 5-18 years) in both epizootics was similar, but significantly younger (P= 0.004) than the 25 non-EHV-1 abortion mares (11.0; 4-24 years) on farm 2. Gestation stage (median; range) of EHV-1 (291.5; 277-313) and non-EHV-1 (211.9; 61-339 d) abortions were significantly different (P=0.001). The post-abortion complications and subsequent reproductive outcomes had no significant association with EHV-1 abortion.

Conclusions: The marked difference in morbidity between the two epizootics may be associated with routes of introduction or intervention strategy dictated by availability of molecular diagnostic techniques. Unexpectedly, EHV-1 was not more commonly associated with post-abortion complications.

Introduction

Equine herpes virus type 1 (EHV-1) is the most important viral cause of equine abortion, both because it is relatively common and because of its potential for epizootic spread, particularly within naïve populations [1; 2; 3; 4]. Under natural conditions, EHV-1 abortion rarely occurs

before four months of gestation: 95% of diagnosed EHV-1 abortions occur in the last third of pregnancy and 75-80% between eight and ten months of gestation [2]. The reported incubation time varies between nine and 121 days and abortion typically occurs without any premonitory signs [2; 4; 5]. Mare factors associated with susceptibility to EHV-1 abortion are largely undefined, but previous reports have suggested that primiparous mares and Lipizzaners are at higher risk [6; 7]. In Thoroughbred mares, age and reproductive status (maiden, barren or foaling) influence the likelihood of pregnancy loss [8] and, although associations between breeds and EHV-1 or infectious abortion in general are difficult to prove, abortion rates of up to 87% during an EHV-1 abortion epizootic have been reported [9; 10; 11].

Following abortion, EHV-1 is cleared from the genital tract, and future breeding capacity is not impaired unless reproductive tract damage has occurred as a result of dystocia [4; 12]. Premature placental separation (PPS) is reported in 1.6% of all foaling's [13], but the likelihood is thought to be higher in the case of EHV-1 abortion or still-birth [4; 14; 15]. The post-abortion incidence of retained fetal membranes (RFM), one of the most common post-partum complications in mares, is reported to be greater than after normal foaling [16]; indeed, a 66.6% rate of RFM in mares that aborted due to EHV-1 was recently reported [15]. On the other hand, no increase in the incidence of post-abortion complications was evident during an extensive abortion epizootic involving unvaccinated Thoroughbred mares [10].

In this study we retrospectively analysed the relationships between epidemiological factors and reproductive outcomes following EHV-1 abortion epizootics on two separate Thoroughbred farms in South Africa. The two epizootics featured divergent environmental and management factors and outbreak interventions. The aim was to improve the understanding of infectious abortion epizootics in horses in order to enhance future prevention and management strategies.

Materials and methods

Background and study design

Aspects of the epidemiology and post-abortion reproductive outcomes in Thoroughbred broodmares were analysed retrospectively for two temporally-unrelated EHV-1 abortion epizootics on farms in geographically separated areas of South Africa. The paddock sizes and stocking density were similar on both farms. Prior to the epizootics, neither of the farms vaccinated broodmares against EHV-1.

Epidemiology

Farm 1: This farm in the Western Cape Province recorded abortions in the period May 10 to September 22, 2007 in nine of a resident population of 30 broodmares confirmed pregnant at the end of the 2006 Southern Hemisphere breeding season. All abortions occurred within the same group of 21 mares located originally in one of two extensive paddocks of approximately eight hectares in area, and in which the farm's resident pregnant broodmares were maintained. No abortions occurred among the nine mares in the second paddock located a considerable distance away. Following the first abortion on May 10 (D0), the affected mare was moved to an isolated quarantine paddock for three weeks while the in-contact mares were sub-divided into smaller groups that were separated geographically to limit the risks of further spread while awaiting the outcome of diagnostic tests. The fetus and associated membranes were immediately sealed in plastic drums to prevent environmental contamination and removed for diagnostic sampling and disposal. A second abortion occurred on D39, one day prior to receiving confirmation that the first abortion was caused by EHV-1, and 40 days after sample submission. The interval between the first (on D0), and last abortion was 135 days. The protracted interval from first abortion to confirmation of EHV-1 biased the intervention strategy and, in all likelihood therefore, the subsequent course of the epizootic. The cause of abortion in all cases was confirmed as EHV-1 by immunostaining of formalin-preserved fetal tissue and membrane samples. No other abortions were recorded during 2007, and the remaining 21 mares foaled uneventfully.

Farm 2*:* This farm, situated in KwaZulu-Natal Province experienced an abortion epizootic between June 20 and July 24, 2009. Eighteen of the 316 pregnant resident broodmares aborted as a result of EHV-1 infection. The suspected instigator of the epizootic was the introduction of a group of eight mares, including six in late-gestation, acquired at a broodmare sale when the resident mares were at least seven months pregnant. Immediately upon arrival, the eight newly acquired mares were quarantined as a group for three weeks, in accordance with the farm's disease prevention protocol. Thereafter, the two non-pregnant mares were introduced into an isolated group of barren mares, while the six pregnant mares were dispersed over three groups, each of approximately 30 pregnant mares, which were kept in grass paddocks ranging from 2.5 to 11 hectares in area. The grouping of pregnant mares was based on anticipated foaling dates, as is customary in Thoroughbred management systems. The first abortion occurred 12 days after the introduction of two newly acquired mares into one of these three groups on June 20 (D0). The fetus and associated membranes were not

recovered, presumably because of scavenging e.g. by the black-backed jackals (*Canis mesomelas*), present in the area. Two days later (D2) three abortions occurred in the same paddock as the first. Ultimately, the 18 mares affected by EHV-1 abortions included two of the six introduced-mares. The fetuses and membranes in these cases were retrieved intact, and samples were submitted to the University of Pretoria's Equine Research Centre (ERC) for diagnostic tests. The results of a real-time-quantitative PCR (qPCR) assay on the fetal tissues were available by D3 of the outbreak, i.e. within 24 h of the second group of abortions. Confirmation that EHV-1 was the cause of abortion in all cases was provided by a duplex qPCR assay for EHV-1 and -4 that employed previously described primers and probes [17], and immunostaining of both fetal tissue and membrane samples.

The rapid etiological diagnosis enabled the farm's management to justify the immediate institution of specific EHV-1 outbreak-control measures aimed at isolating the focus of infection, interrupting virus transmission and reducing the susceptibility of high-risk late-gestation mares [3; 18]. This response was based around a number of key decisions. The first was to vaccinate all pregnant broodmares with an inactivated EHV-vaccine (Pneumabort-K+1B)[a] and to re-vaccinate at two-month intervals until foaling. In addition, the mares were sub-divided, within each paddock, into groups of four to six using electrical tape barriers and maintaining a distance ≥20 m between each enclosed area while ensuring that the groups still had visual and auditory contact until the mares either foaled or aborted. This manner of separation aimed to balance the risks of virus transmission between mares, particularly at the time of foaling or abortion, against additional separation stress that could otherwise conceivably induce viral recrudescence or lead to abortion for other reasons. Night-watchmen maintained constant observation and removed aborting or foaling mares immediately. After delivery, the mares were placed in a quarantine area for three weeks prior to relocation to the farms' barren mare groups at a considerable distance from pregnant mares. After an abortion, in-contact group-mates were immediately relocated to another electrically-fenced enclosure ≥20 m away, precluding direct contact with fetal fluids and reducing the risk of respiratory spread via aerosol [5]. The aborted fetus and fetal membranes were immediately sealed in plastic drums and removed for diagnostic sampling and disposal. An EHV-1-appropriate biosecurity protocol was applied concurrently [3]. The fetus and associated membranes were examined by the farms' veterinarian, and tissue samples were obtained during a standardized autopsy protocol and submitted for laboratory diagnosis as outlined above.

Molecular characterization of viruses

DNA for sequencing was extracted from the supernatant of virus cultures performed on all samples that tested positive for EHV-1 by qPCR. To amplify the ORF 30 region for sequencing, 0.5 µL of extracted DNA was added to 0.5 µL 20 mM dNTP mix, 1.2 µL 25mM MgCl, 2 µL 10xPCR Buffer[b], 0.05 µL Taq polymerase (Super-therm gold DNA Polymerase) and 0.2 µL EHV-1 ORF30 forward (GCG CTA CTT CTG AAA ACG) and reverse (CCA CAA ACT TGA TAA ACA CG) primers, in a total volume of 20 µL. The PCR reaction involved denaturing at 95°C for 10 min, followed by 35 cycles of 95°C for 45 sec, 51°C for 1 min and 72°C for 2 min, before a final cycle of 72°C for 8 min in an ABI 9700 PCR thermocycler[c]. The amplification products were purified with an Invitek PCRapace kit[d] according to the manufacturer's instructions. The sequencing reaction consisted of 2 µL ABI Prism® BigDye® Terminator v3.1 Ready Reaction mix[c], 1 µL sequencing buffer, 1 µL 3.2 pmol forward and reverse primer and 6 µL of purified DNA. The sequencing reactions were run in an ABI 9700 PCR[c] machine according to the manufacturer's instructions. The product was ethanol precipitated and air-dried for 10 min before denaturing using formamide and bi-directional sequencing with an ABI 3130xl Genetic Analyser[c]. EHV-1 and -4 virus isolates grown on equine lung cells were used as positive controls, and pyrogen-free water as negative control.

Reproductive outcomes

The reproductive outcomes recorded in all aborting mares, whether abortion was due to EHV-1 (both farms) or other causes (farm 2 only) were compared to those of the other resident broodmares, both during and following the epizootics. Subsequent to the epizootic-affected seasons, both farms vaccinated all pregnant mares with Pneumabort-K+1B[a] at two-monthly intervals, commencing at five months of gestation and continuing until foaling.

Assignment of mares to the EHV-1 abortion cohort was based on a positive diagnosis *via* immunostaining (farm 1), or both immunostaining and qPCR assay (farm 2) on fetal and fetal membrane samples. Mares aborting due to other causes in the seasons when EHV-1 abortions were reported were classified as non-EHV-1 abortion mares. The sum of these two constituted the 'total aborting mares' cohort. The mares were categorized by age (years) and their reproductive status in the preceding breeding season. Reproductive status allocation was as: i) maiden (never bred previously); ii) barren (bred at least once previously but did not foal during the current season); and iii) foaling (foaled during the current season). The reproductive

variables chosen to describe the pattern and outcomes of abortion were: stage of gestation (median and range at abortion), incidence of RFM and incidence of PPS.

Data analysis

The data sources were the broodmare records maintained by the respective farms' manager and veterinarian. Data was analysed using SigmaPlot V11[e] software. Basic descriptive statistics and between-groups Mann-Whitney Rank Sum Tests were conducted. For all analyses, $P \leq 0.05$ was considered to indicate a statistically significant difference.

Results

Epidemiological data

Farm 1: The initiating events for the first abortion were undefined, but most likely resulted from EHV-1 reactivation in the first aborting mare [19]. Alternatively, because recrudescence and secondary reinfection within a group are difficult to differentiate, the first abortion may have occurred subsequent to horizontal respiratory exposure from an asymptomatic-paddock mate that underwent sub-clinical viral recrudescence in response to an unknown stressor [20; 21]. In either case, after the first abortion, group exposure must have occurred as a result of high virus loads within the aborted fetus, fluids and membranes [21]. At least two additional mares must have been infected at, or before, this first abortion because, even though this initial group was subdivided almost immediately, abortions were subsequently recorded in two of three resultant sub-groups.

Table 1 Reproductive performance of the pregnant broodmare populations during the abortion epizootic in 2007 and the subsequent post-epizootic breeding season on farm 1

Reproductive performance	Mare populations			
	All mares	Total aborting	EHV-1 abortion	Non-EHV-1 abortion
Pregnant prior to epizootic (n)	30	9	9	-
Age - median (range) in years	10.2 (4-19)	8.0 (5-18)	8.0 (5-18)	-
Status at breeding in 2006				
- Foaling	20	6	6	-
- Maiden	5	2	2	-
- Barren	5	1	1	-
Gestation stage at abortion median (range) in days	-	227 (200-291)	227 (200-291)	-
RFM post-abortion or foaling	0	0	0	-
Cycles per pregnancy (mean, range)	1.4 (1-5)	1.8 (1-5)	1.8 (1-5)	-
Cumulative pregnancies (%)	41 (91.1)	8 (100.0)	8 (100.0)	-
Live foals in 2008 (%)	27 (60.0)	5 (62.5)	5 (62.5)	-

EHV-1 equine herpesvirus 1; RFM retained fetal membranes

Farm 2: This epizootic was closely associated with recent introduction and dispersal of pregnant mares into established groups of resident pregnant broodmares. The first abortions occurred soon after the introductions without premonitory clinical signs. Ultimately, EHV-1 abortions occurred in four groups of mares; these included two of the three groups into which acquired broodmares were introduced; the aborting mares included two of the newly-introduced mares.

The initiating pathogenesis may have been associated with social disruption causing stress-induced EHV-1 recrudescence among one or more of the newly introduced or the previously-resident mares [18; 21]. Alternatively, or additionally, virus may have circulated among the introduced group during quarantine prior to dispersal. There was complete agreement between diagnostic methods for all abortions on farm 2 as to whether EHV-1 was implicated or not.

Comparison between farms: Selected reproductive performance parameters were compared for the breeding season in which the epizootics took place and for the following breeding season, for mares that aborted due to EHV-1 or due to other causes. There was no significant difference in the median age of the mares that aborted due to EHV-1 infection between farm 1 and farm 2 (8.0 years), nor was there any difference in the distribution of mare reproductive status or the previous use of EHV-1 vaccination. The most notable mare-level

difference between the affected farms was that the median gestational stage at the time of EHV-1 associated abortion on farm 1 was lower than that of farm 2. This most likely reflected the stage of gestation of the respective mare groups when they were infected with EHV-1. However, other factors including EHV-1 strain differences in virulence or incubation intervals may have contributed. The other significant differences between the two farms were: the duration of the outbreaks (farm 1=135 d; farm 2=34 d), the intervals between the index case and subsequent abortions (farm 1=39 d; farm 2=2 d), and the time taken to confirm a diagnosis (farm 1=40 d; farm 2=2 d).

Molecular characterization of viruses

Retrospectively, both epizootics were confirmed to be associated with abortogenic EHV-1 virus, with the ORF 30 sequence characterized by the G^{2254} genotype for all cases [15].

Reproductive outcomes

The reproductive outcomes in the pregnant mare cohorts are summarized in Tables 1 and 2. On farm 1, there were no recorded cases of non-EHV-1 abortion, PPS or RFM. During the course of 2009, 43 (13.61%) of the pregnant mares on farm 2 aborted. These abortions included 18 due to EHV-1 and 25 that resulted from other causes. There were significant differences in both the median age (P=0.001) and gestational stage (P=0.044) between mares that aborted due to EHV-1 and those that aborted for other reasons. Specifically, EHV-1 abortions were, on average, recorded in younger mares at a later stage of gestation than non-EHV-1 abortions. The cause of non-EHV-1 abortions was diagnosed in ten of 25 (40.0%) cases, and included six (24.0%) ascribed to ascending placentitis, two (8.0%) to twinning, and one each as a result of umbilical cord pathology and placental oedema, respectively. The overall diagnostic rate for all abortions was high, at 65.1%. On farm 1, one mare died acutely as a consequence of a uterine prolapse that presented a few hours after abortion and, on farm 2, one non-EHV-1 abortion mare was euthanized as a result of a rectal prolapse following abortion due to placentitis. Retained fetal membranes were more commonly reported in mares that aborted for reasons other than EHV-1 (Table 2). The frequency of PPS in the current study was 7.3% (21 incidents) which was markedly higher than the historical average of 5.3 events per

year during the previous three seasons from a similar number of foaling mares at the same farms (data not shown).

Table 2 Reproductive performance of the pregnant broodmare populations during the abortion epizootic in 2009 and the subsequent post-epizootic breeding season on farm 2

Reproductive performance	Mare populations			
	All mares	Total aborting	EHV-1 abortion	Non-EHV-1 abortion
Pregnant prior to epizootic (n)	316	43	18	25
Age - median (range) in years	10.5 (3-24)	10.0 (4-24)	8.0† (5-16)	11.0† (4-24)
Status at breeding in 2008				
- foaling	214	25	7	18
- maiden	44	8	4	4
- barren	58	10	7	3
Gestation stage at abortion median (range) in days	-	278.0 (61-339)	291.5* (277-313)	211.9* (61-339)
RFM post-abortion or foaling (%)	16 (4.4)	5 (1.2)	1 (0.6)	4 (16.0)
Cycles per pregnancy (mean, range)	1.7 (1-6)	1.9 (1-4)	1.9 (1-4)	1.8 (1-4)
Cumulative pregnancies (%)	319 (85.1)§	37 (94.9)	17 (94.4)	20 (95.2)
Live foals in 2010 (%)	279 (74.4)	34 (87.2)	16 (88.9)	18 (85.7)

† significant, P= 0.004; * significant, P= 0.001; § five pregnant mares subsequently died; EHV-1 equine herpesvirus 1; RFM retained fetal membranes

Discussion

There are conflicting reports in the literature with regard to the contribution of both age and parity of the mare to the subsequent risk of EHV-1-associated abortion [6; 7]. In the current study, the EHV-1-affected mares in both epizootics were relatively young and evenly distributed over reproductive status categories, thereby partially supporting a previous report of an association of EHV-1 abortion with primiparity [7], but opposing the suggesting of an association with older mares [6].

