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EFFECTS ON SEMEN QUALITY AND ON ESTABLISHMENT OF PERSISTENT EQUINE ARTERITIS VIRUS (EAV) INFECTION IN STALLIONS FOLLOWING EXPERIMENTAL CHALLENGE WITH THE KENTUCKY 84 (KY84) STRAIN

THESIS

A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science
in the College of Agriculture at the
University of Kentucky

By

Juliana Roberta Campos

Lexington, Kentucky

Director: Dr. Udeni B.R. Balasuriya, Professor of Virology

Lexington, Kentucky

2012

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ABSTRACT OF THESIS

EFFECTS ON SEMEN QUALITY AND ON ESTABLISHMENT OF PERSISTENT EQUINE ARTERITIS VIRUS (EAV) INFECTION IN STALLIONS FOLLOWING EXPERIMENTAL CHALLENGE WITH THE KENTUCKY 84 (KY84) STRAIN

Equine arteritis virus (EAV) is the causal agent of equine viral arteritis (EVA), a disease of equids. Following EAV infection, up to 70% of stallions may become carriers and continuously shed the virus in their semen for varying time periods. The long-term carrier stallion has an important role in the transmission and maintenance of EAV in horse populations. Recently, it has been demonstrated a correlation between *in vitro* susceptibility of $CD3^+$ T lymphocytes to EAV infection and establishment of long-term persistent infection among stallions following natural infections. In this study, we investigated whether stallions with *in vitro* EAV susceptible $CD3^+$ T lymphocytes are at higher risk of becoming long-term carriers compared to those with the resistant phenotype following experimental infection with the KY84 strain of EAV. Furthermore, we investigated whether there is a significant effect of EAV infection on semen quality during acute phase of the infection. The data suggested that the establishment of the long-term carrier state seems to be associated with the *in vitro* $CD3^+$ T lymphocyte susceptible phenotypes and that reduced semen quality resulted from the combined effect of fever and scrotal edema observed following EAV infection rather than the direct effect of the virus.

Keywords: Equine arteritis virus, equine viral arteritis, persistent viral infection, carrier stallions, $CD3^+$ T lymphocyte susceptible/resistant phenotypes

Juliana Roberta Campos

November, 15 2012.

EFFECTS ON SEMEN QUALITY AND ON ESTABLISHMENT OF PERSISTENT EQUINE ARTERITIS VIRUS (EAV) INFECTION IN STALLIONS FOLLOWING EXPERIMENTAL CHALLENGE WITH THE KENTUCKY 84 (KY84) STRAIN

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November 15, 2012.

To my parents for their unconditional love and support.
To my husband for his patience and support during this long journey.
To the horses for serving my research.

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CHAPTER ONE

Literature review

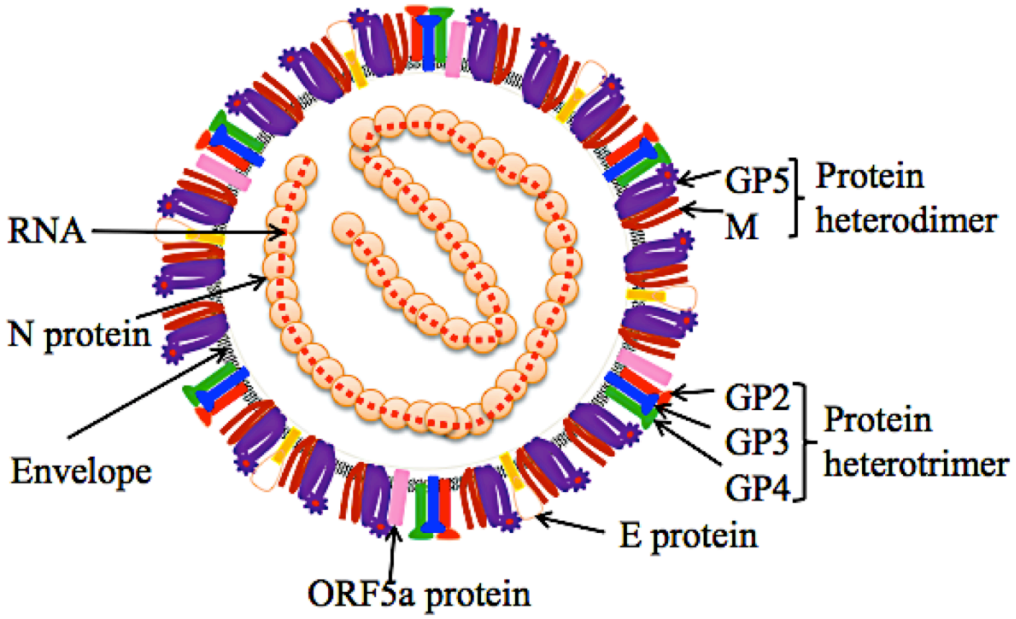
I. THE VIRUS AND THE DISEASE

1.1. EQUINE ARTERITIS VIRUS

Arterivirus is a genus of virus belonging to the family Arteriviridae, which in 1996, was included within the order Nidovirales (Cavanagh, 1997; Snijder and Meulenberg, 1998). Equine arteritis virus (EAV) is the prototype virus in the family *Arteriviridae* (genus *Arterivirus*, order *Nidovirales*). The family *Arteriviridae* (genus *Arterivirus*) also includes porcine reproductive and respiratory syndrome virus simian hemorrhagic fever virus (SHFV) and mice lactate dehydrogenase-elevating virus (LDV) (Cavanagh, 1997; Snijder and Meulenberg, 1998).