The variations in epidemiology and outcome between the two South African epizootics described in this report, even though they affected demographically-similar mare populations, may have been a result of a critical host-factor difference, namely gestational stage at exposure. The outcome may have been further biased, independently or as a co-dependent, by differences in management interventions on the respective farms, including allocation of mares into groups on the basis of gestation stage and the history of introducing high-risk animals on farm 2. On farm 1, the gestation stage at EHV-1 abortion was markedly earlier and the

incubation, inter-abortion intervals and epizootic duration were greater than on farm 2 or in previous reports of epizootics affecting similar-sized groups of unvaccinated late-gestation Thoroughbreds or mares of other breeds [6; 10]. The long interval between presumed exposure and abortion on farm 1 is difficult to explain but may in part have been associated with initial exposure at an early gestational stage less susceptible to immediate abortion, or to viral factors such as abortogenic potential [22]. The previously reported difficulty in recognizing reactivation followed by horizontal spread, because of the absence of obvious clinical signs [20] was also supported. On farm 2, the occurrence of the abortions in a temporal cluster was similar to an Australian Thoroughbred epizootic affecting unvaccinated mares managed on extensive pastures [10]. The abortion rate in the Australian epizootic was, however, much higher. The availability of PCR-based diagnosis for farm 2 allowed a markedly shorter interval to definitive diagnosis, and thereby enabled rapid implementation of an intervention response specific to EHV-1. This justified management decisions that carried significant economic, resource and manpower implications, including the vaccination, constant observation and subdivision of approximately 300 pregnant broodmares. It is tempting to speculate that availability of a validated rapid PCR test was critical in limiting the duration and relative impact of the EHV-1 abortion epizootic on farm 2. An additional difference was the decision to immediately vaccinate all pregnant broodmares. Vaccination in the face of an outbreak had no apparent adverse response, in contrast to anecdotal reports during neurotropic EHV-1 epizootics, and may have helped to limit the spread and extent of the epizootic. Continuous observation of the mares with immediate separation of any aborting or foaling mare and subdivision of affected groups *in situ* by electric tapes almost certainly limited subsequent exposure via fetus, membranes and fluids [5; 22]. The traditional scheduling of broodmare sales in the winter months (coincident with advanced gestation) was highlighted as a risk-factor in Thoroughbreds. A key risk-mitigation strategy would be strict separation of newly-acquired mares from the resident population until after foaling.

The overall abortion rates for the epizootic-associated years were, respectively, within (farm 2) or markedly higher than (farm 1), the reported ranges for Thoroughbreds in South Africa and other countries [2; 23]. The marked differences between the two outbreaks in duration and abortion patterns may have been biased by differences in the outbreak responses which in turn may have been affected by the interval to confirmation of EHV-1. The speed with which the diagnosis was confirmed was a product of the availability (farm 2), or absence (farm 1), of a specific diagnostic qPCR test.

Mares that aborted due to EHV-1 were both significantly younger and aborted significantly later and over a narrower range of gestation than mares that aborted due to other causes. This was probably a product of a number of factors including the general increase in the frequency of abortion with increasing age [24], but also to the range of both infectious and non-infectious aetiologies that could have caused the non-EHV-1 abortions [2; 25].

Both the overall success of diagnosing the apparent cause of abortion and the relatively-high representation of placentitis among the positive diagnoses were similar to other reports [2; 25]. The diagnostic rate was however; appreciably lower than reports that considered non-EHV-1 abortions alone. Moreover, the predilection of placentitis for older mares [26] may have contributed to the higher mean age among the non-EHV-1 abortion cohort.

EHV-1 did not noticeably affect the incidence of RFM, which was similar to that in normal post-foaling mares; this contrasts to a recent report [15]. There was however an increased incidence of RFM in mares that aborted due to causes other than EHV-1, which supported an earlier report of an elevated risk of RFM in aborting mares [16]. PPS was reported in association with both 'normal foaling' and non-EHV-1 abortions, whereas in contrast to previous reports [14; 19; 27] EHV-1 was not associated with PPS. Both farms reported a single mare fatality associated with abortion, one uterine prolapse (EHV-1) and one rectal prolapse (subsequent to placentitis), respectively.

A previously-reported outcomes-based analysis of the post-epizootic reproductive performance on these two farms demonstrated that abortion due to EHV-1 (or due to any other cause) did not predict the pregnancy or foaling rates among the affected mare populations during the subsequent breeding season [12].

Conclusions

This study recorded several novel features, and confirmed some previous associations, of EHV-1 abortion in unvaccinated mare populations, and indicated intervention strategies that may affect the spread of infection. The following preventative and response measures appeared to help: i) subdivide into the smallest practicable groups early in gestation, ii) avoid additions to late-pregnant mare groups, and maintain newly-acquired late-pregnant mares as separate groups until after foaling, iii) investigate all abortions and prioritize EHV-1 diagnosis, iv) rapidly implement EHV-1-appropriate biosecurity measures until an aetiological diagnosis is obtained (availability of a rapid diagnostic test facilitates compliance with these measures), v) submit

appropriate samples for molecular diagnostics (including fetal tissues and membrane samples), vi) if the index abortion occurs in a large group of mares, subdivide as soon as possible to reduce the number of mares exposed to horizontal transmission by any subsequent abortions; vii) minimize separation-associated stress by maintaining group-mates within 'sight and sound' of aborting mares, and vii) institute preventative vaccination in all at-risk pregnant mares.

Manufacturers' addresses

[a] Pfizer Animal Health, Sandton, South Africa

[b] Roche Products, Randburg, South Africa

[c] Lifetech, Johannesburg, South Africa

[d] Celtic Molecular Diagnostics, Cape Town, South Africa

[e] Systat Software Inc, San Jose, California, USA

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CHAPTER 6
The effect of consignment to broodmare sales on physiological stress measured by faecal glucocorticoid metabolites in pregnant Thoroughbred mares

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Abstract

Background: Validation of a method for the minimally-invasive measurement of physiological stress will help understanding of risk factors that may contribute to stress-associated events including recrudescence of equid herpesvirus (EHV), which is anecdotally associated with sales consignment of pregnant Thoroughbred mares. In this study we compared two similar groups of late-gestation Thoroughbred broodmares on the same farm: a consigned Sales group (n=8) and a non-consigned Control group (n=6). The Sales mares were separated from their paddock companions and grouped prior to their preparation for, transport to, and return from the sales venue. Both groups were monitored by sampling at regular intervals from five days prior to until 14 days after the sales date (D0) to measure physiological stress in terms of changes in faecal glucocorticoid metabolite (FGM) concentrations, and for event-related viral recrudescence via daily body temperature measurements and periodic nasal swabs for PCR analysis for EHV-1 and -4 DNA.

Results: In both groups, FGM levels increased post-sales before returning to pre-sales levels. Specifically, FGM concentrations in the Sales mares were significantly higher on D+3 and D+10 than on D-4 and D-3 ($F=12.03$, $P<0.0001$, Post hoc: $P=0.0003$-0.0008) and in the Control group FGM concentrations were higher on D+10 than D-4 ($F=5.52$, $P=0.004$, Post hoc: $P=0.005$). Interestingly, mean FGM levels in Control mares were significantly higher at four of the five sampling points ($t=5.64$-2.25, $p=0.0001$-0.044). Only one (Sales) mare showed PCR evidence of EHV-1 shedding.

Conclusions: Using FGM to measure physiological stress was supported by the increases observed in all mares after Sales consignment, including those not consigned to the sale. Monitoring FGM levels therefore represents an appropriate, minimally-invasive method for future studies to assess the contribution of physiological stress to EHV recrudescence in horses transported to sales or equestrian events.

Keywords: Pregnant mare; Thoroughbred; Faecal glucocorticoid metabolites; Non-invasive hormone measurement

Background

The physiological stress response in horses involves various metabolic, immunological and neuro-endocrine mechanisms [1; 2; 3; 4; 5]. Moreover, chronic exposure to stress may result in

immunosuppression and an increased susceptibility to disease [1; 5]. The interpretation of serum cortisol levels, which are commonly employed as an indicator of stress in horses [3; 4; 5], is however complicated by the effects of episodic fluctuations and pulsatile secretion of this hormone [1; 2; 4; 6; 7; 8]. To avoid obtaining non-representative samples, assessment of adrenocortical function can be standardized using faeces as a hormone matrix for measuring time-averaged response to a stressor [9; 10; 11]. Monitoring changes in faecal glucocorticoid metabolite (FGM) output offers several advantages in that faeces are easily collected with minimal handling of the animal and the FGM concentrations measured reflect cumulative secretion and elimination of hormones over several hours [8]. Enzyme immunoassays (EIA) have been developed and validated for monitoring changes in FGM output in various species, including the horse [9; 12]. The lag time between stress-related plasma hormone release and the associated appearance of the signal in faeces is approximately 24-48 h [4] and the changes in FGM concentrations do not appear to be significantly influenced by changes in grass or other food intake [2]. Faecal FGM measurement therefore represents a practical, non-invasive method to monitor adrenocortical endocrine function in horses, overcoming potential shortcomings of serum cortisol assays.

It has been suggested that one of the key events in the pathogenesis of equid herpesvirus (EHV) abortion is stress-associated reactivation of latent infection followed by transfer and replication of the virus in the upper respiratory epithelium and local lymph nodes. Virus is subsequently shed via nasal secretions, while cell-associated viraemia also occurs [13; 14]. In pregnant mares, transfer of abortogenic virus to the placenta, resulting in placentitis and abortion has been reported [15; 16; 17]. Stress-associated reactivation of latent EHV-1 is one proposal advanced for why EHV-1 abortion is still seen, albeit, sporadically in vaccinated broodmare groups [13; 17; 20]. The risk factors for viral recrudescence (including potential stressors) are however poorly understood [18]. The few reported studies investigating potential relationships between specific stress factors and viral reactivation and shedding are limited by the absence of any data concurrently verifying the physiological effects of the presumed stressor [18; 19]. Relocations, including sales' consignment and the introduction of new mares into established groups of pregnant Thoroughbred broodmares are anecdotally associated with subsequent EHV-associated sequelae including abortion. In such cases, it is assumed that the relevant potential stressors include social, environmental and management-associated cues [13; 17; 20].

During the course of routine sales consignment of pregnant broodmares, we investigated changes in faecal glucocorticoid metabolite (FGM) measurements, while concurrently monitoring for any event-related viral reactivation by daily monitoring of body temperature and molecular detection of the presence of viral nucleic acid for EHV by real-time quantitative PCR [14; 21] in samples recovered from the upper respiratory tract by nasal swabbing.

Results

FGM levels of both Sales and Control mares showed an overall increasing trend of between the pre-sales period and the initial post-sales period, before returning to pre-sales levels again (Figure 1). For the Sales group, FGM levels were significantly lower on both D-4 and D-3 when compared to respective FGM concentrations on D+3 and D+10 ($F=12.03$, $P<0.00001$, power of performed test = 0.999, $\alpha=0.05$, Post hoc: $P=0.0003$-0.0008). A significant increase in FGM concentrations between D-4 and D+10 was also found for the Control group ($F=5.52$, $P=0.004$, power of performed test = 0.899, $\alpha=0.05$, Post hoc: $P=0.005$). Somewhat unexpectedly, the mean FGM concentrations for Control mares were higher than in Sales mares, with a significant difference between the two groups at four of the five of the individual sampling points ($t=5.64$-2.25, $P=0.0001$-0.044).

Only two of the daily recordings of body temperatures exceeded the lower threshold defined to indicate pyrexia, namely ≥38.0°C, with one incident in each of two Sales mares. In addition, only one incident of viral shedding of EHV-1 was detected by PCR during the course of this study (Ct=34.7), and this in a Sales mare on D+14. Over the complete range of data points, FGM levels and rectal temperatures were weakly but significantly correlated ($r=0.238$, $n=130$, $P=0.006$) (Figure 2).

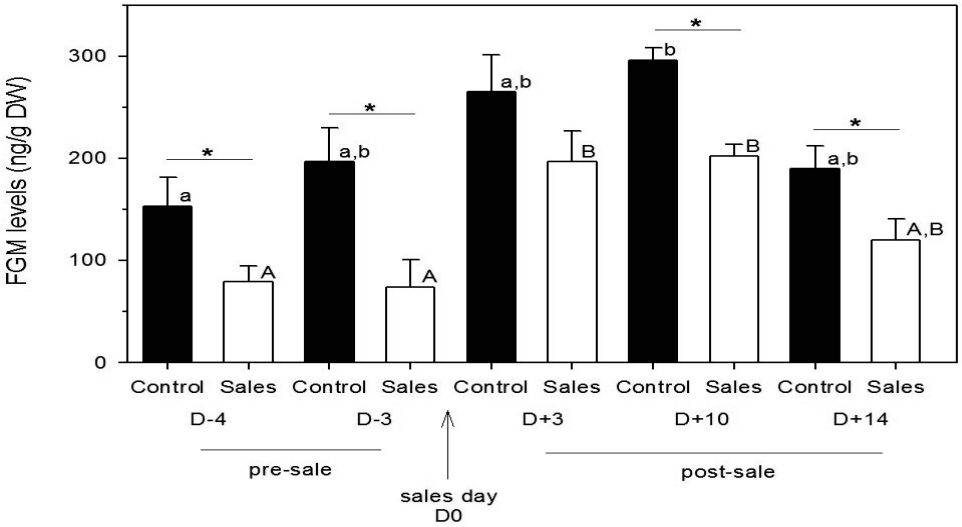

Figure 1 The effect of sales consignment on physiological stress measured by faecal glucocorticoid metabolites (FGM) in pregnant Thoroughbred broodmares

FGM concentrations (mean +SEM) of mares in the Control (n=6) and Sales groups (n=8) at the selected sampling points D-4, D-3, D+3, D+10 and D+14 with respect to the day of the Sales. Asterisks indicate statistically significant differences between the two groups at a respective sampling point, determined using t-tests. Different superscripts (capital letters = Sales group; lower case letters = Control group) indicate statistically significant differences between sampling points within the respective group, determined using one-way repeated measures ANOVA.

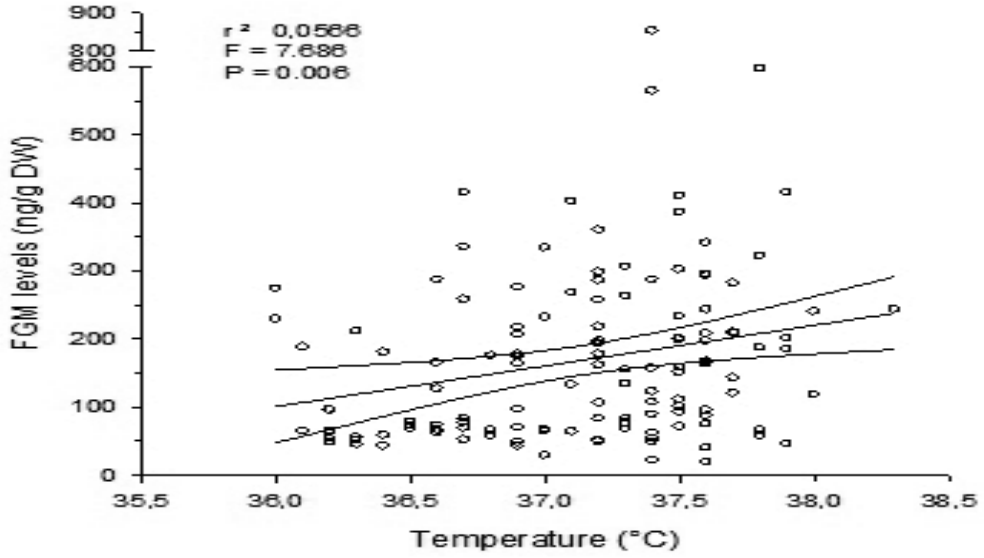

Figure 2 The relationship between faecal glucocorticoid metabolites (FGM) and body temperature measurements in pregnant Thoroughbred broodmares consigned for sale

Relationship between FGM levels and body temperature (r=0.238, n=130, P=0.006) determined in 26 pregnant Thoroughbred broodmares of >7 months gestation, monitored up to seven times over a selected 19 day pre- to post-sale period.

Discussion

The utility of FGM levels as a means of monitoring stress associated with sales consignment, or indeed other potential stressors, among pregnant broodmares was supported by the current data. The major stressful events (i.e. transport to, individual stabling and sales' ring appearance, and return from the sales) on day 0 were associated with a significant rise in FGM levels three days later, which would account for the expected lag-time of 24-48 h before the respective changes in glucocorticoid concentrations would appear in the faeces [1; 4]. An interesting, if slightly unexpected, finding was that mares that were not consigned for sale showed significantly higher pre-sales FGM concentrations than the Sales mares at nearly all time points, and a rise in FGM concentrations in the days after their herd mates had been taken to the sales, that reached statistical significance on D+10. Although it is difficult to separate the individual stressors that contributed to the observed response, this suggests that social disruption (in this case removal of Sales mares from the settled groups and subsequent removal

from visual and olfactory contact for approximately 24 h) may be a key stressful event among settled groups of horses; this echoes a previous study investigating the effects of social instability on chronic stress [29]. The raised FGM concentrations in non-consigned (control) mares may have been influenced by a 'neighbour' stress effect, given that the consigned mares remained within sight and sound of their previous group mates on all except the day of sales. Alternatively or additionally, it is probable that removal of an animal from a group disrupted the social order and resulted in a potential stress-related period during which a new hierarchical equilibrium ('pecking order') was established. The comparatively high FGM levels in non-consigned mares subsequent to disruption of their group stability indicate that the paradigm of increased abortion-risk in pregnant mares as a result of social stress should also include consideration of the susceptibility of mares in a group from which others have been removed. It is, however, less easy to interpret why the Sales mares did not show higher FGM levels in the pre-sales period as a result of removal from their group and introduction into a new, albeit smaller group.

A weak but statistically significant association between body temperature and FGM levels was observed but is difficult to interpret, not only because the incremental rises in FGM with increasing body temperature are modest but also because of the potentially confounding effect of some of the stressful events (e.g. transportation) which could result in mild hyperthermia concomitant with stress. Future observations in larger populations may clarify whether there is a biological association between FGM and body temperature, or whether the correlation detected represents parallel increases (stress and hyperthermia) induced by a specific stressful event. The present study's limited success in associating stressful events with clinical pyrexia and associated EHV shedding was similar to previous studies [18; 21]. This was not particularly surprising and most probably related to the small sample population, the relatively short sampling period, sampling frequency, the effects of vaccination and, not least that stress-induced reactivation of latent EHV infection is almost certainly an uncommon event.

Conclusions

This study described a novel approach to quantify physiological stress in Thoroughbred broodmares during late gestation after their exposure to potential stressors during a series of events anecdotally-associated to predispose to EHV recrudescence and associated abortion. The study provided useful preliminary data to support the value of FGM monitoring as a

minimally-invasive but reliable method for monitoring stress when investigating EHV reactivation, or indeed other events proposed to be a result of medium to long-term stress, in horse populations.

Methods

The study was approved by the University of Pretoria's Animal Use and Care Committee (V068/05).

Animals

Twenty-six Thoroughbred broodmares of ≥7 months gestation, aged from 3-14 years and resident on a farm in KwaZulu-Natal Province, South Africa were included in this study during the southern hemisphere winter of 2010. The mares were distributed among routinely-managed groups of approximately 30 pregnant mares, grouped based on anticipated foaling dates. Mares were kept permanently outdoors in paddocks of 4.6-11.7 ha in area that provided free access to kikuyu grass (*Pennisetum clandestinum*) pasture. All mares were routinely vaccinated with an inactivated EHV-1 vaccine at five, seven and nine months of gestation. Eight mares were destined for consignment at an annual sale of broodmares and designated as the 'Sales group'. Six mares, of a similar stage of pregnancy but not destined for sales consignment were selected as the Control group. Twelve additional mares were monitored to increase the number of data points for analyzing the relationship between FGM levels and body temperature.

Study design

The Sales mares were removed from their original respective groups approximately one month prior to the sales and transferred to a common, separate paddock within sight and sound of their original paddock group-mates. The key events identified as potential stressors in Sales mares were: temporary removal for pregnancy confirmation by trans-rectal palpation and ultrasound five days prior to the sale (D-5), washing and grooming two days prior to the sale (D-2), transportation by road to the sales venue 30 km away with a journey time of approximately one hour on the morning of the sale (D0), being stabled individually at the venue prior to and after the short duration of the auction process in the sales ring, returning to the farm on the

same day *via* the manner and route described above, and upon return being maintained as a single, separately managed group as a biosecurity precaution until foaling.

Sample collection and analyses

The broodmares were all accustomed to routine, frequent gynaecological examinations, including trans-rectal palpation, and were monitored using minimal manual restraint within their paddocks between D-5 to D+14 by daily recording of rectal temperature and recovery of a faecal sample. Faecal samples were collected daily between 08h00 and 10h00 by manual extraction using a lubricated latex-gloved hand inserted *per* rectum. The glove with homogenized faecal material inside was tied, labelled with an indelible marker pen and removed for immediate storage at -20°C until hormone extraction and assay.

In addition nasal secretions were collected by swabbing at five points: D-4, -3, +3, +10 and +14. The nasal secretions, obtained by insertion of a sterile cotton-tipped swab[a] *via* the *external nares*, were placed in a pre-labelled plastic sleeve and stored at -20°C prior to molecular detection of viral nucleic acid using qPCR.

Faecal extraction and hormone analysis

Frozen faecal samples were lyophilized, pulverized, and sifted using a metal mesh strainer to remove fibrous material [22]. Approximately 0.05 g of the faecal powder was then extracted by vortexing for 15 min with 80% ethanol in water (3ml). Following centrifugation for 10 min at 1500 g, supernatants were transferred into micro-centrifuge tubes and stored at -20°C until analysis. Faecal extracts were measured for immunoreactive FGM concentrations using an EIA that detects 11, 17 dioxoandrostanes, and previously validated for monitoring adrenocortical endocrine function in a range of mammals including horses [12; 23; 24]. Serial dilutions of faecal extracts gave displacement curves which were parallel to the standard curve of the assay. Sensitivity of the assay at 90% binding was 3.0 pg/well. Intra- and Inter-assay coefficients of variation, determined by repeated measurements of high- and low-value quality controls, ranged between 5.2% and 13.7%. The cross-reactivity's of the antibody and the assays performed on microtiter plates were previously reported [25; 26].

PCR for EHV

Nasal swabs were agitated for 5 s in 0.5 ml of 0.1 M PBS (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[a] and a Kingfisher 96 Magnetic Particle Processor[b] according to the manufacturer's protocol. Subsequently, a duplex PCR for EHV-1 and -4 was performed using previously described primers and probes [27]. Briefly, 17 µl of a mastermix 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[c] 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.

Data and statistical analyses

Differences in FGM levels between two sets of data were examined by t-test, after confirming between-group equivalence of variances. Differences in hormone concentrations between more than two sets of data were examined by one-way repeated measures ANOVA, followed by post hoc analysis using Tukey's test. All tests were two-tailed, with significance set at $P \leq 0.05$. In cases of all-pairwise multiple comparison procedures, the α-level was adjusted by applying a previously described procedure [28]. The relationship between the two variables (FGM levels and body temperature) was examined using Pearson's product-moment correlation test. Statistical analysis was performed using SigmaPlot 12[d].

Endnotes

[a] Life Technologies™, Carlsbad, CA, USA.

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[c] Kapa Biosystems, Cape Town, South Africa

[d] Systat Software Inc., San Jose, CA, USA

Abbreviations

ANOVA, analysis of variance; DNA, deoxyribose-nucleic acid; EHV, equid herpesvirus; EHV-1, equine herpesvirus type 1; EIA, enzyme immune-assay; FGM, faecal glucocorticoid metabolite;

PBS, phosphate-buffered saline; qPCR, quantitative real-time polymerase chain reaction; RNA, ribose-nucleic acid

Competing interest

The authors declare they have no competing interests

Authors' contributions

MS conceived and participated in the design of the study, organised and participated in the data collection and interpretation and drafted the manuscript. AB participated in the data collection and study execution. SG performed the faecal extraction and EIA. AlG participated in the design of the study, participated in the data collection and interpretation and in the PCR analysis. TS participated in data interpretation and drafting of the manuscript. AG participated in the study execution, interpretation, draft of the manuscript and performed the statistical analysis. All authors read and approved the final manuscript.

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CHAPTER 7
Detection of equine herpesvirus-4 and physiological stress patterns in young Thoroughbreds consigned to a South African auction sale

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Abstract

Background: The prevalence of equine herpesviruses 1 and 4 (EHV-1 and -4) in South African Thoroughbreds at auction sales is currently undefined. Commingling of young Thoroughbreds from various populations together with physiological stress related to their transport and confinement at a sales complex, may be associated with shedding and transmission of EHV-1 and -4. This prospective cohort study sampled 90 young Thoroughbreds consigned from eight farms, originating from three provinces representative of the South African Thoroughbred breeding demographic to a sales complex. Nasal swabs for quantitative real-time polymerase chain reaction (qPCR) assay to detect EHV-1 and -4 nucleic acid and blood samples for enzyme-linked immunosorbent assay for EHV-1 and -4 antibodies were collected from all horses on arrival and departure. Additional nasal swabs for qPCR were obtained serially from those displaying pyrexia and, or nasal discharge. Daily faecal samples were used for determination of faecal glucocorticoid metabolite (FGM) concentrations as a measurement of physiological stress and these values were modelled to determine the factors best explaining FGM variability.

Results: EHV-4 nucleic acid was detected in 14.4% and EHV-1 from none of the animals in the study population. Most (93.3%) and very few (1.1%) of this population showed antibodies indicating prior exposure to EHV-4 and -1 respectively. Pyrexia and nasal discharge were poor predictors for detecting EHV-4 nucleic acid. The horses' FGM concentrations increased following arrival before decreasing for most of the remaining study period including the auction process. Model averaging showed that variation in FGM concentrations was best explained by days post-arrival and transport duration.

Conclusions: In this study population, sales consignment was associated with limited detection of EHV-4 nucleic acid in nasal secretions, with most showing prior exposure to EHV-4 and very few to EHV-1. The physiological stress response shown by most reflected the combination of stressors associated with transport and arrival and these are key areas for future investigation into management practices to enhance health and welfare of young Thoroughbreds during sales consignment.

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

Background

Equine respiratory infection is an important 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 [1; 2]. Equine herpesviruses 1 and 4 (EHV-1 and -4) are important agents associated with infectious upper respiratory tract disease (IURD) [3-5]. Few surveillance studies to detect these viruses are reported in healthy horse populations [6; 7].

Risks associated with IURD are multifactorial, including host, environmental, management and pathogen-specific factors [8; 9]. 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 [4; 8-13]. Physiological stress is arguably one of the more important risk factors, with associations reported between EHV-1 and -4 recrudescence and shedding and various exogenous stressors, as well as experimental corticosteroid administration [14-16]. Stress may further increase the susceptibility of naive animals to new infections [14]. Transport and the subsequent confinement, handling and management at sales events may contribute to physiological stress [11; 13; 14; 17]. 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 [9; 11; 18]. 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 [13; 17].

Measurement of faecal glucocorticoid metabolite (FGM) concentrations to monitor adrenocortical function in horses provides a practical, non-invasive and feedback-free alternative to glucocorticoid (e.g. cortisol) determination in blood, saliva, urine or milk [18; 19]. 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 h in horses [19; 20]. Fluctuations in FGM concentrations have been reported for determining stress responses in horses, including animals consigned to sales and following short, medium and long-distance road transport [18; 21; 22].

The effects of sales consignment on detection of viral nucleic acids and physiological stress responses among young Thoroughbreds have not been reported previously. This study aimed to detect EHV-1 and -4 nucleic acids in nasal secretions of young Thoroughbreds at a South

African auction sale. It also aimed to determine which factors among: EHV-4 nucleic acid detection, clinical signs of respiratory disease, transport and preparation for auction explained variability in FGM concentrations, as an indicator for physiological stress.

Methods

The study was approved by the Animal Ethics Committee of the University of Pretoria (Study V040-13).

Animals

The study population included 90 (51 colts, 39 fillies) of the 358 two-year old Thoroughbred horses catalogued for an annual auction sale in South Africa. 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 (Farm 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 (Farm 6, 7 and 8) in KwaZulu-Natal Province. This selection was representative of the South African Thoroughbred breeding demographic.

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 with metal grids enabled nose-to-nose contact between horses in adjacent stables. Horses were periodically removed from their stables into adjacent walkways for showing or grooming and mixed randomly at communal facilities during routine daily activities including in-hand exercise and washing. Daily care activities of the horses, including feeding, were performed by the staff of the respective farms according to each farm's protocol. All enrolled horses were vaccinated against equine influenza virus, 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.

Study design

A prospective cohort study was performed during the late southern hemisphere winter at the Thoroughbred Breeders Association National Two Year Old Sales in Germiston, Gauteng Province of South Africa. Horses travelled by road transport and arrived at the sales complex on various dates. Their residence period therefore varied between four and nine days. The auction occurred over two days (15 and 16 August 2013), with each horse's date of auction regarded as the end-point of clinical data collection for that horse, prior to its departure. For half of the horses, FGM data were also collected on the day post auction. For each horse, the period from its day of arrival until the third day post-arrival was defined as the 'adaptation phase'. For each horse, the period including the day prior to and the day of its auction was defined as the 'auction phase'. These phases were defined *a priori* to allow comparison of data from horses with varying periods of residence.

Sample collection and analyses

Sample collection

At arrival: One nasal swab, a blood sample and a faecal sample were collected from each horse within 24 h of arrival. Nasal secretion samples were collected using a 15 cm metal shaft rayon-tipped swab[a] advanced into either of the horse's nostrils and gently rotated against the mucous membranes for collection of nasal secretion and epithelial cells. Following collection the swab was replaced in its sterile, dry plastic tube and refrigerated at 4-6°C until delivery to the Veterinary Genetics Laboratory, University of Pretoria. Laboratory processing of nasal swabs was performed within 48 h of collection. One 8.5 ml BD Vacutainer® SSTTM II Advance Plus serum tube[b] was filled with blood from each horse by means of jugular venipuncture. Blood samples were refrigerated at 4-6°C following collection, until delivery to the Immunocontraception Laboratory, University of Pretoria. A 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 h of collection and kept frozen until delivery to the Endocrine Research Laboratory, University of Pretoria.

Daily monitoring: From arrival until departure horses were monitored twice daily, between 06h00-09h00 and 15h00-18h00 with rapid digital thermometers (Thermoval®[c]) for pyrexia, defined as a rectal temperature ≥38.5°C. Horses were additionally monitored once daily, between 06h00-09h00, for the presence of an obvious nasal discharge.

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: One nasal swab, a blood sample and a faecal sample were collected as described from each horse following their auction, within 24 h prior to their departure.

Laboratory analyses

Quantitative real-time polymerase chain reaction (qPCR) for EHV-1 and -4 deoxyribonucleic acid (DNA): 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 qPCR for EHV-1 and -4 was performed using previously described primers and probes [23]. 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. EHV-1 and -4 reference viral cultures were obtained from the Equine Virology Research Laboratory, University of Pretoria. Aliquots of nucleic acid extracted from these reference virus cultures were included on each plate as positive controls with nuclease free water being included as a negative control. The qPCR 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.

Enzyme-linked immunosorbent assay (ELISA) for EHV-1 and -4 antibodies: Each serum sample was tested against the glutathione-S-transferase (GST) fusion proteins of EHV-1 glycoprotein G, EHV-4 glycoprotein G and against GST only, as previously described by Crabb and Studdert [24] and validated by Gilkerson *et al* [25]. 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. 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 for EHV-1 and -4 antibodies were included on each plate. All antigens and positive control samples used in these assays were obtained from the Centre for Equine Virology, University of Melbourne.

Faecal extraction and hormone analysis: Frozen faecal samples were lyophilized, pulverized and sifted using a metal mesh strainer to remove fibrous material [26]. 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 enzyme immunoassay that detects 11, 17-dioxoandrostanes, previously shown to provide reliable information on adrenocortical function in various mammals, including horses [27-29]. 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%. The assay was performed as previously described using antibodies for which cross-reactivity's have been reported [30; 31].

Statistical analyses

To determine which factors best explained variability in physiological stress in our study animals, we modelled the natural-log-transformed FGM concentrations from 655 faecal samples in linear mixed models, fitted with the 'identity' link function, using *lmer* in Package 'lme4' in R [32]. The global model included six standardized fixed effects: days post arrival; auction phase (including the preparation day one day prior to auction and the auction day); transport duration; pyrexia; nasal discharge; EHV-4 DNA detection. Transport duration ranged from 6-22 h (median: 15.25 h). Repeated measures were modelled as random effects: horse identity (1|horse); farm identity (1|farm). Candidate models were evaluated with Akaike's Information Criterion (AIC_c) [33]. Model averaging was performed using Akaike weights (w_i) of all candidate models [34]. Goodness of fit of parameter estimates was assessed using 85% confidence intervals and Ω_0^2 for the global model [35; 36]. Collinearity among covariates was assessed with variance inflation factors (with *a priori* values of >5 deemed questionable and >10 deemed unacceptable correlation).