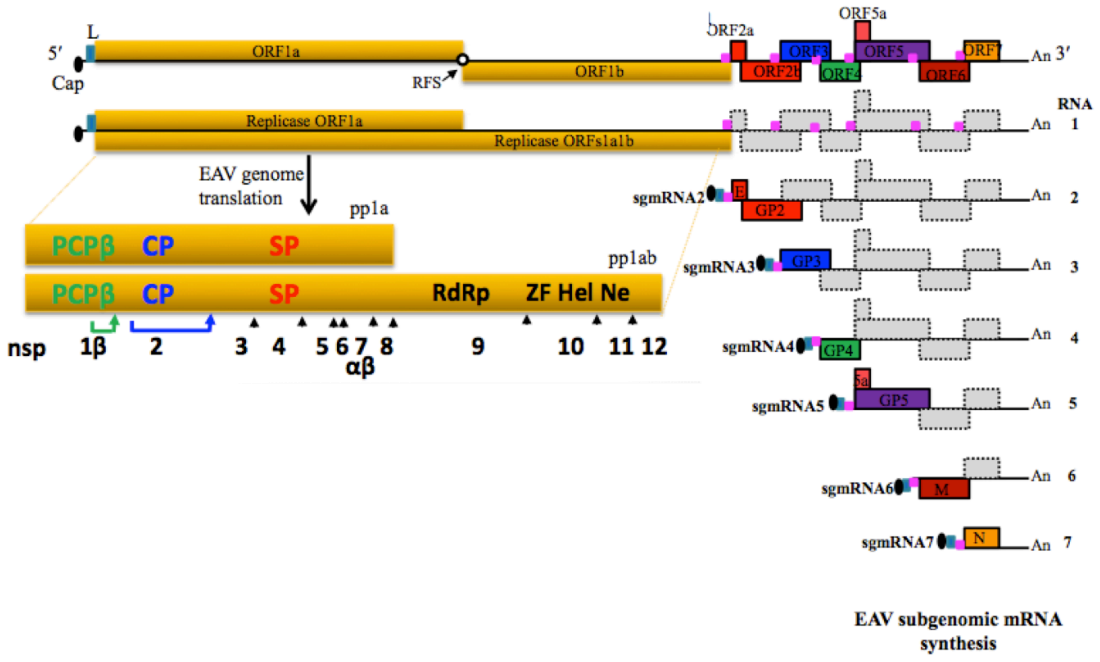
The EAV virion is an enveloped, spherical, 50-nm to 65-nm particle with an isometric core that contains a single-stranded, positive-sense ribonucleic acid (RNA) (Maess et al., 1970; van der Zeijst and Horzinek, 1975; den Boon et al., 1991; Wieringa et al., 2004). The EAV genome is encapsidated by a nucleocapsid protein (N) of 14 kDa, which forms an icosahedral core structure. A lipid-containing envelope with small surface projections, which contains seven proteins (E, GP2, GP3, GP4, ORF5a protein, GP5, and M), surrounds the nucleocapsid (Figure 1.1.1.) (Snijder and Meulenberg, 1998; Firth et al., 2011).

Figure 1.1.1. Schematic representation of equine arteritis virus (EAV) particle (Balasuriya, 2012).



The EAV genome length varies between 12,704 to 12,731 bp among different strains (den Boon et al., 1991; Cavanagh, 1997; Snijder and Meulenberg, 1998; Zhang, Timoney, et al., 2010), which includes a 5' leader sequence with 224 nucleotides, and ten open reading frames (ORFs 1a, 1b, 2a, 2b, 3, 4, 5a, 5b, 6, and 7), and a poly (A) tail at the 3'end (Figure 1.1.2.) (den Boon et al., 1991; van Dinten et al., 1997; Snijder et al., 1999; Snijder and Meulenberg, 2001; Firth et al., 2011).

Figure 1.1.2. Schematic representation of the genomic organization of equine arteritis virus (EAV) (Balasuriya, 2012).



ORFs 1a and 1b occupy about three fourths of the most 5'-proximal end of the genome and encode two replicase polyproteins (pp1a and pp1ab). ORFs 1a and 1b are overlapping and their expression involves ribosomal shifting (Vries et al., 1992). Pp1a and pp1b have identical N-terminals, but due to the ribosomal frame shifting, the C-terminals are distinct (den Boon et al., 1991). These two precursor proteins are extensively processed after translation into at least 13 non-structural proteins (nsp1-12, including nsp7 α/β) by three virus-encoded proteases (nsp1, nsp2 and nsp4) (Snijder and Meulenberg, 1998, 2001; Ziebuhr et al., 2000).

The seven envelope proteins (E, GP2 [previously known as G_S], GP3, GP4, GP5a, GP5 [G_L], and M) and the nucleocapsid protein [N] make up the structural proteins of the EAV virion and are respectively encoded by ORFs 2a, 2b, 3, 4, 5a, 5b, 6 and 7 located at the 3'-proximal quarter of the EAV genome (Vries et al., 1992; Snijder and Meulenberg, 1998; Snijder et al., 1999; Firth et al., 2011). These structural proteins are expressed from a 3'-coterminal nested set of six subgenomic viral messenger RNAs (sgmRNA) and not from the genomic RNA (de Vries et al., 1990). The 3'-end of the sgmRNAs of EAV have the same 3'-end as genomic RNA, but the 5'-end is variable (Miller and Koev, 2000). Three of the minor envelope proteins (GP2, GP3, and GP4) in the EAV virion are covalently associated and form a heterotrimer; whereas, the two major envelope proteins (M and GP5) form a disulfide-linked heterodimer (de Vries et al., 1995; Wieringa et al., 2004). It has been shown that the major GP5 and M proteins, and the N protein are essential for virus particle formation, but not the E and the GP2, GP3, and GP4 minor proteins (Wieringa and Vries, 2004). In contrast, all envelope proteins are essential for the production of infectious progeny virus, and elimination of ORF5a by reverse genetics resulted in defective progeny virus, with small plaque phenotype and significant reduced virus titer (Wieringa et al., 2004). The amino terminal ectodomain of GP5 protein contains the known major neutralization determinant of EAV (Balasuriya et al., 1993, 1997, 2000, 2004; Deregt et al., 1994; Chirnside, de Vries, et al., 1995; Glaser, de Vries, and Dubovi, 1995). It has been demonstrated that heterodimerization of GP5 with M proteins is critical for the post-translational modification (glycosylation) and conformational maturation of neutralization determinants in the GP5 protein to form EAV epitopes that induces neutralizing antibodies in mice, and horses (Balasuriya et al.,

2004; Zhang, Timoney, Maclachlan, and Balasuriya, 2008). Although considerable variation occurs in the sequence of the GP5 protein of field strains of the virus, there is only one known serotype of EAV and all strains evaluated so far are neutralized by polyclonal antiserum raised against the virulent Bucyrus strain of EAV (Timoney and McCollum, 1993a; Balasuriya and MacLachlan, 2004). Even though there is only one recognized serotype of the virus, not all strains are neutralized to the same degree by antiserum raised against various strains of EAV (Balasuriya et al., 1997, 1998; Balasuriya et al., 1999; Hedges et al., 1999; Zhang et al., 2010).