Results

EHV nucleic acid detection

No EHV-1 DNA was detected, however EHV-4 DNA was detected in nasal secretions of 13/90 (14.4%) horses originating from 7/8 participating farms (Tables 1 & 2). A total of 21 swabs positive for EHV-4 DNA were obtained from these 13 horses. Repeated incidents of EHV-4 DNA detection occurred in 4/13 (30.8%) horses. Both the longest period of continuous detection and the longest interval between consecutive detection events in an individual horse, were four days. Nasal swabs from 1/90 (1.1%) and 7/90 (7.8%) horses were positive for EHV-4 DNA on day of arrival and departure, respectively. Details of the temporal pattern of EHV-4 nucleic acid detection during the observation period are shown in Table 1. The EHV-4 qPCR-results for 13 horses from which EHV-4 DNA was obtained in their nasal secretions, are shown in Table 2.

EHV-4 nucleic acid detection and clinical signs

All 13 horses from which nasal swabs positive for EHV-4 DNA were obtained showed either nasal discharge alone, or both pyrexia and nasal discharge during the observation period (Table 3). However, 65/77 (84.4%) horses in which EHV-4 DNA was not detected also showed one or both of these clinical signs (Table 3). Duration of pyrexia was less than 24 h in 7/8 horses with concurrent detection of EHV-4 DNA.

EHV-1 and EHV-4 serology

Upon arrival at the sales complex, 1.1% and 93.3% of the study population showed serological evidence of prior exposure to EHV-1 and -4, respectively. No instances of seroconversion were recorded between arrival and departure. Only one horse (Horse E) of the 13 from which qPCR-evidence of EHV-4 DNA was detected in its nasal secretions, was EHV-4-seronegative on arrival and remained seronegative on departure seven days later.

FGM concentrations

None of the covariates in our models exhibited unacceptable collinearity: all variance inflation factors were <2.2. Several models contained a similar amount of information or explained a similar amount of variability in FGM's (i.e. 'best models' with AICc <10, Table 4), suggesting that model averaging was an appropriate approach. Based on model averaging, the covariates that best explained variation in FGM concentrations were days post arrival, transport duration, and

pyrexia, which all had large standardized effect sizes and differed from zero (Figure 1). Days post arrival was selected in all of the best candidate models, but transport duration was almost as important (Figure 1) and was only left out of two of the best models (Table 4). Although the parameter estimate for pyrexia differed from zero, this covariate had a relatively smaller standardized effect size than those of the transport-associated parameters (Figure 1) and it had relatively lower importance (it was not selected in the second best candidate model, Table 4). The auction phase, EHV-4 DNA detection, and nasal discharge parameters all had either high variability in parameter estimates or a small effect size (Figure 1). The global model explained 31% of variation in FGM concentrations, with Ω_0^2=0.31.

Post hoc graphical analysis of the FGM data supported the results of the model averaging and suggested that median FGM concentrations for the eight farms increased (with increased variability) after arrival, before decreasing in concentration and variability (to approximately 40 ng/g dry weight) for most of the remainder of the study period (Figure 2). During the adaptation phase, FGM concentrations were 64% higher and 93% higher on the day of arrival and one day after arrival, respectively, when compared to three days after arrival (Figure 2A, and represented by the largest effect size in Figure 1). No discernible increase in FGM concentrations was associated with the auction phase (Figure 2B, and represented by small effect size with a confidence interval overlapping zero in Figure 1).

Table 1 Proportion of EHV-4 nucleic acid qPCR-positive nasal swabs obtained from 90 Thoroughbreds at an auction sale

Farm of consignment	Province of origin	Consigned horses (n)	Sample dates (August 2013)								
			8	9	10	11	12	13	14	15*	16*
1	**Western Cape**	13		0/13	1/8	0/9	0/9	0/9	0/9	0/12	0/5
2	**Western Cape**	9		0/9	2/5	0/5	0/5	0/6	1/7	1/8	1/5
3	**Western Cape**	8	0/8	0/5	0/5	0/5	1/6	1/7	1/7	1/8	1/5
4	**Eastern Cape**	13			1/13	0/4	0/4	0/7	0/7	3/12	0/4
5	**Eastern Cape**	5			0/5	0/1	0/3	1/3	2/4	1/5	0/1
6	**KwaZulu-Natal**	10				0/10	0/0	1/3	0/5	0/8	0/3
7	**KwaZulu-Natal**	26		0/26	0/16	0/16	0/17	0/18	0/19	1/25	0/9
8	**KwaZulu-Natal**	6						0/6	0/1	0/6	0/2
Number (%)		90	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

Table 2 qPCR-results from 13 Thoroughbreds with detection of EHV-4 nucleic acids in their nasal secretions

Horse identity	Farm of consignment	Province of origin	Sample dates (August 2013)								
			8	9	10	11	12	13	14	15	16
A	1	Western Cape		-	39.34*	-	-	-	-	-	
B	2	Western Cape		-	38.21*	-	-	-	-	38.80*	38.41*
C	2	Western Cape		-	37.20*	-	-	-	-	-	-
D	2	Western Cape		-	-	-	-	-	38.27*	-	-
E	3	Western Cape	-	-	-	-	34.87*	-	-	-	
F	3	Western Cape	-	-	-	-	-	34.17*	35.64*	33.56*	29.58*
G	4	Eastern Cape			36.98*	-	∞	-	-	38.70*	
H	4	Eastern Cape			-	n/s	n/s	n/s	n/s	38.94*	
I	4	Eastern Cape			-	n/s	n/s	n/s	n/s	34.29*	
J	5	Eastern Cape			-	n/s	-	38.18*	38.24*	33.79*	
K	5	Eastern Cape			-	n/s	-	-	37.35*	-	
L	6	KwaZulu-Natal				-	n/s	38.25*	-	-	
M	7	KwaZulu-Natal		-	-	-	-	-	-	38.41*	
Number of EHV-4 qPCR-positive nasal swabs			0	0	4	0	1	3	4	7	2

horse not present at sales complex; - EHV-4 qPCR-negative; * EHV-4 qPCR-positive with Ct- (cycle threshold) value; n/s no swab collected; ∞ sampling not possible

Table 3 Detection of EHV-4 nucleic acid and clinical signs recorded for 90 Thoroughbreds at an auction sale

EHV-4 nucleic acid	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 Models with Akaike weights (wi) >0 and ΔAICc <10, modelling faecal glucocorticoid metabolite concentrations in horses

Model: log(FGM)~	log*L*	*K*	AICc	Δ	w_i
days + duration + discharge + EHV-4 + pyrexia + (1|farm) + (1|horse)	-364.6	9	747.8	0.0	0.48
days + duration + discharge + EHV-4 + (1|farm) + (1|horse)	-366.5	8	749.5	1.7	0.21
days + duration + discharge + EHV-4 + pyrexia + auction + (1|farm) + (1|horse)	-364.4	10	749.5	1.7	0.21
days + duration + discharge + EHV-4 + auction + (1|farm) + (1|horse)	-366.4	9	751.2	3.5	0.08
days + discharge + EHV-4 + pyrexia + (1|farm) + (1|horse)	-369.6	8	755.5	7.7	0.01
days + discharge + EHV-4 + pyrexia + auction + (1|farm) + (1|horse)	-369.2	9	756.9	9.1	0.01

FGM faecal glucocorticoid metabolites; EHV-4 equine herpesvirus type 4

Individual horses (1|horse) and farms (1|farm) were random effects in all models. Fixed effects were days post arrival (days), auction phase (auction), duration of transport (duration), pyrexia, nasal discharge (discharge), and EHV-4 DNA detection.

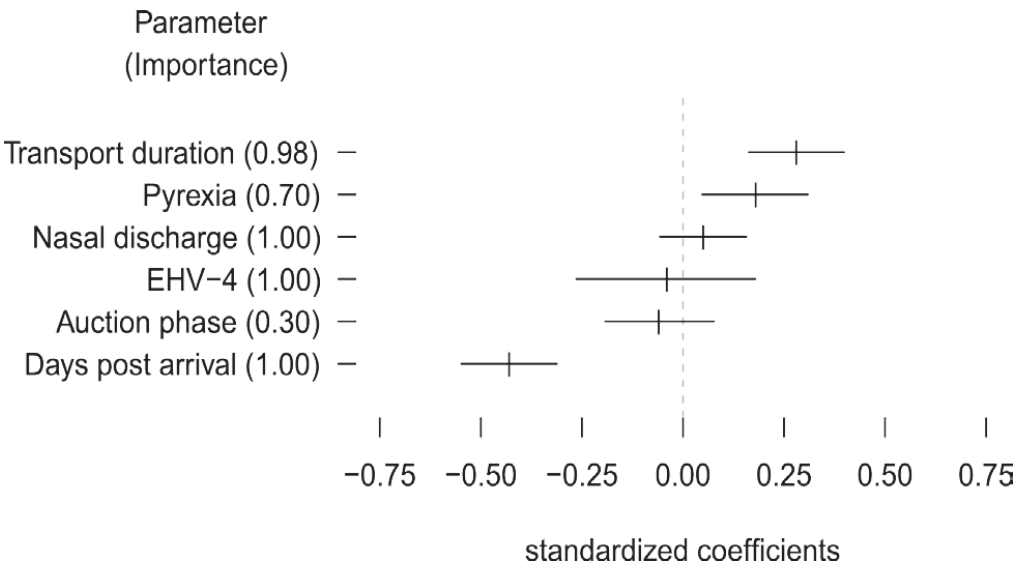

Figure 1 Model-averaged standardized parameter estimates with 85% confidence intervals for covariates explaining faecal glucocorticoid metabolite (FGM) concentrations in horses. Importance indicates the sum of Akaike weights for all models containing the parameter.

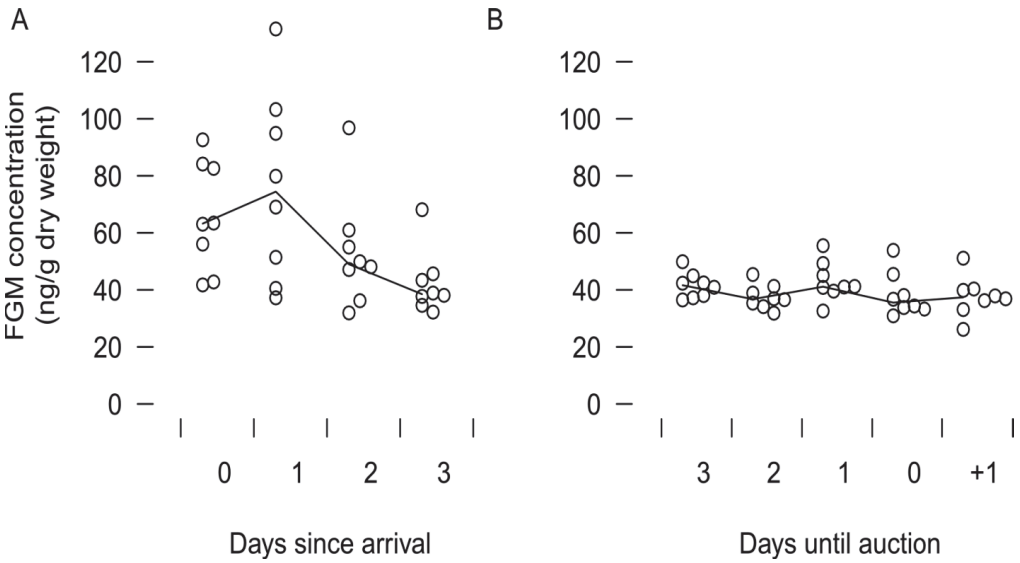

Figure 2 Faecal glucocorticoid metabolite (FGM) concentrations of consigning farms (n=8) during the adaptation (**A**) and auction (**B**) phase. Circles represent median FGM concentrations for horses from different farms on each day. Lines connect daily medians of the eight farm median FGM values. Day '+1' in panel B indicates the day post-auction for which faecal samples were obtained from half of the study's horses. With a gut passage time of approximately 24 h, FGM values reflect physiological stress responses to stimuli experienced on the previous day.

Discussion

Detection rates of EHV-4 and -1 DNA in nasal secretions from the study population horses were higher and lower, respectively, than those reported in populations showing clinical signs of respiratory disease [4; 5]. It was previously suggested that EHV-1 rarely circulates outside breeding populations inclusive of young foals [13]. In the present study, EHV-4 DNA detection included single and repeated, continuous or interrupted events and the relatively low rate of detection upon sales arrival was similar to a previous report [13]. Most EHV-4 detection events coincided with the dates of the two auction days, peaking on the first of these days. Both viral recrudescence and horizontal spread of primary infection may have contributed to the increased detection of EHV-4 DNA observed between arrival and departure [14; 15]. The study population showed a greater seroprevalence of EHV-4 compared to EHV-1, reflecting that prior exposure on the farms of origin was almost universal or only in a few individuals, to EHV-4 and -1 respectively. This seroprevalence was similar to previous reports from different populations, including two-year old racehorses in Australia [14; 37; 38]. EHV-4 DNA was detected in the nasal secretions of 12 horses despite the presence of EHV-4 antibodies, most likely as a result of

viral recrudescence. In the case of Horse E, EHV-4 DNA detection likely resulted from recent primary EHV-4 infection with nasal shedding of virus and serum sample collection prior to establishment of a detectable antibody response. The implications of EHV-1 and -4 DNA detection at sales departure and the associated risks of viral shedding and transmission at subsequent destinations, including training facilities, warrant further investigation.

In this study we observed a similar prevalence of pyrexia and a higher prevalence of nasal discharge than previously reported among EHV-4-positive horses [4]. Clinical signs was a poor indicator for viral nucleic acid detection, with several horses that showed clinical signs being negative for EHV-4 DNA, contrasting with a reported association between clinical signs of respiratory disease and EHV-4 detection in foals [40]. A pyrexia duration <24 h in the majority of EHV-4 qPCR-positive horses supported the utility of twice daily rectal temperature monitoring for suspected clinical cases [9; 39]. 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. The combination of pyrexia and nasal discharge was reported in association with molecular evidence of lesser characterised respiratory viruses EHV-2, EHV-5, equine adenovirus-1 and equine rhinitis B [41]. The current study's discrepancy in the prevalence of clinical signs and detection of EHV-1 and -4 warrants further investigation of the association of IURD with alternative infectious agents during sales consignment.

Sales consignment was associated with an elevation in FGM concentrations shortly after arrival. This presumably reflected a cumulative series of stressful events associated with transport and sales arrival, and gradually decreased as horses became accustomed to environmental and routine changes. The covariates that best explained the variation in FGM concentration in order of importance were the number of days post-arrival, transport duration and pyrexia. The EHV-4 DNA detection threshold on qPCR was lower than that reported by Diallo *et al* [23] which may explain the observation that EHV-4 DNA was not an important covariant in our model. Notably, the auction process itself did not appear to initiate any prolonged physiological stress.

Practicalities precluded monitoring of case-matched horses on the farms of origin for EHV-1 and -4 DNA detection and FGM alterations.

Conclusions

EHV-4 DNA was detected in nasal secretions of some young Thoroughbreds consigned to a South African auction sale. Most of these horses had been exposed to EHV-4 and very few to EHV-1 prior to their arrival at the sale. The combination of stressors associated with their transport and arrival was associated with most horses showing a physiological stress response. These, other stressors and commingling inherent to the current worldwide consignment process increase the risk association with IURD in young horses. The transport and arrival phases are key areas for future investigation into management practices to reduce the impact of physiological stress on the health and welfare of young Thoroughbreds during sales consignment.

Endnotes

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

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

[c] Paul Hartmann AG, Heidenheim, Germany

[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

Abbreviations

AIC: Akaike's information criterion; Ct: cycle threshold; DNA: Deoxyribose-nucleic acid; EHV: equine herpesvirus; EHV-1, -2, -4, -5: equine herpesviruses 1, 2, 4, 5; ELISA: Enzyme-linked immunosorbent assay; FGM: Faecal glucocorticoid metabolites; GST: glutathione-S-transferase; PCR: polymerase chain reaction; IURD: Infectious upper respiratory tract disease; qPCR: Quantitative real-time polymerase chain reaction; RNA: Ribose-nucleic acid.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

MB participated in the study design, organised and participated in the data collection and interpretation and drafting the manuscript; PP participated in the design of the study, assisted in the data collection and interpretation and drafting the manuscript; AG participated in the design of the study, data interpretation and statistical analysis and drafting the manuscript;

PL participated in the design of the study, data interpretation, drafting the manuscript and performed the statistical analysis; AG participated in the design of the study, data interpretation and drafting the manuscript; and MS conceived and participated in the design of the study, data collection and interpretation and drafted the manuscript. All authors read and approved the final manuscript.

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CHAPTER 8
Summarising discussion

8.1 The threat of the equine herpesviruses to equine health and reproductive performance

The threat posed by the equine herpesviruses is shaped primarily by adaptations evolved over millennia to evade the natural hosts' immune response and establish life-long latent infection. Latency interspersed with periodic cycles of (often clinically-silent) reactivation and viral shedding helps ensure infection of susceptible hosts, particularly young foals, and maintains the presence of the virus in the population. In breeding populations, the major consequences of herpesvirus infections are sporadic or epizootic late-gestation abortion and highly-fatal neonatal disease, with a potentially devastating impact in terms of economic and genetic losses. Herpesvirus infections can also result in sporadic or epizootic respiratory or neurological disease in horses. Even without epizootics, constant veterinary and management interventions and international regulatory vigilance against the threat of disease significantly impact the efficiency and economics of the international horse breeding industry.

The fact that elimination of these ubiquitous pathogens is unlikely and that available vaccines, despite increasingly widespread use, offer limited disease protection at best, underlines the importance of enhancing currently-available, and developing novel, interventions to reduce the impact of herpesviruses in the field. Epidemiological investigations of natural outbreaks, particularly in unvaccinated populations, are important because they help improve our understanding of herpesvirus pathogenesis, which is currently heavily-reliant on experimental challenge studies. The analysis of epidemiological data from EHV disease outbreaks combined with recent developments in molecular diagnostic techniques has the potential to guide the development of more effective intervention strategies.

From a personal perspective, this thesis reflects a distillation of my cumulative experiences of EHV-1 abortion outbreaks garnered over the past decade, and attempts to formulate the lessons learned into an enhanced approach to mitigate the impact of this virus on breeding farms. The experiences have included epizootics on Thoroughbred farms as geographically distant as northern California, the Western Cape and KwaZulu-Natal Provinces of South Africa, as well as a teaching herd of Nooitgedacht ponies at the University of Pretoria's Veterinary Faculty at Onderstepoort, Gauteng Province, South Africa. The development of this thesis was driven to a large extent on an evolving understanding of these complex pathogens, with the central question of each chapter shaped by its predecessor as new research questions arose. The original aim was to better understand the contribution of pregnancy loss to reproductive

performance. This was hitherto largely unreported in the South African context, with the exception of early studies by Van Niekerk, Marlow and their respective co-workers [1-3] that addressed factors contributing to pregnancy attrition. Dr Marlow's meticulous recording of reproductive data during a working lifetime as a veterinarian in the Eastern Cape Province was particularly informative. These, largely unpublished, data sets indicated a significant impact of both early and late pregnancy loss on foaling rates. His considerable contribution to the South African Thoroughbred industry included initiating a stud health programme to monitor sexually-transmitted diseases. Notably, the first study in this thesis, described in Chapter 3, was co-authored by Dr Marlow.

8.2 The impact of infectious abortion on reproductive performance in horses

8.2.1 Reproductive performance in broodmares

Chapter 3 presents a retrospective analysis of 25-years of nationally-reported Thoroughbred breeding records from South Africa, with a comparison of reproductive outcomes to contemporaneous data from other countries. The study aimed to quantify the influence of pregnancy losses on reproductive performance at a population level. This required access to a large data set obtained over a protracted interval. The only available data source meeting these criteria was the national Thoroughbred registry, albeit with inherent limitations on accuracy given that it is based primarily on breeders' returns [4; 5]. As no internationally-recognised standardised measures of equine reproductive performance were available, we decided that the most meaningful parameter (at least retrospectively and at the population level) describing a successful reproductive outcome was the foaling rate, defined as the production of a live foal at term.

The South African reproductive performance data showed similar trends over a similar period to those reported from larger and smaller Thoroughbred populations in the USA and Germany, respectively. The mare cohort from the last decade surveyed showed a significant improvement in live foaling rate compared with earlier cohorts. The more recent cohort's annual foaling rates were consistently >60%. This improvement is similar to reports from other countries over a comparable period and presumably reflects the considerable technological and therapeutic advances made in reproductive medicine over the last 30 years, with the additional contribution of a stud health scheme to reduce the impact of infectious disease. The improvements are most visible in terms of higher pregnancy rates and a decline in the

proportion of mares remaining barren at the end of the season. Notably, twin pregnancy was reduced to a negligible contributor to pregnancy loss, demonstrating that it is now effectively managed in practice by early detection and manual reduction to a singleton. Despite the considerable advances, the incidence of late pregnancy attrition showed little change. Furthermore, the live foal rate might arguably have been expected to show an even more marked improvement over earlier cohorts, given the scale of improvements in pregnancy rates. In this respect, improvements in the live foal rates appeared to be almost entirely attributable to improved pregnancy rates with little improvement in subsequent pregnancy loss rates.

8.2.2 Predicting the influence of abortion on subsequent reproductive performance

The observation that pregnancy loss rates are little changed despite considerable improvements in conception rates, prompted the next research question. This aimed to define the contribution of pregnancy loss, in particular infectious abortion, to overall reproductive performance. The importance of defining the factors that contribute to pregnancy loss is well recognised and, in particular, identifying factors that can be categorised as avoidable is felt to be key to further improvement [4; 6]. Age and reproductive status (i.e. maiden, barren or foaling) are mare factors that have previously been identified as the most important predictors of reproductive success. This appears to be particularly true of Thoroughbred breeding systems, e.g. in the UK, Sweden, USA and New Zealand [7; 8; 9; 10; 11], where stallion and management-level factors are reported to play relatively minor roles; the logical conclusion is that attempts to improve mare-level management are central to optimising reproductive performance [4; 5]. The incidence of pregnancy loss, has been reported to increase with both increasing mare age and a history of having ended the previous season as barren [7-9]. By contrast, mare factors associated specifically with susceptibility to EHV-1 abortion are poorly defined, with the possible exceptions of apparent predispositions of primiparous mares and those of the Lipizzaner breed [12; 13]. Bivariate analysis of the effect of predictors such as age and status on reproductive performance are, however, insufficient to account for the complexity of interactions between the multiple factors that can affect reproductive outcome. This may contribute to misleading conclusions with regard to the two parameters affecting reproductive outcome, namely establishing pregnancy and pregnancy loss. This was demonstrated by the second study in which interpretation using bivariate analysis of the most common predictor variables suggested that pregnancy as an outcome was apparently either

unaffected, or somewhat contra-intuitively, enhanced in mares that had suffered previous abortion.

Chapter 4 describes the development of novel predictive models for the relative influences and interactions of variables associated with EHV-1 and other causes of abortion on important reproductive outcomes. This prospective cohort study also aimed to define whether or not EHV-1 abortion in mares compromised their subsequent reproductive performance. The data used to develop these models were derived from reproductive examinations of approximately 400 Thoroughbred mares categorised by their age and reproductive status following the two unrelated EHV-1 abortion epizootics on South African farms described in Chapter 5. Two logistic regression models were developed to predict the probabilities of first establishing pregnancy and then foaling successfully in the following season. Multivariable models were applied to hypothetical Thoroughbred broodmares characteristic of the international demographic to illustrate the relative influences and interactions of selected reproductive variables on the probabilities of these two key outcomes. Additionally, the number of matings and month of last breeding attempt to establish pregnancy were used as indices to describe reproductive efficiency. Early embryonic death was shown to be an important predictor of all the primary reproductive endpoints, including pregnancy, foaling and reproductive efficiency. By contrast, abortion, due to EHV-1 or other causes, did not influence whether a broodmare either became pregnant or foaled, but was a predictor of poor reproductive efficiency in terms of number of matings required to establish pregnancy and of pregnancy being established late in the season. Fewer than 50% of aborting mares became pregnant after a single breeding compared to nearly 65% of mares that had not aborted. However, approximately 50% of aborting mares became pregnant during the first month of the season, and it was interesting that breeding in this first month increased the probability of establishing a pregnancy in barren mares. In agreement with previous reports, increased mare age was an important predictor of reproductive outcome and, in particular, was associated with a reduced likelihood of pregnancy and foaling and of decreased reproductive efficiency [5; 8; 12]. In contrast to previous reports however, reproductive status did not influence the likelihood of pregnancy, although it did influence breeding efficiency and was associated with a late last breeding [9; 11; 12].

The development of this model for predicting reproductive success enhanced our understanding of the complex interactions and relative influences of reproductive variables that influence both pregnancy establishment and loss. The future application of this model in

larger populations of broodmares will hopefully support its use to support informed decisions by breeders and veterinarians involved in Thoroughbred breeding worldwide.

8.2.3 Infectious abortion and reproductive performance

During an EHV-1 epizootic year, a farm in KwaZulu-Natal Province recorded a 13.6% (43/316) abortion rate amongst its resident mares. There were 18 (41.8%) EHV-1 and 25 (58.1%) non-EHV abortions, with a 65.1% overall diagnostic rate. A presumptive cause was identified in 40% of non-EHV-1 abortions, with 6 (24%) due to bacterial placentitis. Both the diagnostic success rate and the relatively high incidence of placentitis were similar, although the diagnostic rate for non-EHV-1 abortion was lower, than in other reports [14; 15]. Two years previously, 9/30 (30%) pregnant mares had aborted due to EHV-1 on a Western Cape Province farm. Interestingly, the gestational stages of mares affected during the earlier epizootic were much earlier, and the inter-abortion intervals and epizootic duration longer, than in the later epizootic or, indeed, in other previously-reported epizootics affecting similar sized groups of unvaccinated mares [13; 16]. The median age of the EHV-1 aborted mares (8.0; 5-18 years) was similar on both farms, but significantly younger than among the 25 non-EHV-1 abortion mares (11.0; 4-24 years) on the one farm. This may have been a product of various factors including the generally increased frequency of abortion in older mares, but also the greater range of aetiologies, infectious and otherwise [14; 15].

In these two case series, EHV-1 did not apparently affect the incidence of either retained fetal membranes (RFM) or premature placental separation (PPS), in contrast to previous reports [17-20]. There was however, an increased risk of both RFM and PPS among the non-EHV-1 abortion mares. A single post-EHV-1 abortion mare fatality was recorded, subsequent to uterine prolapse similar to a previous report [21].

Retrospectively, all cases in both epizootics were associated with EHV-1 ORF30 G_{2254}, i.e. the neuropathogenic genotype virus. The variations between the two epizootics are therefore more likely to have resulted from a host-factor difference, namely gestational stage at the time of exposure, and may have been further biased by management interventions.

The logistic regression model described in Chapter 4 was derived from data recorded during these two epizootics and demonstrated that abortion due to either EHV-1 or any other cause was not predictive of subsequent pregnancy or foaling rates in the affected mare populations.

Abortion from any cause was however predictive of increased effort required and late time of establishing a pregnancy in an affected mare.

8.3 The epidemiology and pathogenesis of epizootic EHV-1 abortion

The role of infectious abortion and in particular the recognition of EHV-1 as undisputedly the most important viral pathogen contributing to reproductive losses, *via* abortion and neonatal death, in many countries is reviewed in Chapter 2. Chapter 5 described the course of events and reproductive outcomes during two EHV-1 abortion epizootics affecting unvaccinated Thoroughbred mares in South Africa. A retrospective analysis of the data recorded during the epizootics identified epidemiological risk factors to formulate an appropriate intervention strategy to assist in future threatened epizootics.

8.3.1 Risk factors for epizootic abortion

Previous reports have associated several epidemiological risk factors with both sporadic and, in particular, epizootic EHV-1 abortion; a selection of the most recent reports are summarised in Chapter 2. By considering the salient features of herpesvirus biology, the risk factors can be further categorised as being either associated with novel introduction of abortogenic virus or alternatively (and possibly more frequently) with recrudescence of abortogenic virus from within a population. The differentiation of exogenous introduction from reactivation of infectious virus, with any degree of certainty, is however very difficult. A further complication to the unlocking of the epidemiological puzzle is virus-associated factors, particularly variations in viral epitope or degree of pathogenicity that may independently or co-dependently influence disease outcome.

The epidemiological data obtained from the two South African epizootics investigated suggested that they represented the two different routes of introduction of infectious virus. The index abortion in the first epizootic occurred within a single large group of 21 resident pregnant mares of varying gestation stage, apparently without any recent introductions or movements, i.e. within a 'closed herd' scenario. In the second, abortions were reported subsequent to recent acquisition of several mares, most in late gestation, from a recent broodmare sale. Upon arrival, these mares were initially housed together for a three-week isolation period, prior to dispersal among several established groups each of approximately 30

resident mares in late gestation. The dispersal and grouping were based on anticipated foaling dates, as commonly performed in many Thoroughbred systems. The first abortion followed shortly after dispersal, with subsequent abortions recorded in mares from this and several other groups. The epidemiological interpretation was more complicated than in the previous case. Several potential sources of and responses to abortogenic virus were possible: *de novo* introduction of virus into a naïve population; re-introduction of virus (possibly a highly abortogenic strain) into a previously-exposed population; stress-related recrudescence of abortogenic virus within the resident population provoked by social disruption of established groups of resident mares by the introductions. These two case series shared at least two recognised epidemiological risks, the unvaccinated status of the mares and maintenance of large groups of susceptible mares. The second epizootic was additionally associated with social disruption resulting from recent introductions into established groups, late gestation status of the affected mares and an inadequate quarantine protocol subsequent to dispersal. The mare level effects observed showed that younger mares with an even distribution over all reproductive status categories had an increased risk of EHV-1 abortions that partially supported but also opposed previous reports [12; 13]. The variation in mares' gestational stage between the two epizootics at exposure may have been critical to their marked variations in spread and outcome. The epizootic in which mares were at an earlier gestation stage was characterised by much longer inter-abortion intervals and longer total duration of the epizootic, with 30% of pregnant mares aborting. The epizootic affecting later-gestation mares showed a 'cluster' pattern with 5.7% of pregnant mares aborting over a shorter duration. The interventional differences on the two farms may have either independently or co-dependently further biased the outcomes.

8.3.2 Effects of interventions to mitigate abortion

Importance of (improved) diagnostics

Interventions thought to affect the outcomes during an EHV-1 epizootic reportedly include the time to initial response. That interval may in turn be co-dependant on diagnostic method, and has been suggested to bias the outcome. The definitive laboratory diagnosis of EHV abortion can be performed by various methods including virus isolation, histopathology and PCR. Reducing the time from initial disease manifestation to establishment of a diagnosis is greatly enhanced if there is compliance with a protocol of submission of samples from every abortion.

This is additionally dependant on the availability of a laboratory offering appropriate testing, which may additionally be affected by geographical location and proximity. The current gold-standard is a laboratory offering real-time (quantitative) polymerase chain reaction (RT-qPCR) assays for EHVs. The introduction of various PCR methods, as reviewed in Chapter 2, has greatly enhanced diagnostic speed and accuracy by detection of even minute quantities of viral nucleic acid. This permits enhanced sensitivity and specificity in addition to its versatility for a greater range of sample types, e.g. organs, aspirates and swabs. PCR also imposes fewer limitations on transport criteria, such as temperature and duration which are important practicalities that increase its geographical and temporal range. The lower associated costs and greater potential sample throughput following nucleic acid extraction are all significant benefits at the laboratory level. Finally, and perhaps most importantly, the resulting rapid turn-around time affords a more dynamic interventional ability during the management of disease outbreaks.

The application of PCR to epidemiological investigations has provided a truer reflection of the prevalence of EHVs. In addition, development of nested and duplex assays have enabled discrimination between viral species, in particular EHV-1 and -4 and of genotypic and strain differences within EHV-1. In parallel with the recognition of atypical EHV-1 abortion (EHV-negative fetus), the ability of PCR to detect viral nucleic acids in or on fetal membranes is currently unmatched by other techniques, and has significantly increased the scope of EHV-1 detection.

Our findings supported previous reports that suggested a link between the diagnostic interval from observing an index case to the outcome of an abortion epizootic [16; 19; 21]. The data from essentially homogeneous populations suggested that, although additional host and virus factors may also have affected outcome, an early diagnosis and PCR-based testing, or a combination of the two were important parameters affecting the outcome of an epizootic. In the later compared with the earlier epizootic, there was both a markedly shorter diagnostic interval and availability of qPCR testing. A diagnosis was obtained *via* duplex EHV-1 / -4 qPCR on the day of sample submission at a laboratory approximately 500 km away, this was within three days of the first abortion. The qPCR assay results were in complete agreement with those obtained later by duplicate testing of all samples by histopathology with immunofluorescent staining. This rapid, accurate reporting enabled a confident justification of a comprehensive, targeted EHV-1 specific intervention. This included immediate blanket vaccination and other interventions within a large population, complicated by extensive dispersal of potential in-

contacts within numerous groups over a geographically-large site. This approach was chosen with reference to previous reports of devastating outcomes affecting epidemiologically and demographically-similar populations, and in the earlier epizootic included in the study.