A number of studies have shown that different strains of EAV vary significantly in their pathogenicity, with different clinical outcomes upon experimental inoculation of horses (Balasuriya et al., 1999; McCollum and Timoney, 1999; Balasuriya et al., 2002; Balasuriya et al., 2007).

EAV is inactivated immediately by common and lipid solvents (ether and chloroform), disinfectants and detergents. It has been reported that tissue culture fluid containing the experimentally adapted derived virulent Bucyrus strain of EAV survives approximately 75 days at 4^0C, 2 to 3 days at 37^0C, and 20 to 30 minutes at 56^0C (McCollum et al., 1961). The virus is stable at -70^0C for years without loss of infectivity (Crawford and Henson, 1973). Lyophilized virus is highly stable at -20^0C and moderately stable at 4^0C (Harry and McCollum, 1981).

1.2. EQUINE VIRAL ARTERITIS

1.2.1. History

Equine viral arteritis (EVA) is an acute, contagious, respiratory and reproductive disease of equids caused by EAV. EAV was first isolated from a lung of an aborted fetus in 1953 following an extensive outbreak of respiratory disease and abortion in a Standardbred breeding at Bucyrus, Ohio, U.S.A. (Doll et al., 1957b; Doll, Bryans, and Knappenberger, 1957). Although descriptions of a disease in late 18th and early 19th centuries was very likely EVA, this was referred as "infectious or epizootic cellulitis" (Hodgins and Haskett, 1907), "influenza erysipelatosa," "Pferdestaupe," "Rotlaufseuche" (Balasuriya and MacLachlan, 2007) "pinkeye" (Mohler, 1917) and "equine influenza" (Huidekoper, 1923), EVA was characterized as a new disease only in the 1950s, after the causal agent was isolated and the vascular lesions found on histological examination of acutely infected cases was characterized (Doll et al., 1957a; Doll et al., 1957b; Doll, Bryans, and Knappenberger, 1957). The virus obtained from the Ohio outbreak was referred as the Bucyrus strain from which the prototype strain of EAV was isolated with the *American Type Culture Collection* catalogue designation VR-796™ (American Type Culture Collection, 1992). Outbreaks of EVA have been reported worldwide (Switzerland, Austria, Poland, Italy, the United Kingdom, Spain, the Netherlands, Canada and the U.S.A.) after the identification of the causal agent of the disease (Jaksch et al., 1973; Clayton, 1987; Autorino et al., 1992; Nowotny, 1992; Weiis et al., 1994; Monreal et al., 1995; Balasuriya, 2012). Several outbreaks of the disease took place in the U.S.A since the outbreak reported in 1953, in Ohio. Major outbreaks have occurred during the 1900s in: Kentucky (1977 – Standardbred racetracks and 1984 – Thoroughbred breeding

farms), Illinois (1993 – Thoroughbred racetracks) and Pennsylvania (1996 – a single Warmblood breeding farm) (Balasuriya, 2012). The 1984 EVA Kentucky outbreak was the first recorded in the Thoroughbred population in the U.S.A., and it generated widespread interest and major concern to the equine industry. Epidemiological data collected from that outbreak strongly suggested the occurrence of long-term persistence of EAV in stallions and the importance of the carrier stallion in the dissemination and perpetuation of the virus within the horse population (Timoney and McCollum, 1985). There had been a number of recent outbreaks reported in Europe, North America and South America. These include: (1) the 2006/2007 multi-state occurrence of EAV in the U.S.A. affecting initially American Quarter Horses and subsequently spreading to horses of 11 different breeds located in 9 different states (Timoney et al., 2008; Zhang et al., 2010); (2) the extensive 2007 event in France that affected draft and saddle horses and involved 8 mortality cases including 1 fetus, 5 young foals and 2 mature horses (Pronost et al., 2010; Miszczak et al., 2012); and (3) the 2010 outbreak in Argentina affecting mainly sport-horses with abortion rates reaching about 50% and 38 stallions becoming seropositive for EAV (Committees Infectious Diseases of Horses, 2010).

1.2.2. Seroprevalence, distribution and transmission

Serologic surveys have shown that EAV infection is distributed worldwide and have been reported in the horse populations in North and South America, Europe, Africa, Asia, Australia and New Zeland (Jaksch et al., 1973; McCollum and Bryans, 1973; Moraillon and Moraillon, 1978; de Boer et al., 1979; Huntington et al., 1990; Timoney and McCollum, 1993b; Monreal et al., 1995; Huovilainen and Ek-Kommonen, 1998;