Interventions to reduce exposure to virus during epizootics

Interventions to prevent or limit equine herpesvirus epizootics target the three major sources and routes of infectious virus transmission; respiratory shedding by an actively-infected horse, recrudescence of latent virus in a carrier and post-abortion environmental contamination. Aerosol transmission from a contaminated environment in the period following an abortion is a critical risk consideration for susceptible mares grouped together. Transmission is facilitated by the large amounts of abortogenic virus present and the inquisitive behaviour of mares. This was arguably the most important mode of transmission in Chapter 5, perhaps in combination with viral recrudescence from one or more carriers within a large 'closed' group. In support of this suggestion, are the lack of premonitory signs prior to the initial abortion and subsequent rapid response following removal of the affected mare, fetus and membranes and concurrent subdivision and removal of the exposed mares into three smaller and geographically-separated groups. Additional interventions included application of a 'DISH' infection control protocol and biosecurity measures to contain infection and limit exposure. Despite intervention, subsequent abortions occurred within two of three new groups, the first after a relatively prolonged interval of 39 d. These observations combined with other reports suggests that there is a very short window (essentially hours) between abortion and intervention if viral transmission is to be prevented [23; 24]. The protracted interval until subsequent abortions in this case may reflect viral and host factors, including abortogenic potential or mare susceptibility given their exposure at a comparatively early gestational stage.

The later epizootic affected a demographically similar population of unvaccinated mares. This epizootic was epidemiologically-linked to introductions of newly-acquired mares into established groups of resident mares at a similar (late) gestation stage. Despite possible variations in initiation and epidemiological features, the outcomes of the latter epizootic differed markedly particularly in terms of morbidity and duration. These differences may have been associated with differences in interventions during the second epizootic as a result of experience gained during the earlier epizootic. The differences that we consider to have been crucial include the availability of qPCR, enabling more rapid diagnosis and targeted response,

and immediate vaccination of the entire pregnant mare population in the face of the outbreak. Additional interventions that may have contributed include enhanced measures to limit potential exposure and to limit the risk of recrudescence of latent virus. This involved a labour-intensive constant observation of all mares to enable almost-immediate isolation of a post-abortion mare with removal from the immediate environment of group-mates. The group mates were then immediately subdivided into smaller groups. Separation was achieved using electrified tape to create temporary, adjacent enclosures. This allowed group-mates to remain within sight and sound of each other despite an appropriate intervening 'sanitary' barrier, and aimed at reducing physiological stress due to social group disruption which has previously been implicated in the recrudescence of latent virus.

The reluctance to immediately vaccinate at risk mares in the first epizootic was based on anecdotally-reported risks of neurological complications. Vaccination was however used in the later epizootic as a means to reduce susceptibility. However, the efficacy of vaccination during that epizootic is difficult to quantify, and the possibility that the lower abortion rate obtained in the later compared with the earlier epizootic was influenced by vaccination must be interpreted with caution. Vaccination may have contributed by acting as a booster in the case of previously-vaccinated broodmares, with the presumed rapid anamnestic response reducing further spread [23]. On the other hand, it is questionable whether vaccination in the face of an EHV-1 outbreak really exacerbates the risk of neurological disease; this was not substantiated in our experience where both outbreaks were retrospectively found to be associated with a neuropathogenic EHV-1 virus.

8.4 Physiological stress and recrudescence of infectious EHV-1 and EHV-4

Defining the frequency of EHV-1 recrudescence in latently-infected horses is complicated because it is only detectable if accompanied by clinical symptoms, viraemia or shedding. Experimentally, EHV-1 reactivation has been induced and was accompanied by clinical and laboratory evidence of infection; reactivation of EHV-4 has also been induced experimentally, albeit without detection of clinical signs. There is however little concrete in-the-field evidence of reactivation induced by either physiological stress or immunosuppression, even though it is proposed to be a major source of infectious virus. Indeed, the risk-factors and stressors associated with viral recrudescence are poorly understood, but reported to include various environmental and management stressors such as transport, underlying disease,

hospitalisation, overcrowding, inclement weather and disruption of social hierarchy [25]. There are also no documented measurements of physiological stress accompanying detection of infectious virus to support the proposed mechanism initiating recrudescence.

Chapter 6 describes a prospective study to validate a method for the minimally-invasive measurement of physiological stress to help improve our understanding of stress-associated recrudescence of EHVs. Physiological stress responses in horses involve various metabolic, neuro-endocrinological and immunological mechanisms, and chronic stress is thought to result in immunosuppression and thereby increase susceptibility to disease. The consignment of pregnant broodmares to sales is a feature of the Thoroughbred industry worldwide and has been anecdotally associated with a heightened risk of subsequent EHV-abortion, similar to the epizootic described in Chapter 5. This is presumed to be the result of exposure to one or more potential stressors at a susceptible stage of gestation. The prospective study included two groups of vaccinated late-gestation Thoroughbred broodmares, one group consigned to a bloodstock sale and a matched group of non-consigned control mares. The mares were monitored for physiological stress and viral reactivation associated with the events leading up to, during and following the actual sales. Physiological stress was monitored via faecal glucocorticoid metabolite (FGM) concentrations in serial faecal samples to give a time-averaged global indication of level of response to potential stressors. Event-related viral reactivation was monitored by daily body temperature measurements and serial nasal swabbing to detect viral nucleic acid using a duplex EHV-1 / -4 RT-qPCR assay. A significant rise in FGM levels was measured from both groups of broodmares in the interval corresponding with preparation, transport to and from and the sales. An unexpected but interesting observation was the greater elevations in FGM shown by control mares, presumably as a consequence of pre-sales separation from group mates destined for sales consignment. This suggests that social disruption may be a key stressful event among settled groups of mares. On the other hand, there was very limited evidence of event-related viral reactivation: only a single incident of EHV-1 detection by PCR in one consigned mare, and a few incidents of pyrexia. This limited association between stressful events, clinical signs and viral detection may have been biased by the small sample population, the sampling duration and frequency, and the effects of vaccination. The results also support previously reported studies that stress-induced reactivation of latent EHV infection is probably an uncommon event [26-28]. The study did at least provide data to support the use of FGM measurements as a practical, non-invasive and

reliable technique for monitoring adrenocortical function when studying EHV reactivation or events associated with medium to long-term stress in horses.

The prospective study described in Chapter 7 applied the model previously developed for non-invasive measurement of physiological stress to a population of horses chosen for a higher likelihood of stress-associated reactivation and transmission of EHV. This involved targeted surveillance to detect EHV-1 and -4 in a population of healthy young Thoroughbreds consigned for auction. The prevalence of EHV-1 and -4 in South African horses has not previously been reported. Indeed, there are surprisingly few reports of EHV-1 and -4 prevalence worldwide, despite these viruses being amongst the most-commonly identified pathogens in upper respiratory tract infections, which is in turn a major contributor to clinical disease and economic loss in the international Thoroughbred racing industry. Although the risk associations for respiratory infection are multifactorial, physiological stress has been proposed to be associated with both recrudescence and transmission of latent EHV infections, and its immunosuppressive effect in naïve animals is thought to increase their susceptibility to infection. Higher detection rates for the EHVs have been reported in juvenile horses and during the winter months [23; 28-30]. However, limited detection rates were reported following long-distance transport of horses to sales and competition events in North America [25; 29]. Sales are characterised by transport, confinement and management of large intermingled groups of horses from diverse sources, potentially providing an ideal environment for viral shedding and transmission, exacerbated by the potentially stressful disruption of established social groups.

The event selected was an annual sale of two-year old horses during late winter at a central venue in South Africa. The study population (n=90) included both colts and fillies representative of the South African Thoroughbred breeding demographic, having been enrolled from eight farms located in three provinces. The horses were transported *via* road over distances ranging from approximately 500-1500 km, with associated journey times of 6-22 h, to the sales venue where they were resident for approximately 4-9 d prior to the actual auction on the last two days. None of the horses were previously vaccinated against EHV. All were monitored for physiological stress responses during their residence by daily faecal sampling for FGM concentrations. The potential for event-related reactivation accompanied by viraemia and detection of nasal shedding of EHV-1 and -4 was monitored on arrival, at departure and serially using a combination of blood sampling (for type-specific EHV-1 / -4 ELISAs), nasal swabbing (for duplex EHV-1 / -4 RT-qPCR) and twice-daily observation for pyrexia and nasal discharge.

The serological samples obtained on arrival and departure showed that almost all (98%) and very few (2%) horses had prior exposure to EHV-4 and -1, respectively. In addition, a few horses showed increased titres on departure, possibly associated with both viral recrudescence and horizontal spread of infection. Equine herpesvirus 4, but not EHV-1, nucleic acid was detected in approximately 14% of the study population with repeated incidents in nearly 1/3 of these animals for up to four consecutive days. Equine herpesvirus 4 DNA was detected in the nasal secretions of nearly all of these positive horses despite the presence of EHV-4 antibodies, most likely as a result of viral recrudescence. Detection of EHV-4 showed an initial peak soon after arrival at the sales complex followed by a second, higher peak during the interval associated with auction preparation. From a clinical perspective, pyrexia and, in particular, nasal discharge were poor indicators of EHV-4 infection.

Transport-associated elevations in FGM concentrations were observed in the first few days following arrival at the sales venue in horses from all farms, independent of distance and duration of the journey. Thereafter, FGM concentrations decreased, presumably because the horses adapted or became accustomed to the novel environment and management stimuli. The auction process itself had no noticeable effect on FGM concentrations. This suggests that management practices should be adjusted to reduce the impact of travel and the sales environment on physiological stress.

The study's findings were similar to previous studies with low or absent detection of EHV-1 in apparently healthy adult horse populations, but serological evidence of high EHV-4 prevalence. The circulation of EHV-4 during sales consignment despite the presence of antibodies in shedding horses also suggests that preventative isolation following sale and movement to a final destination may be beneficial in limiting viral transmission. This destination is often a training facility with associated intermingling, high stocking rates and associated stressors that may further exacerbate the potential for viral reactivation and transmission. As none of the study population was vaccinated, the value of strategic vaccination prior to sales consignment remains undefined and warrants further investigation.

This population of young Thoroughbreds may arguably reflect the essentially universal exposure to EHV-4 on breeding farms. The absence of serological evidence in a representative sample of horses from these farms may further suggest that EHV-1 circulation on South African farms is uncommon.

8.5 Recommended interventions during EHV-1 abortion epizootics

This thesis presents a compelling argument for the value of prompt diagnosis and epidemiologically-informed intervention in ameliorating abortion outbreaks. Chapters 5 and 6 report observations supporting various interventions that appeared to mitigate the potentially disastrous outcomes of an EHV-1 abortion epizootic. The general aims of intervention during an infectious disease outbreak are to reduce both exposure to, and horse-to-horse transmission of, infectious virus within a population. In the event of a herpesvirus-associated disease outbreak, the potential for recrudescence of latent infectious virus is an additional consideration. The outcomes described in Chapter 5 may have been biased by inclusion of interventions targeting reactivation in addition to those more-commonly advocated to reduce exposure to infectious virus. The availability of qPCR-based diagnostic tests appeared to influence outcomes. Although establishing a definitive association between physiological stress and viral reactivation was problematic, a link was suggested by the studies in Chapters 6 and 7. These studies examined broodmares and young horses exposed to stressors anecdotally associated, albeit independently, with both physiological stress and viral reactivation.

Reducing exposure to an infectious pathogen within a population is predicated by the recognition of its presence, with the interval to an interventional response as a dependent variable, which is in turn dependent on diagnostic modality. Thereafter, the interventional focus shifts to isolation and containment of the infectious focus and preventing its spread both within and outside the affected population. Epidemiologically, three main sources of infective EHV-1 are recognised; nasopharyngeal shedding of lytic virus from actively-infected horses, shedding of virus within tissues and fluids associated with abortion, and recrudescence and shedding of endogenous virus by a latently-infected carrier. Horse-to-horse transmission requires relatively intimate contact with virus-containing secretions to allow access via the upper respiratory tract mucosae, but allow for short distances traversed by airborne virus and transfer from contaminated fomites.

Mitigating the effects of environmental contamination with infectious virus post-abortion is vital. Subdividing an already exposed group of mares to further contain an infectious focus is probably of limited efficacy unless accompanied by their rapid post-abortion removal from the contaminated environment to minimize the risk and duration of contact with virus. In the first epizootic described in this thesis, despite only a few hours passing from initial abortion to removal and subdivision of in-contact mares, intervention failed to prevent subsequent abortions within the smaller, reconstituted groups. This observation informed and justified the

considerable management effort and labour requirements to provide constant observation of all pregnant broodmares in the later epizootic. This strategy facilitated immediate separation of any aborting mare from her exposed group mates, and their rapid removal from an infected environment and further subdivision into groups of two or three mares. A novel modification was the use of easily-erected electrified tape barriers to provide effective sanitary barriers while maintaining sight and sound contact between former group mates to reduce the potential risk of viral reactivation due to social disruption.

The generally held caveat associated with vaccination in the face of an epizootic, was not supported by our findings [31]. That is, no initiation of neurological disease was observed despite retrospectively identifying a neuropathogenic EHV-1 genotype in both epizootics. The difference in using vaccination after the first abortion between the two case series (both affecting unvaccinated broodmares) may have contributed to the marked variation in their outcomes. The rapid and comprehensive vaccination programme within 24 h of initial diagnosis in the second outbreak may have been beneficial in reducing viral challenge and its impact within that population.

A prerequisite for improving outcomes includes compliance with a protocol that assumes that all late gestation abortions are due to EHV-1 until proven otherwise, with the prompt application of appropriate infection control and biosecurity measures. This response includes submission of appropriate diagnostic samples from both fetus and fetal membranes. Currently, the most rapid diagnostic turn-around time is offered by qPCR assays. Response protocols are ideally developed with consideration of the individual farm's environment, layout, horse population and staffing.

8.6 Recommendations for prevention of epizootic EHV-1 abortion

Formulating an appropriate preventative strategy for EHV-1 abortion must *a priori* consider the key epidemiological features associated with EHV-1. These are characterised by establishment of early and probably life-long latent infections; moreover, on breeding farms mares and foals act as reservoirs of infection for previously unexposed, susceptible horses via periodic, generally asymptomatic, viral recrudescence with shedding. Environmental transmission is only central to outbreaks typically associated with closely-confined horse populations.

There is little doubt that vaccination, despite its inherent limitations and associated costs and management implications is of prophylactic value in diminishing the impact of EHV-1

abortion. The regular vaccination of all pregnant broodmares should be a feature of any preventative strategy.

Interventional efforts aimed at reducing reactivation of latent virus are difficult to monitor but offer possibly the best chance of influencing the outcome. Our data supported several previously reported key risks associated with initiation of abortion epizootics. The features that potentially enhance transmission of abortogenic virus include unvaccinated populations of susceptible mares maintained in relatively large groups, and inadequate post-arrival quarantine protocols. Risks that could be associated with viral recrudescence include introducing high-risk mares into similarly-susceptible populations of resident mares, with consequent disruption of established groups.

The value of routine-submission of diagnostic samples for all abortions was supported by our observations. This value was however in part dependent on the availability of qPCR diagnostics primarily because of the more rapid turn-around times.

The roles of quarantining and isolation facilities in attempting to contain an infectious focus was highlighted by Chapter 5. A routinely-applied 30 d quarantine for all introductions appeared to be inadequate to protect resident mares from exposure to abortogenic virus, particularly considering the wide range of incubation intervals and varying risk-susceptibility for EHV-1 abortion depending on gestation stage. The quarantine process itself may be associated with additional risks. Post-arrival quarantining of newly-acquired and heavily-pregnant mares as a mixed group may exacerbate risks and enhance spread of infectious virus amongst them prior to further dispersal through the herd. Abortogenic virus may circulate sub-clinically within this group, perhaps via stress-induced reactivation associated with exposure to stressors prior to (e.g. sales environment and transportation) or shortly post-arrival (social or environmental perturbations). The risks of introducing late-gestation mares into groups of similar pregnancy stage resident mares would support recommending isolation of newly acquired pregnant mares from resident mares until after foaling.

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CHAPTER 9
Nederlandse samenvatting

Inleiding

De equine herpesvirussen (EHVs) zijn mede-geëvolueerd met hun gastheren tot het punt waarop ze - heden ten dage - alomtegenwoordig zijn in gedomesticeerde en wilde populaties van paardachtigen. Twee van de EHVs, namelijk equine herpesvirus 1 (EHV-1) en equine herpesvirus 4 (EHV-4) zijn daarbij klinisch, economisch en epidemiologisch verreweg de belangrijkste. Vanwege hun hoge prevalentie vormen deze pathogenen wereldwijd een aanmerkelijk risico voor de gezondheid en het welzijn van paarden populaties. De meest ernstige gevolgen van EHV-1 en -4 infecties zijn abortus in een laat stadium van de dracht, neonatale sterfte en myeloencephalopathie, vooral wanneer deze symptomen leiden tot een epidemie zoals nog wel eens wordt gezien na een EHV-1 infectie.

Bij het in stand blijven van een EHV-1 en -4 infectie binnen een populatie spelen langdurige, wellicht levenslange, latente infecties bij asymptomatische paarden een belangrijke rol. Door periodieke reactivering van zo'n reservoir kunnen nieuwe, vatbare paarden geïnfecteerd worden.

Voor een beter begrip van de ziekte en voor de ontwikkeling van strategieën om de risico's en gevolgen van deze belangrijke virale pathogenen te beperken is meer kennis nodig. Met name is een beter inzicht van belang in gastheer gerelateerde risicofactoren welke predisponeren voor een nieuwe uitbraak van de infectie. Daarbij kan onder andere gedacht worden aan fysiologische stress maar ook aan milieu gerelateerde risicofactoren.

Doel van het onderzoek

Het onderzoek was er in eerste instantie opgericht om inzicht te krijgen in de incidentie en de oorzaken van voortijdige beëindiging van de dracht bij het Zuid-Afrikaanse paard. Hoewel de initiële drachtpercentages van de merries in de afgelopen decennia zijn verbeterd, zijn de verliezen gedurende de dracht over deze periode niet of nauwelijks vooruitgegaan zodat veulenpercentages niet werden verbeterd. Deze verliezen bleken de belangrijkste oorzaak van de mate van inefficiëntie van het voortplantingsproces. De focus van het onderzoek werd derhalve verlegd naar besmettelijke abortus waarvan EHV-1 onbetwist de belangrijkste virale ziekteverwekker is. Een beter inzicht in de epidemiologie van herpesvirus abortus en een betere onderbouwing van relevante preventie- en interventiestrategieën bleek noodzakelijk.

Met name is ook de lange termijn invloed van deze strategieën op het uiteindelijke reproductieproces van belang.

De volgorde van de hoofdstukken in dit proefschrift weerspiegelt het verleggen van de initiële focus op de betekenis van het afbreken van de dracht als oorzakelijke factor bij een inefficiënte voortplanting naar, meer specifiek, de impact van EHV abortus en de rol van latente herpesvirus infecties bij een abortus epidemie. Ook werd de rol van fysiologische stress onderzocht bij reactivering van latente infecties. Het laatste hoofdstuk geeft de algemene conclusies van het onderzoek en beschrijft een weloverwogen interventie strategie om de gevolgen van EHV infecties op het voortplantingsproces te beperken.

Deze retrospectieve en prospectieve studies zijn uitgevoerd bij Zuid-Afrikaanse volbloedpaarden, de resultaten zijn derhalve in grote lijnen toepasbaar op overeenkomstige volbloedpopulaties.

Equine herpesvirussen in context

De literatuur over de belangrijkste EHVs, namelijk EHV-1 en -4 wordt - vanuit veterinaire voortplantingsperspectief - besproken in **hoofdstuk 2**. De aandacht gaat daarbij met name uit naar de epidemiologische karakteristieken en de pathogenese van EHV-1 op het gebied van de voortplanting en verder naar latente infecties en nieuwe uitbraken van dit virus vanuit deze laatste bronnen. De gegevens uit studies en rapporten uit vele landen en over een periode van tientallen jaren zijn samengevat in een tabel. Ook zijn epidemiologische risicofactoren samenhangend met epidemische EHV-1 abortus uitbraken in tabelvorm weergegeven. Het belang van de verbetering van laboratoriumdiagnostiek (met name door toepassing van moleculaire methoden) en van interventie en preventie strategieën, waaronder vaccinatie, zijn geëvalueerd.

Efficiëntie in de paardenfokkerij

In **hoofdstuk 3** wordt een overzicht gegeven van de voortplantingsresultaten binnen de Zuid-Afrikaanse Volbloed fokkerij van 1975 tot 1999. Deze basisgegevens verschaften de mogelijkheid om het effect te bestuderen van door EHV-1 veroorzaakte abortus op de uiteindelijke voortplantingsresultaten binnen subgroepen van deze populatie. Een verbetering van het percentage levend geboren veulens (ter kwantificeren van verliezen tijdens de

graviditeit op populatie niveau) was bijna geheel toe te schrijven aan stijging van het initiële drachtpercentage terwijl het verliespercentage gedurende de dracht nauwelijks verbeterde. Overeenkomstige trends over een vergelijkbare periode werden gerapporteerd voor grotere en kleinere volbloed populaties in de Verenigde Staten en in Duitsland.

Equine herpesvirussen en hun rol bij besmettelijke abortus

Het voorspellen van de invloed van abortus op de voortplantingsresultaten

Het belang van het definiëren van de factoren die bijdragen aan het afbreken van de dracht is alom bekend. Met name het identificeren van vermijdbare factoren is van groot belang om in de toekomst de resultaten te kunnen verbeteren. Tweewegsvariantie-analyse van het effect van variabelen zoals leeftijd en voortplantingsresultaten vanuit het verleden is echter niet voldoende om de complexiteit van de interacties te beoordelen tussen de vele, verschillende factoren die het voortplantingsresultaat kunnen beïnvloeden. In **Hoofdstuk 4** wordt de ontwikkeling van een model beschreven waarbij gebruik wordt gemaakt van voortplantingsgegevens van ongeveer 400 volbloed merries op Zuid-Afrikaanse bedrijven zoals leeftijd en voortplanting status na EHV-1 abortus epidemieën. Dit model dient om de relatieve invloed van en interactie tussen variabelen te evalueren die verband houden met een EHV-1 abortus in vergelijking met andere oorzaken van vroegtijdige beëindiging van de dracht. Tevens dient het model ook om vast te stellen of een door EHV-1 geïnduceerde abortus een negatief effect heeft op de toekomstige fertiliteit. Twee logistische regressie modellen werden ontwikkeld om de kansen op dracht en het vervolgens met succes veulenen in het volgende seizoen te voorspellen. Multivariabele modellen werden toegepast om de relatieve invloeden en interacties van geselecteerde voortplanting variabelen te beschrijven op deze twee belangrijke parameters. Deze modellen werden getest op een hypothetische populatie volbloed fokmerries met bekende internationale demografische kenmerken. Bovendien werden het aantal dekkingen en de laatste maand van dekking gebruikt als indices om reproductieve efficiëntie te bepalen.

Vroeg embryonale sterfte (EED) bleek een belangrijke voorspeller voor primaire voortplanting criteria zoals de dracht, het veulenen en de efficiëntie van het voortplantingsproces. Daarentegen had abortus, door welke oorzaak dan ook en in het bijzonder door besmettelijke abortus ten gevolge van EHV-1, geen invloed op de vraag of een

fokmerrie of drachtig werd of een veulen bracht. Abortus voorspelde wel dat voor de betreffende merries een extra inspanning nodig was om ze in de tweede helft van het voortplantingsseizoen toch nog drachtig te krijgen. In overeenstemming met eerdere publicaties, was bij de ouder wordende merrie de kans op dracht en een veulen minder groot en hetgeen duidt op verlaagde efficiëntie van de voortplanting. In tegenstelling tot eerdere rapporten had echter de voortplantingsstatus geen invloed op de kans op dracht, maar wel op de efficiëntie van het voortplantingsproces.

De ontwikkeling en gebruik van dit model voor het voorspellen van het voortplanting resultaat vergroot onze kennis van de complexe interacties en de relatieve invloed van de voortplantingsvariabelen die zowel de kans op dracht als op het vroegtijdig beëindigen daarvan beïnvloeden. Gebruik van dit model bij grotere groepen volbloed fokmerries zal wereldwijd fokkers en dierenartsen ondersteunen om weloverwogen beslissingen te nemen in de fokkerij.

De epidemiologie en pathogenese van epidemische EHV-1 abortus

Hoofdstuk 5 beschrijft een retrospectieve studie van epidemiologische en voortplanting gegevens van twee zowel geografisch als in de tijd gescheiden EHV-1 abortus epidemieën met een onderling sterk verschillende morbiditeit bij niet-gevaccineerde fokmerries. De eerste vond plaats op een boerderij in de provincie West-Kaap, waar 9 van de 30 (30%) drachtige merries aborteerden door een EHV-1 infectie. De tweede betrof, twee jaar later, een boerderij in de provincie KwaZulu-Natal waar abortus werd geconstateerd bij 13,6% (43/316) van de merries: 18 (41,8%) EHV-1 en 25 (58,1%) niet-EHV gerelateerde abortussen. De studie had tot doel om de verschillen in de epidemiologische kenmerken en interventies te identificeren van invloed op de sterk uiteenlopende uitkomsten van de twee epidemieën. De resultaten worden gebruikt om preventie en interventie strategieën te verbeteren bij eventuele nieuwe EHV-1 abortus epidemieën.

Door rekening te houden met de meest opvallende kenmerken van de biologie van het herpesvirus kunnen verschillende epidemiologische risicofactoren in verband worden gebracht met een epidemische EHV-1 abortus. Deze worden verder gecategoriseerd als hetzij een nieuwe introductie van het virus of alternatief (en mogelijk vaker) als een reactivering van het virus binnen een populatie. Het onderscheid tussen deze categorieën, met enige mate van zekerheid, is echter zeer lastig. De bij ons onderzoek verkregen epidemiologische gegevens duiden er op dat - hoewel er in beide gevallen sprake was van een grote groep niet-

gevaccineerde vatbaar zijnde merries - de introductie van besmettelijke virus op verschillende manieren plaats vond. In het eerste geval was er sprake van een 'gesloten kudde' scenario en vond de epidemie plaats binnen een populatie drachtige merries in verschillende stadia van de dracht zonder dat dieren werden verplaatst of aan de kudde waren toegevoegd. In het tweede geval vonden abortussen plaats na de introductie van een aantal recent aangekochte merries die in een late fase van de dracht waren en verder afkomstig van verschillende bedrijven. De verdeling van de aangekochte merries over de subgroepen van het eigen bestand gebeurde op basis van de verwachte veulen data, een werkwijze die gebruikelijk is bij veel volbloed fokkerijen. In dit geval waren derhalve verscheidene potentiële infectiebronnen mogelijk: *de novo* introductie van het virus in een naïeve populatie of herintroductie van het virus in een eerder blootgestelde populatie of stress-gerelateerde reactivatie van het virus binnen de populatie veroorzaakt door een verstoring van de rust binnen bepaalde merrie-groepen door de recente introducties. Deze tweede epidemie betrof merries in een latere fase van de dracht maar daarnaast was er sprake van een inadequaat quarantaine protocol bij de introductie.

In de twee ziekte uitbraken bleek dat op merrie niveau jongere merries een hoger risico liepen, waarbij de abortus in alle stadia van de dracht kon optreden en verder gelijkmatig verdeeld bleek over de verschillende fases van de dracht. De mediane leeftijd van het EHV-1 aborterende merries (8,0; 5-18 jaar) was vergelijkbaar op beide boerderijen, maar aanzienlijk jonger dan onder de 25 niet-EHV-1 aborterende merries (11,0; 4-24 jaar) op één van de boerderijen. Equine herpesvirus type 1 had geen invloed op de incidentie van het aan de nageboorte blijven staan of op het voortijdige loslaten van de placenta, ook was er slechts één geval van uterusprolaps postpartum. Het stadium van de dracht waarin de merries zich bevonden ten tijde van de initiële blootstelling kan van cruciaal belang zijn geweest voor het waargenomen verschil in het spreidingspatroon van de uitbraak en het eindresultaat van de twee epidemieën. De eerste epidemie betrof merries in een eerder stadium van de dracht en werd gekenmerkt door een wat langere duur en grotere intervallen tussen abortussen, terwijl de tweede epidemie wat latere dracht stadia betrof en een 'cluster' patroon liet zien van aborterende merries over een kortere periode.

Verder complicerend in deze epidemiologische puzzel zijn virus geassocieerde factoren, waaronder variaties in viraal epitoop of de mate van pathogeniteit die al of niet afhankelijk van elkaar het ziekteverloop kunnen beïnvloeden. Daar beide epidemieën werden veroorzaakt door het neuropathogene EHV-1 ORF30 G_{2254} virus, zijn de verschillen tussen de beide epidemieën daarom waarschijnlijk het resultaat van een gastheerfactor verschil, namelijk

drachtstadium ten tijde van de besmetting maar kunnen ook verder zijn beïnvloed door verschillen in management interventies.

Interventies waren gericht op de drie meest waarschijnlijke infectie bronnen en de routes van virus overdracht: respiratoir door een paard met een actieve infectie, reactivatie van een latent virus bij een drager en omgevingsverontreiniging nadat een abortus heeft plaats gevonden. Dat laatste was misschien wel de meest belangrijke factor in beide epidemieën mogelijk in combinatie met virale reactivatie bij 1 of meer dragers binnen een 'geïsoleerde' groep. Onafhankelijk van mogelijke verschillen bij het begin van de uitbraak of in epidemiologische karakteristieken kunnen de verschillen in morbiditeit en duur van de epidemie zijn beïnvloed (op zich zelf staand of in samenhang) door verschillen in management interventies, mede door opgedane ervaring bij de eerste uitbraak. Cruciale verschillen waren beschikbaarheid van qPCR testen, waardoor een snellere diagnose en gerichte respons mogelijk waren en verder een onmiddellijke vaccinatie van de gehele drachtige merrie populatie bij de uitbraak.

Hoewel additionele gastheer en virus factoren ook de uitkomst beïnvloed kunnen hebben wijzen onze bevindingen in deze eigenlijk vergelijkbare populaties op een verband tussen benodigde tijd voor het stellen van de diagnose na het eerste ziektegeval enerzijds het verloop van de daar op volgende abortus epidemie anderzijds. Bij de tweede uitbraak was de diagnose sneller gesteld door de beschikbaarheid van een duplex EHV-1 / -4 qPCR test. Dit maakte onderbouwde EHV-1 specifieke maatregelen mogelijk waaronder vaccinatie van de populatie.

De weerstand tegen het onmiddellijk vaccineren van merries bij de eerste uitbraak berustte op een anekdotische gerapporteerd risico van neurologische complicaties. De effectiviteit van de vaccinatie tijdens de latere epidemie, waaronder een lager abortus percentage, is echter moeilijk te kwantificeren. Anderzijds was het risico van neurologische afwijkingen na vaccinatie bij van een EHV-1 uitbraak niet onderbouwd, zeker niet wanneer beide uitbraken retrospectief in verband werden gebracht met een neuropathogeen EHV-1 virus.

Aanvullende ingrepen die mogelijk bijgedragen hebben zijn verbeterde hygiënische maatregelen om eventuele blootstelling te limiteren en maatregelen om het risico van een reactivatie van latent virus te beperken zoals voortdurende observatie van alle merries en bijna-onmiddellijke isolatie van een aborterende merrie en verwijdering uit de directe omgeving van de kuddegenoten. Kuddegenoten werden onmiddellijk onderverdeeld in kleinere groepen en gescheiden met behulp van schriklint om tijdelijke, aangrenzende behuizingen te creëren. Hierdoor konden dieren uit de zelfde sociale groep in het zicht en op

gehoorsafstand van elkaar blijven ondanks tussenliggende 'sanitaire' barrières. Zo werd fysiologische stress door ontwrichting van de sociale groep zoveel mogelijk voorkomen, een stress die reactivatie van latent virus kan veroorzaken.

Fysiologische stress en reactivatie van EHV-1 en EHV-4

Hoofdstuk 6 beschrijft een prospectieve studie naar de relatie van fysiologische stress met reactivatie van EHVs waarvoor in de praktijk aanwijzingen zijn. Risico-en stressfactoren zijn onderliggende ziektes, transport, opname in een kliniek, teveel dieren in een beperkte ruimte, slechte weersomstandigheden en verstoring van de sociale hiërarchie. Veiling van een paard gaat vaak gepaard met een aantal van deze factoren. Als het merries betreft in een daarvoor kwetsbaar stadium van de dracht wordt dit anekdotisch geassocieerd met een verhoogd risico op latere EHV-abortus.