Newton et al., 1999). Iceland and Japan are free of EAV infection (Balasuriya, 2012). The seroprevalence of EAV infection varies not only between countries but also between horse populations of different breeds and ages within the same country (McCollum and Bryans, 1973). Studies have shown that there is a marked disparity in seroprevalence of EAV infection between Standarbreds (78 to 84%) and Thoroughbreds (<5%) in the USA, with the infection being considered endemic in the Standarbred horse population in this country (McCollum and Bryans, 1973). A serologic survey done with 226 imported horses from several countries to California have demonstrated that the prevalence of EAV infection appears also to be high in European Warmbloods (Hullinger et al., 2001). Antibodies against EAV have been found in: donkeys and mules in Brazil (Bello, 2006) and South Africa (Paweska and Barnard, 1993; Paweska et al., 1995, 1997); and donkeys in Italy (Ramina et al., 1999). In addition, experimental inoculation of donkeys with EAV caused clinical disease, shedding of the virus in the semen of males and seroconversion to EAV (McCollum et al., 1995; Paweska et al., 1995). EAV-specific neutralizing antibodies were also detected in 12 of 51 (24%) zebras of the Burchell's zebra population in the Serengeti National Park, Tanzania (Borchers and Wiik, 2005), but not in 266 park or captive zebras from 9 different countries such as South Africa, Kenya, Zimbabwe, Botswana, Germany, Netherlands, Australia, Canada and the USA (Paweska et al., 1997). Antibodies to EAV were also found in a herd of alpacas in Germany. A case of late abortion in an alpaca from the same cohort was reported and genome fragments of EAV could be detected in the fetal tissues by using reverse-transcription polymerase chain reaction (RT-PCR) (Weber et al., 2006).

Transmission of EAV occurs mainly by the respiratory (Doll, Bryans, and Knappenberger, 1957; McCollum et al., 1971) and venereal routes (Timoney et al., 1986, 1987). Transmission via respiratory route requires direct contact between susceptible horses and acutely infected animals or their contaminated body secretions as urine, vaginal secretions, feces, aborted fetuses and their membranes and, masturbates of stallions (Doll, Bryans, and Knappenberger, 1957; McCollum et al., 1971; Fukunaga et al., 1981; McCollum and Timoney, 1984; Cole et al., 1986; Timoney and McCollum, 1993a; Guthrie et al., 2003). Although virus is shed in tissues and bodily fluids, the amount and duration of the virus shed is greater into the respiratory tract and it varies from around 7 to 14 days during acute infection (McCollum et al., 1971). Acutely or chronic persistently infected stallions play a major role in the venereal transmission of EAV to mares, during natural breeding and artificial insemination with fresh, cooled and frozen semen (Timoney et al., 1986, 1987; Balasuriya et al., 1998; Balasuriya, Hedges, et al., 1999). Persistently infected stallions shed virus only in semen and 85 to 100% of mares bred to these long-term carrier stallions seroconvert to EAV within 28 days (Timoney and McCollum, 1985, 1993a; Timoney et al., 1986, 1987). Other less common modes of dissemination of EAV include vertical (transplacental) and indirect transmission. Although rare, exposure of pregnant mares to EAV late in gestation can result in transplacental transmission of the virus and congenital infection of the fetus (Vaala et al., 1992). Indirect spread of EAV via contaminated fomites, such as shanks, artificial vaginas, twitches, apparel or personnel, has been reported and it is thought to play a minor role in the transmission of the virus (Collins et al., 1987; Timoney and McCollum, 1993a; Glaser et al., 1996; Balasuriya et al., 1999). Other sources of EAV can

be an infected teaser stallion or a nurse mare (Timoney, 1984; Timoney and McCollum, 1993a). Recently, it was demonstrated under experimental conditions, that embryo transfer from a donor mare inseminated with EAV infective semen to naive recipient mares, could represent a risk of EAV transmission (Broaddus et al., 2011).

1.2.3. Clinical Signs

The outcome of EAV infections in equids varies from subclinical to influenza-like signs in adult horses, abortion in pregnant mares, interstitial pneumonia and pneumoenteritis in young foals (Johnson et al., 1991; Vaala et al., 1992; Timoney and McCollum, 1993a; Del Piero et al., 1997). Most infections with EAV results in an unapparent illness, especially in mares bred to persistently infected stallions (Timoney et al., 1987; Timoney and McCollum, 1993a). The development of clinical signs following natural or experimental infection with EAV can vary considerable in severity among individual horses and outbreaks (Timoney, 1984; Timoney and McCollum, 1993a). This variability depends on a number of factors such as strain and dose of virus, route of infection, age and physical condition of the animal at the time of exposure, environmental conditions, and route of exposure (Timoney and McCollum, 1993a; McCollum and Timoney, 1999; Balasuriya, 2012). Fever (up to 41^0C) develops after an incubation period of 3-14 days (6-8 days in the case of venereal exposure) and may persist for 2-9 days. Typical signs of EAV may include pyrexia, depression, anorexia, leukopenia, nasal and/or ocular discharge, conjunctivitis, rhinitis, urticarial, periorbital or supraorbital edema, edema of the limbs, stiffness of gait, preputial and scrotal edema in stallions, edema of the mammary glands and abortion in pregnant mares (Doll, Bryans, and

Knappenberger, 1957; McCollum et al., 1971, 1995; Timoney and McCollum, 1993a; Glaser et al., 1996). The most consistent clinical findings of EVA are fever and leukopenia. Regardless of the severity of clinical signs, usually all naturally infected adult horses recover spontaneously from infection. However, infected neonates and young foals up to few months of age can die from a severe interstitial pneumonia or a rapidly progressive pneumoenteric syndrome (Golnik et al., 1981; Carman et al., 1988; Vaala et al., 1992). Abortion due to EAV is reported to occur between 3 and 10 months of gestation and may occur late in the acute phase of the disease or early in the convalescent phase of EVA, following natural or experimental infection of horses. Abortion rates between 10 and 60% have been reported in the case of natural outbreaks of the disease and are not necessarily preceded by clinical signs of EVA. Experimental infection with the Kentucky 1984 strain of EAV lead to 71% abortion in pregnant mares (Doll, Bryans, and Knappenberger, 1957; Cole et al., 1986; Johnson et al., 1991; Timoney and McCollum, 1993a). However, it appears that different strains of EAV differ in their virulence and abortigenic characteristics. Horses experimentally inoculated with virulent strains of EAV (e.g., the VB, recombinant VB, and KY84) develop severe lymphopenia with a high-titer viremia (McCollum and Timoney, 1984; MacLachlan et al., 1996; Balasuriya et al., 2002; Balasuriya et al., 2007). In contrast, horses inoculated with the attenuated modified live virus (MLV) strain or with other avirulent strains (e.g. 030H and CA95) develop a mild lymphopenia with a low-titer viremia (Doll et al., 1968; McCollum, 1969; Balasuriya et al., 1999; Patton et al., 1999).