In de studie werden twee groepen gevaccineerde volbloed fokmerries onderzocht in een laat stadium van de dracht: één aangeboden op een veiling en een tweede gelijkwaardige groep controle merries. De merries werden onderzocht op fysiologische stress en virale reactivering in verband met de gebeurtenissen in de aanloop naar, tijdens en na de daadwerkelijke veiling. Fysiologische stress werd onderzocht door bepaling van fecaal glucocorticoïden metaboliet (FGM) concentraties in opeenvolgende mest monsters. Een mogelijk virus reactivering, verband houdend met de veranderende omstandigheden, werd onderzocht door het dagelijkse temperaturen en seriële nasale bemonstering om viraal nucleïnezuur te detecteren met behulp van een duplex EHV-1 / -4 RT-qPCR assay. Een significante stijging van FGM niveaus werd waargenomen bij beide groepen in de periode ter voorbereiding voor de veiling, en rondom het vervoer voor en na de veiling. Een onverwachte maar interessante observatie was de sterkere verhoging in FGM bij de achtergebleven controle merries, vermoedelijk als gevolg van vertrek van kuddegenoten. Dit wijst er op dat sociale ontwrichting een belangrijke stressvolle gebeurtenis kan zijn bij een stabiele merriegroep. Er zijn slecht beperkte anekdotische aanwijzingen voor EHV-1 virus reactivering gerelateerd aan stress al of niet gepaard gaande met koorts. Deze studie benadrukt het belang van FGM metingen als een praktische, niet-invasieve en betrouwbare techniek voor evaluatie van de bijnierschors functie bij onderzoek naar EHV reactivering of naar gebeurtenissen die met de middellange tot lange termijn stress bij paarden in verband worden gebracht.

Er zijn verrassend weinig publicaties over EHV-1 en -4 prevalentie, ondanks dat deze virussen een van de meest geïdentificeerde pathogenen zijn in de bovenste luchtwegen bij jongere paarden en een belangrijke bijdrage leveren aan klinische ziekte en aan economisch verlies bij de internationale volbloed rensport. De prevalentie van EHV-1 en -4 bij Zuid-Afrikaanse paarden was niet bekend. Hoewel het risico op luchtweginfectie multifactorieel is, wordt fysiologische stress in verband gebracht met reactivering en overdracht van latente EHV infecties.

In de prospectieve studie beschreven in **hoofdstuk 7** wordt het in het vorige hoofdstuk vermelde model toegepast op een groep paarden met een relatief grote kans op stress-geassocieerde reactivering en overdracht van EHV. Gerichte frequente bemonstering werd gedaan ter detectie van het EHV-1 en -4 in een populatie van 90 niet-gevaccineerde maar gezonde jonge (<3 jaar) volbloed paarden aangeboden ter veiling tijdens de wintermaanden. De paarden werden over de weg naar de veiling locatie vervoerd over afstanden variërend van ca. 500-1500 km, met reistijden van 6-22 uur. Daar verbleven ze 4-9 dagen voorafgaand aan de tweedaagse veiling. Alle paarden werden gecontroleerd op fysiologische stressreacties tijdens hun verblijf door middel van FGM concentratiebepaling in dagelijkse mestmonsters. De kans op reactivering, viremie en nasale uitscheiding van EHV-1 en -4 door stress in deze periode werd onderzocht. Daartoe werd bij aankomst, bij vertrek en tussenliggend serieel bemonsterd door bloedafname (voor een typespecifieke EHV-1 / -4 ELISA), nasale bemonstering (voor een duplex EHV-1 / -4 RT-qPCR test) en tweemaal daagse controle op koorts en neusuitvloeiing.

Transport-geassocieerde FGM concentraties waren verhoogd in de eerste dagen na aankomst, onafhankelijk van afstand en tijdsduur van de reis. Daarna namen FGM concentraties af, waarschijnlijk omdat de paarden zich aanpasten aan de nieuwe omgeving en het veranderde management.

Uit serologisch onderzoek bleek een hoge prevalentie van EHV-4 en slechts een beperkte aanwezigheid van EHV-1 op de Zuid-Afrikaanse bedrijven. Nucleïnezuur van EHV-4 (maar niet van EHV-1) werd gedetecteerd in nasale secreties van ongeveer 14% van de populatie, vaak meerdere keren bij hetzelfde dier, ondanks de aanwezigheid van EHV-4 antilichamen. Deze detectie was waarschijnlijk het gevolg van virale reactivering. Preventieve isolatie na veiling en transport naar de eindbestemming zou virusoverdracht dus kunnen beperken. Noch koorts noch neusuitvloeiing waren goede indicatoren voor een EHV-4 infectie.

Aanbevolen maatregelen tijdens een EHV-1 abortus epidemie

Dit proefschrift levert een overtuigend bewijs voor de waarde van een snelle diagnose en een epidemiologisch onderbouwde interventie ter bestrijding van abortus uitbraken. De in hoofdstuk 5 beschreven resultaten kunnen zijn beïnvloed door de beschikbaarheid van zowel qPCR-gebaseerde diagnostische tests en door interventies ter voorkoming van reactivering. Laatstgenoemde zijn essentieel bij herpesvirus-geassocieerde ziekte-uitbraken naast de algemeen aanbevolen maatregelen ter vermindering van blootstelling aan infectieus virus. Bevindingen bij de eerste uitbraak toonden de waarde aan van het verminderen van virusverspreiding na een abortus en van het minimaliseren van de risico's en duur van interactie tussen virus en gezonde, vatbare merries. Bij de tweede epidemie werden daarom alle drachtige fokmerries voortdurend geobserveerd. Een aborterende merrie werd snel uit de groep verwijderd, de mogelijk aan het virus blootgestelde groepsgenoten werden uit de besmette omgeving gehaald en onderverdeeld in kleinere groepen. Nieuw daarbij was het gebruik van schriklint om effectieve sanitaire barrières te creëren met behoud van visueel en geluid contact tussen de dieren ter voorkoming van mogelijke stress gerelateerde virale reactivering door volledige onderlinge scheiding van de dieren. In strijd met de standaard aanbeveling om niet te vaccineren werd bij de tweede uitbraak direct uitgebreid gevaccineerd hetgeen gunstig kan zijn geweest voor beperking van de virale besmetting en de impact ervan op de populatie.

Het aantonen van een verband tussen fysiologische stress en virale reactivatie is moeilijk, maar aanwijzingen hiervoor komen voort uit de studies beschreven in de hoofdstukken 6 en 7

Aanbevolen maatregelen ter preventie van een EHV-1 abortus epidemie

Een preventieve strategie voor EHV-1 abortus moet a priori rekening houden met de belangrijkste epidemiologische kenmerken zoals vroege en waarschijnlijk levenslange latente infecties met periodiek, over het algemeen asymptomatisch, virale reactivatie met virusverspreiding. Virus verspreiding in de directe omgeving is vooral van groot belang als de paarden in een beperkte ruimte en in nauw contact met elkaar zijn gehuisvest. Het effect van maatregelen ter vermindering van reactivering van latent virus is moeilijk te evalueren maar is van zeer groot belang voor een goed eindresultaat.

Onze gegevens onderschrijven de eerder gemelde belangrijkste risico factoren die van betekenis zijn bij een uitbraak van een abortus epidemie. Dit zijn onder andere niet-gevaccineerde populaties van vatbare merries die in relatief grote groepen worden gehouden, terwijl bovendien bij nieuw aangevoerde dieren onvoldoende aandacht wordt besteed aan quarantaine protocollen. Risico's voor een virale reactivering zijn onder meer de aanvoer van hoog risico dragende merries in overeenkomstige gevoelige reeds aanwezige stabiele merrie groepen met als gevolg sociale onrust binnen de groepen.

Het leidt geen twijfel dat regelmatige vaccinatie van alle drachtige fokmerries een onderdeel zou moeten zijn van een strategie ter preventieve van EHV-1 abortus.

Onze bevindingen toonden het grote belang nog eens aan van routinematig monsteronderzoek voor de diagnosestelling bij een abortus, zeker nu de qPCR techniek beschikbaar is.

De rol van quarantaine en isolatiefaciliteiten, in het geval van een infectieus agens, werd benadrukt door de bevindingen in hoofdstuk 5. Het routinematig toepassen van een 30-daagse quarantaine periode voor alle nieuw aangevoerde merries bleek echter onvoldoende om reeds aanwezige merries te beschermen tegen het virus, vooral gezien de brede spreiding van incubatie-intervallen en variërende risico-gevoeligheid voor EHV-1 abortus, afhankelijk van het stadium van de dracht. Het quarantaine proces kan op zich zelf risico's vergroten door voorafgaande verspreiding van besmettelijke virus onder aangevoerde drachtige fokmerries voordat deze toegevoegd aan en verdeeld worden over de reeds aanwezige populatie. Het risico van introductie van merries aan het eind van de dracht in groepen van merries in een overeenkomstig stadium zou niet genomen moeten worden. Introductie zou pas gedaan moeten worden na het veulenen.

CHAPTER 10
Acknowledgements

This thesis represents an arrival following a journey that has featured the challenges, travails and pleasures traditional to the accounts of such experiences. To continue with the analogy, the route was made notable by the companionship, instruction, encouragement and acts of kindness received from many individuals and although without being named, I wish to sincerely acknowledge them all.

The journey began at an early age initiated by my abiding interest in animals and nature in general. This was enabled *via* easy access to wide-open spaces growing up in South Africa and encouraged through a series of much loved household dogs and cats, hand-raised birds and the cattle, horses and sheep on a family farm. My interests were supported mostly without reservations by my parents, Shirley and Peter. I will always be grateful for their encouragement and the opportunities they afforded me in arriving at an important early stage on my travels in obtaining a veterinary degree. My subsequent and somewhat circuitous route was given a general sense of direction through my interest in equine veterinary medicine greatly stimulated by exposure as a veterinary student to several outstanding teachers and veterinary practitioners. Choosing between divergent routes was resolved perhaps serendipitously by a posting during my period of National Service shared between the Theriogenology Department of the Veterinary Faculty and the Defence Force' stud farm in the Karoo. My strong clinical interest in horses and their breeding was readily fulfilled as was, unexpectedly, the prerequisite for a research involvement that featured the stallion and sperm biology. I chose to follow the path towards clinical specialisation in equine reproduction and stud medicine and still continue along this way, acquiring as I go some new skills and many more novel experiences. The path that my research has subsequently taken would have been impossible to map. The vagaries of geography, funding and opportunities have led to projects as diverse as colic, receptor biology, gynaecology, cryobiology and more recently population management, immunocontraception and infectious disease affecting reproduction. I have diverged from the horse to the less familiar in lions, cheetahs, elephants and wild dogs and am currently planning the way ahead to donkeys, monkeys and rhinos.

My travels have taken me through landscapes as diverse as the ordered green fields and hedgerows of the East Anglian countryside, the soaring mountains of the North West United States and the arid landscapes of Namibia. Throughout this meandering journey with its memories of chasing cows across Norfolk fields, elephants in Etosha and bears in South Dakota has been the company of many individuals from the Cape Province to California with whom I have worked alongside and whom I now regard as friends. In their good company I have

enjoyed Zinfandel in California, tequila in Monterrey, beer in Ghent and gone racing at Turffontein, played polo in Argentina, followed the banks of the Danube and swum in the Caribbean.

Along the way there have been a host of other people's beloved pets, internationally-renowned bloodstock and the exotic ranging from a heroic gorilla to cheetahs. They have provided the inspiration for undertaking and continuing along this journey and are all remembered and acknowledged.

The road I chose to travel took me to a stud farm breeding Arabian horses and so also to Debby who has since shared my journey and all its experiences, most notably with the birth of our daughter Rivka. My travels will hopefully continue and have already brought much happiness and fulfilment, none more so than having Debby and Rivka to accompany me.

CHAPTER 11
Curriculum Vitae

Martin Lance Schulman, the eldest child of Peter and Shirley Schulman was born on 21st November 1959 in Johannesburg. He was schooled at Houghton Primary School and Highlands North Boys High School where he matriculated in 1976. In 1977 he obtained a place at the University of the Witwatersrand where he graduated in 1980 with a BSc degree majoring in Physiology and Zoology. He commenced his studies in 1981 for a veterinary degree at the University of Pretoria's Faculty of Veterinary Science at Onderstepoort and a BVSc was awarded in 1986.

Two years of national service followed during which he was posted to both the Veterinary Faculty at Onderstepoort and the Defence Force's Stud Farm at De Aar. His responsibilities included investigating modifications for applications under field conditions to stallion semen cryopreservation and artificial insemination of mares. This experience encouraged him to further his knowledge through post-graduate study by enrolling for a BVSc (*Hons*) degree at the University of Pretoria which he obtained in 1989.

In September 1988 he was appointed as a Clinical Assistant in the Department of Theriogenology at the Veterinary Faculty at Onderstepoort, serving in that capacity until July 1990. This position required registration for the MMedVet (*Gyn*) which included course work majoring in Theriogenology with ancillary subjects and a research dissertation. The degree was awarded in December 1991 by the University of Pretoria on examination and submission of a dissertation entitled '*A triple staining method to determine the acrosomal integrity and viability of cryopreserved stallion sperm'*. In June 1991, Martin married May (Debby) Deveraux Voorendyk before relocating to the United Kingdom where he spent several years in rural veterinary practise in East Anglia. On returning to South Africa in 1993, Martin registered as a Specialist Theriogenologist with the South African Veterinary Council and then practiced in his private capacity primarily involved in equine and small animal reproduction. A daughter, Rivka Deveraux Schulman was born in January 1995.

In 1995 Martin was appointed as a Senior Lecturer in the Department of Companion Animal Studies at the Veterinary Faculty of the Medical University of South Africa. This appointment was held until amalgamation with the Veterinary Faculty of the University of Pretoria in 1999. His responsibilities were the didactic and clinical instruction of veterinary students in equine and small animal reproduction and provision of the equine clinical service for the Veterinary Hospital. During this period he developed new curricula in his areas of teaching responsibility and developed his clinical expertise in equine frozen semen AI and embryo transfer. Research outputs included case reports and a research publication in collaboration with the Equine

Research Centre (ERC) of the University of Pretoria. In his private capacity he provided a consultant service to various breeders and referring veterinarians, particularly to Thoroughbred farms in KwaZulu-Natal, the Northern Cape and the Western Cape Provinces. His involvement continues to the present in particular for his expertise in stallion breeding soundness evaluations, sub-fertile mare referrals and abortion investigations.

In February 1999, he accepted a transfer to the Reproduction Department of the Faculty of Veterinary Science of Pretoria University and in 2006 was appointed as an Associate Professor in the Section of Reproduction. He is engaged in teaching, research, post-graduate leadership and clinical duties in the Veterinary Academic Hospital.

Commencing in 2000 he assumed sole responsibility for the undergraduate and post-graduate teaching modules in Equine Reproduction. He served as Co-ordinator for equine clinical studies from 2000-2012 and played a leading role in development of two successive equine clinical teaching curricula for the BVSc programme.

He has mentored and instructed a series of clinical assistants, residents and post-graduate students since his appointment in the role of Promotor or Co-promotor to MSc, MMedVet and PhD candidates in both the Departments of Production Animal Studies and Companion Animal Clinical Studies.

His current research focus is twofold and includes infectious diseases affecting equine reproduction and immunocontraception of domestic and wildlife species. He was a co-developer of the Veterinary Population Management Laboratory established in 2014. International collaborations include UC Davis (USA), Utrecht University (Netherlands), University of Ghent (Belgium), UC Dublin (Ireland) and Ross University (St Kitts and Nevis). Local collaborators include the Council for Scientific and Industrial Research (CSIR) and colleagues at the Veterinary Faculty of UP. His publications record includes a growing list of papers published in peer-reviewed scientific journals and international conference and meeting proceedings.

He has held appointments as Visiting Professor in Equine Reproduction in the Department of Population Health and Reproduction at the University of California, Davis in 2005 and 2011. During these appointments he was responsible for didactic and clinical training of undergraduate veterinary students in equine reproduction and the mentorship of residents appointed to the Equine Reproduction Service of the Veterinary Teaching Hospital.

He is actively involved and consulted both locally and internationally regarding various aspects of equine reproduction and stud medicine. Professional veterinary registrations

include SAVC specialist registration, MRCVS and Namibian Veterinary Council. In 2011 the Director, Department of Agriculture, Forestry and Fisheries appointed him as an expert to assist the State in the eradication of CEM which has produced regulatory changes and successful containment of the outbreak. He is the elected South African representative on the International Committee for Animal Reproduction (ICAR). He was appointed to the Executive Committee of the South African Equine Veterinary Association (SAEVA) in 1996 and currently continues as SAEVA's Stud Health Committee Chairman. In this role he has been convenor of a series of Courses in Stud Management for breeders and veterinarians in association with the Thoroughbred Breeders Association and the National Horse Racing Authority, various CPD initiatives for veterinarians and participation in lectures and seminars for the equine industry. He has produced numerous articles for the lay-press on various aspects of equine and general veterinary reproduction including wildlife species as well as immunocontraception and infectious diseases.

A lifelong interest in African wildlife, horses and equestrian sports helps provide the impetus for his professional activities.