Acutely infected stallions shed virus in their semen and it has been shown that they experience a period of temporary subfertility believed to be the result of increased

testicular temperature and scrotal edema, rather than a direct effect of the virus in the semen. Reduction in the stallions libido during the acute phase of EAV infection have been reported, in addition to the changes in the semen quality (decreased motility, sperm concentration and percentage of morphologically normal spermatozoa) that persisted for up to 6-7 weeks, (Neu et al., 1992). However, no long-term effects on stallions fertility have been observed on long-term carrier stallions (Timoney and McCollum, 1993a). Mares bred to persistently EAV infected stallions appear not to experience any short- or long-term fertility problems (Raz et al., 2006).

Although mortality is very rare in horses following natural infection with field strains of EAV, it has been reported that the highly virulent horse adapted Bucyrus strain of EAV can cause up to 60% mortality in adult horses (MacLachlan et al., 1996).

1.2.4. Persistent infection and the carrier state in stallions

The establishment of persistent infection in the reproductive tract of the infected stallions is a unique feature of EAV infection. The recognition of an asymptomatic carrier stallion as a natural reservoir of EAV was first described in late 1800's when it was noted that a healthy stallion could infect mares with equine influenza or "pink-eye" at the time of natural breeding but could not infect animals in close contact (Pottie, 1888; Reeks, 1902). It was known that those stallions could be infective for 2 or more years and mares infected by them were able to transmit the disease to other contact animals via the respiratory route (Pottie, 1888; Reeks, 1902). However, the EAV carrier state was better elucidated only after the 1984 epizootic affecting the Thoroughbred horse population in Central Kentucky, in which a high percentage of the naturally infected stallions were

confirmed to shed EAV in their semen by isolation of the virus from the semen and/or transmission of the disease to test-bred mares. Epidemiological data collected from that outbreak strongly suggested the establishment of a long-term persistence of EAV in stallions and the importance of the carrier stallion in the dissemination and perpetuation of the virus within the horse population (Timoney and McCollum, 1985). In 1986, Timoney et al. (Timoney et al., 1986) confirmed the chronic carrier state in 30-35% of the stallions naturally infected during 1984. Subsequent studies confirmed the existence of persistently infected stallions for many years, perhaps life long, with the frequency of the carrier state varying somewhere between 30-60% in stallions exposed to EAV via the respiratory route (Timoney et al., 1987; Neu and Timoney, 1988; Timoney and McCollum, 1993a).

The virus persists in the male reproductive tract, specifically in the accessory glands with highest titers being found in the ampulla of the vas deferens (Neu and Timoney, 1988; Little et al., 1991), despite the presence of high titer neutralizing antibodies in serum (Timoney et al., 1986; Timoney et al., 1987; Neu and Timoney, 1988; Little et al., 1991). Virus is not present in the nasopharyngeal secretions, blood, urine, or other body secretions of long-term carrier stallions. Isolation of EAV from the pre-ejaculatory fluid of persistently infected stallions was not successful and thus, virus is believed to be associated with the sperm-rich fraction of the ejaculate. In addition, assessment of the shedding status of a putative carrier stallion was best undertaken with entire ejaculates rather than dismount samples. However, dismount samples are a feasible alternative choice in the case when entire ejaculates cannot be obtained (Timoney, McCollum, and Roberts, 1986; Timoney et al., 1987; Little et al., 1991). Stallions can

become carriers and continuously shed the virus in their semen for varying time periods, from weeks (convalescent or short-term carriers) to years (chronic or long-term carriers) (Timoney et al., 1986; Timoney, McCollum, and Roberts, 1986; Timoney et al. 1987; Neu and Timoney, 1988). Intermittent virus shedding or the presence of a latent period of EAV has never been demonstrated. Persistently infected stallions can stop shedding virus in their semen years following infection with EAV with no evidence of reversion to a shedding state at a later date (Timoney and McCollum, 1993a). The mechanism of EAV persistence in the reproductive tract of stallions is unclear, however it has been shown that establishment and maintenance of infection is testosterone dependent (Little et al., 1992; Holyoak et al., 1993; McCollum et al., 1994). Surgical castration of long-term carrier stallions resulted in either viral clearance or significant reduction of virus shedding in their semen. In contrast, castrated persistently infected stallions that received supplementation of testosterone subcutaneously kept shedding virus in their semen for as long as non-castrated carrier stallions (Little et al., 1992). It was suggested that high levels of testosterone are necessary for maintenance of the virus in the reproductive tract of stallions but not for viral replication and persistence of EAV for an intermediate period of time (months). Prepubertal and peripubertal colts experimentally inoculated with EAV remained infected in the reproductive tract for a considerable period of time, even though testosterone levels in these animals are lower than in stallions. These findings have confirmed that the virus can replicate in the male reproductive tract for an intermediate period of time in the absence of circulating concentrations of testosterone equivalent to those found in sexually mature stallions. However, long-term persistent infection does not appear to occur in colts exposed to EAV before the onset of puberty (Holyoak et al.,

1993). Similarly, persistence of EAV has never been reported in mares, gelding, or fetuses (McCollum et al., 1994; Timoney and McCollum, 1993; Timoney et al., 1986; Timoney and McCollum, 1987b). Thus, persistently infected stallions can be divided into three groups based on the duration of virus excretion in semen: (1) the short-term or convalescent carrier state (few weeks following clinical recovery); (2) the intermediate carrier state (3 to 7 months); and, (3) the long-term carrier state (years to lifetime) (Little et al., 1992; Holyoak et al., 1993; Timoney and McCollum, 1993a).

One of the explanations for long-term persistence of EAV infection in the reproductive tract of mature stallions could be the immunosuppressive activity that testosterone is believed to have. Several *in vitro* studies have suggested that testosterone inhibits lymphocyte proliferation, cytokines production, and macrophage activity (Muehlenbein and Bribiescas, 2005). In addition to the immunosuppressive activity, testosterone plays an important role in the maintenance of susceptible host cell population of the male reproductive tract (Amann, 2011a), which supports viral persistence, and it is an important mechanism of viral immune system evasion (Alcami and Koszinowski, 2000). Another important function of testosterone in peripheral blood is to ensure normal function of all accessory sex glands (Thompson et al., 1979), which in turn, contribute most of the fluid to the ejaculate (seminal plasma). Seminal plasma contains immunosuppressive factors, which are believed to protect sperm from immunological damage and prevent sensitization of the female reproductive tract to sperm antigens following coitus, which is fundamental to the process of reproduction itself. But this benefit is not without risk; the local immune response to bacteria and viruses might be inhibited and, therefore, accessory cells might be prevented from recognizing bacterial or

viral antigens (James and Hargreave, 1984). However, EAV mostly persists in the ampulla of the vas deferens, which is located distally to the other accessory glands of the stallion. Thus, the immune suppressive factors of seminal plasma would have to come from the fraction of seminal plasma produced by the ampulla to have an immune suppressive effect on virus replication, which seems unlikely to happen. These could possibly be EAV strategies to evade the immune system and establish persistent infection in the reproductive tract of stallions.

Recently, other explanations for viral persistence in the reproductive tract of stallions have been suggested in addition to viral testosterone dependence. It is assumed that virus persists by continuously re-infecting susceptible tissues in the reproductive tract together with constant evolution of antigenic variants of the virus (Balasuriya et al., 1999; Hedges et al., 1999; Balasuriya, 2004). Until 2008, there was no information available about host and viral factors involved in the establishment and maintenance of EAV infection, other than testosterone. Thus, Zhang *et al.* developed an *in vitro* model system in an attempt to elucidate the mechanism of EAV persistence. Reverse genetics studies using an EAV infectious cDNA clone showed that amino acid changes in the structural proteins of the virus (E, GP2, GP3, GP4, and GP5) were responsible for the establishment of persistent infection in HeLa cells (Zhang et al., 2008). Previous studies, in which EAV isolated from the semen of long-term carrier stallions was sequenced, have shown that genetic variants emerge during the course of long-term persistent infection in stallions (Hedges et al., 1999; Balasuriya, 2004).

Sequence analysis of strains isolated form the semen of EAV carrier stallions have shown the emergence of novel genotypic and phenotypic variants (Balasuriya et al.,

1998; Balasuriya, et al., 1999; Hedges et al., 1999; Patton et al., 1999; Balasuriya, 2004; Zhang et al., 2010; Miszczak et al., 2012). EAV, as a RNA virus, mutates frequently and therefore exists not as a single genotype, but rather, as a heterogeneous population of related viral genomes known as quasispecies that facilitates its adaptability and survival (Cavanagh, 1997; Domingo, 1997). It has been suggested that, perhaps, major outbreaks of EVA are the result of the emergence and selection of specific variants of the virus present in the semen of carrier stallions, however the mechanism of selection remains unclear (Balasuriya et al., 1999). High rates of nonsynonymous amino acid changes were observed in regions of the GP3 and GP5 structural proteins of EAV isolated from infective semen (Hedges et al., 1999; Balasuriya, 2004) indicating that those proteins are under constant selective pressure during the establishment and persistence of EAV infection in the reproductive tract of carrier stallions (Balasuriya et al., 1997; Hedges et al., 1999). Variation in the GP5 protein was found to have occurred in the specific V1 variable region critical for neutralization of the virus by some monoclonal antibodies and polyclonal equine antisera, which correlates with the emergence of novel variants with distinct neutralization phenotype during persistent infection (Balasuriya et al., 1997, 2004; Hedges et al., 1999; Balasuriya and MacLachlan, 2004). However, all variants that arise during persistence of EAV infection are neutralized by polyclonal equine sera from the same carrier stallion, therefore, immune evasion may not be entirely responsible for the establishment and maintenance of the virus in the reproductive tract of carrier stallions (Hedges et al., 1999; Balasuriya, 2004). In contrast, EAV that circulates during outbreaks associated with horizontal and vertical transmission, generally, is relatively genetic stable (Hedges et al., 1999; Balasuriya, 2004). Interestingly, the molecular

epidemiologic and genetic characterization of the EAV isolates collected for an extended period of time (9-10 months) during the multi-state occurrence of EVA in 2006-2007, in the USA, showed a high mutation rate and genetic diversification of the strain involved in this disease event, even though the principal mode of transmission of EAV in this outbreak was horizontal and vertical. That event provided a unique opportunity to assess the genomic stability of the virus during lateral and vertical transmission over an extended period of time. Data from that study suggested that the EAV genome remains genetically relatively stable over a short time period. However, genomic variability tended to increase if horizontal and vertical transmission took place over a more extended time period. In addition, it was the first time that a study was based on a great number of stallions (n=8) and on the evaluation of the full-length genomic sequences of 18 EAV isolates collected sequentially from those stallions. From the results, it was evident that EAV evolved genetically during persistent infection, but the evolution rate varied according to the individual stallion and the period of viral shedding of particular stallion. The longer the shedding period, the more accumulated mutations were observed in the virus genome.

1.2.5. Pathogenesis

Most of the information on the pathogenesis of EVA is derived from experimental inoculation of horses but the exact mechanism of EVA pathogenesis remains to be determined (Doll et al., 1957b; Estes and Cheville, 1970; McCollum et al., 1971; Prickett et al., 1972; MacLachlan et al., 1996).

EAV has been isolated from a variety of tissues and body fluids, such as, spleen, liver, kidneys, bronchial and mesenteric lymph nodes, endothelium and mesothelium, buffy coat, reproductive tract of colts and stallions, and semen, starting from 1 to 2 dpi following experimental inoculation (Estes and Cheville, 1970; McCollum et al., 1971, 1994; Breese and McCollum, 1972; Neu and Timoney, 1988; Neu et al., 1992; Holyoak et al., 1993; Del Piero, 2000).

Virus can be isolated from the nasopharynx, buffy coat, and serum or plasma for varying period of times following intranasal exposure. The disappearance of virus from serum coincides with the development of EAV-specific neutralizing antibodies, which occurs approximately between 6 and 9 dpi (McCollum et al., 1971; Summers-Lawyer et al., 2011; Balasuriya, 2012). Apart from its incidental recovery from the buffy coat and its isolation from the reproductive tract of colts and stallions (Neu and Timoney, 1988; McCollum et al., 1994), EAV is generally not detectable in most tissues after 28 days following infection.

Following approximately 1 day post respiratory infection, EAV replicates in the respiratory tract epithelium and pulmonary macrophages (McCollum et al., 1971; MacLachlan et al., 1996; Del Piero, 2000; Balasuriya, 2012). After 2 days post-infection (dpi), the virus proliferates and gains entry into the bronchial lymph nodes and is disseminated to circulating monocytes and endothelial cells (McCollum et al., 1971; Del Piero, 2000). Virus is then systemically distributed throughout the body (viremia) (McCollum et al., 1971; MacLachlan et al., 1996; Del Piero, 2000; Balasuriya, 2012). Endothelial cells and macrophages are the principal sites of virus replication, but EAV also replicates in selected epithelia, mesothelium, and smooth muscle cells of the tunica

media of smaller arteries, venules, and may also infect the myometrium (Coignoul and Cheville, 1984; Johnson et al., 1991; Del Piero, 2000; Balasuriya and MacLachlan, 2004). A characteristic panvasculitis involving the small arteries and, to a lesser extent, the small veins throughout the body, is observed. Initially the endothelial cells of the intima are compromised and then the tunica media, followed by the adventitia (Doll et al., 1957a). The clinical manifestations of EVA reflect the hemorrhage and edema that occur around affected vessels caused by the increased vascular permeability and leukocyte infiltration from the generation of chemotactic factors (Estes and Cheville, 1970). The vascular lesions of EVA are likely the result of the direct effect of viral replication and virus-induced inflammatory cytokines on vascular endothelium, rather than the result of immune-mediated injury (Moore et al., 2003; Balasuriya, 2012). *In vitro* EAV infection of macrophages and endothelial cells demonstrated an increase in the transcription of genes encoding proinflammatory mediators (Moore et al., 2003). This study strongly suggested that cytokine mediators produced by endothelial cells and machrophage have a central role in the pathogenesis of EVA.

Recently, it has been demonstrated that the clinical outcome of EVA infection is determined by host genetic factors (Go, et al., 2012). Go *et al*. showed that clinical signs and viremia burdens are greater in horses that have their $CD3^+$ T lymphocytes resistant to *in vitro* EAV infection compared to horses that have susceptible $CD3^+$ T lymphocytes. In addition, a significant difference between the two groups was found in terms of proinflammatory and immunomodulatory cytokine mRNA expression.

The pathogenesis of EAV-induced abortion is controversial. Doll *et al*. concluded that abortion is the result of infection of the fetus by the virus rather than from secondary

influences such as, fever, toxemia, or debility of the mare, since it occurs during the early convalescent period of the disease (Doll, Bryans, and Knappenberger, 1957). The aborted fetuses contain higher concentration of virus than the dams, which suggests that viral replication occurs in the fetus. Fetal stress activates the fetal-hypothalamic-pituitary axis and may cause abortion (MacLachlan et al., 1996). However, other authors, based upon light and electron microscopic studies of tissues from the genital tracts, placentae and fetuses of mares experimentally infected with EAV, suggested that abortion may result from a necrotic myometritis observed in the uterus of the EAV infected mares rather than fetal infection with the virus (Coignoul and Cheville, 1984). Another possible explanation for abortion caused by EAV infection is an impaired utero-placental blood flow due to generalized vascular necrosis (Doll et al., 1957a) or due to mechanical compression on blood vessels caused by edema in myometrium or from tissue distension by loss of myometrium tonicity (Vaala et al., 1992). In addition, it has been suggested that a decrease of progesterone production by the injured placenta and local release of prostaglandins could also be one of the mechanisms involved in EAV infection abortion (Coignoul and Cheville, 1984).

Fetuses aborted following natural or experimental infection with EAV frequently do not show any gross or histopathological lesions (Doll et al., 1957b; Doll, Bryans, and Knappenberger, 1957; Coignoul and Cheville, 1984; McCollum and Timoney, 1984). Fetuses are usually partially autolyzed at the time of expulsion (Doll et al., 1957b; Doll, Bryans, and Knappenberger, 1957). The placenta typically is grossly unremarkable and expelled with the fetus, however placentitis characterized by vasculitis has been

described during an outbreak in Thoroughbred horses that occurred in Oklahoma in 1987 (Johnson et al., 1991).

1.2.6. Immune response of horses to equine arteritis virus infection

The immune response to viral infection comprises innate and adaptive defenses. The innate immune response (native or natural immunity) is the first line of defense of the organism and acts very quickly, within minutes to hours after viral infection. It is constantly present and thus, previous exposure to the virus is not required to activate this mechanism. The innate defense has an important role in the detection of invading pathogens and in limiting their spread; it also plays an important role in activation of the adaptive immune response (Racaniello, 2009; MacLachlan and Dubovi, 2011).

In contrast, the adaptive immune response (acquired immunity) is the second line of defense and is responsible for total clearance of pathogens. While the innate immunity is activated immediately, the adaptive immunity takes longer to develop. A key feature of the adaptive immune system is memory. Repeat infections by the same virus are met immediately with a strong and specific response that usually effectively stops the infection with less reliance on the innate system. Immunological memory confers long-term protection against associated disease. Adaptive immunity involves cellular and antibody (humoral) effector mechanisms, mediated respectively by T- and B-lymphocytes (Racaniello, 2009; MacLachlan and Dubovi, 2011).

A. Innate immune response

The primary entry of EAV following natural infection of horses is either the respiratory or reproductive tracts (Timoney and McCollum, 1993a). Thus, the initial defense following EAV respiratory or venereal infection of horses is provided by the innate immune system of the mucosa lining the respiratory and genital tracts, respectively (Balasuriya and MacLachlan, 2004). The innate immune response of horses to EAV infection is not well elucidated, and so, it is assumed to be similar to what occurs during viral infection of other species (Balasuriya and MacLachlan, 2004; MacLachlan et al., 2008).

In response to viral infection, virus-infected cells in the mucosa, infiltrating macrophages, and natural killer (NK) cells, release various antiviral proinflammatory cytokines such as interferons type I INF-β, type I INF-α, and type II INF-γ, respectively, as well as tumor necrosis factor (TNF-α) and interleukins (such as IL-12). These cytokines confer antiviral resistance (INF-α and β) and initiate the adaptive immune response (INF-γ) (Balasuriya and MacLachlan, 2004; MacLachlan et al., 2008). However, it has been observed that EAV (Go et al., 2012 unpublished data) and porcine reproductive and respiratory syndrome virus tend to inhibit Type I Interferon production (Wang and Christopher-Hennings, 2012).

The role of innate immunity in the protective response of horses to EAV infection has not yet been elucidated. In a recent *in vitro* study it was shown that cytokine mediators (IL-1b, IL-6, IL-8, and TNF-α) are produced by EAV infected equine cells, such as endothelial (EC), alveolar machrophages, and blood-derived machrophages. This study showed that those EAV-induced, machrophage-derived cytokines are capable of

inducing E-selectin (recruitment of leukocytes to the site of injury) expression by equine ECs, and thus, suggested that these cytokines contribute to the characteristic vascular injury observed during severe EAV infection. The EAV virulent strains produced higher amount of cytokines compared to the EAV avirulent strains (Moore et al., 2003).

B. Humoral immune response

The humoral immune response to EAV is characterized by the development of complement-fixing (CF) and virus-specific neutralizing (VN) antibodies (Timoney and McCollum, 1993a). Complement-fixing antibodies develop 1 to 2 weeks after EAV infection of adult horses, peak after 2 to 3 weeks, and decline to disappear by 8 months. Neutralizing antibodies are also detected within 1 to 2 weeks after exposure, however peak at 2 to 4 months, and persist for years (Timoney and McCollum, 1993a; Balasuriya and MacLachlan, 2004; Summers-Lawyer et al., 2011). Several studies on the mechanism of EAV neutralization have shown that EAV neutralizing activity is complement dependent (Radwan and Burger, 1973a, 1973b; Radwan et al., 1973; Fukunaga and Imagawa, 1993). EAV neutralization is believed to occur in two steps: (1) irreversible binding between EAV and specific antibody to produce an infectious virus-antibody complex (sensitized virus), and (2) inactivation of this complex by either complement or anti-IgG (Radwan and Burger, 1973a). It has been shown that a decrease in EAV neutralizing activity of horse, hamster, guinea pig, and rabbit antisera following heat inactivation could be restored after addition of fresh complement (Radwan and Burger, 1973b). In addition to these finding homologous complement was more efficient in the neutralization of sensitized virus when compared with heterologous complement

(Radwan et al., 1973). Later, other authors determined that the use of 10% guinea pig complement in viral suspensions was optimal for enhancement of serum neutralization antibody response in serum neutralization tests in the diagnosis of EVA (Fukunaga and Imagawa, 1993). The same authors conclude that the requirement of complement for efficient virus neutralization is dependent on the virulence of the strain. Complement dependent EAV neutralizing activity is associated with the IgG fraction of the sera, but not with the IgM fraction (Radwan and Burger, 1973b; Fukunaga and Imagawa, 1993).

A long-lived virus-neutralizing antibody response is induced following viral infection or vaccination of horses with the modified live attenuated strains or inactivated virus vaccines and is protective against clinical disease (Doll et al., 1957b; McCollum, 1970; Fukunaga et al., 1992; Timoney and McCollum, 1993a; Balasuriya and MacLachlan, 2004), but not always against re-infection with the virus (McCollum, 1969). Appearance of neutralizing antibodies in serum (6-9 days post infection) coincides with the disappearance of virus from the circulation of infected horses (McCollum et al., 1971; Balasuriya, 2012). However, in chronically infected stallions, the virus persists in the reproductive tract despite the presence of high neutralizing antibody titers in their serum (Neu and Timoney, 1988; Timoney et al., 1987, 1986).

Foals born to immune (seropositive) mares acquire EAV-specific antibodies following ingestion of colostrum and are protected against clinical EVA following intranasal inoculation with virulent EAV at 6 days of age. Neutralizing antibodies appear in the blood serum of the newborn a few hours after passive transfer, peak at 1 week of age, and the levels decrease gradually to extinction between 2 and 6 months of age (McCollum, 1976; Hullinger et al., 1998). Virus neutralizing antibodies are not detected

in foals' sera prior to nursing, and thus, passive immunity is believed to not occur through the placenta (McCollum, 1976). Foals that receive colostrum should be vaccinated only after the maternal acquired antibodies disappear because these antibodies in their serum are capable of neutralizing the vaccine virus (McCollum, 1976).

The humoral immune response to structural EAV protein is variable and depends on the EAV strain the animals were infected with, the interval after infection when sera are collected, the individual horses responses, and the serological assay used (Hedges et al., 1998; MacLachlan et al., 1998; Nugent et al., 2000). The humoral immune response to EAV is mainly directed to the major envelope proteins GP5, M, and to the N protein (Chirnside, et al., 1995; Kheyar et al., 1997; Hedges et al., 1998; MacLachlan et al., 1998). The conserved C-terminal domain of the M protein contains linear epitopes, which are recognized by all EAV-specific horse anti-sera (Jeronimo and Archambault, 2002). The M protein was the most consistently recognized by convalescent sera from EAV-infected horses, other than carrier stallions, while response to the N and GP5 protein was variable (Hedges et al., 1998; MacLachlan et al., 1998). However, resistance to reinfection is assumed to be mediated by neutralizing antibodies directed against the GP5 protein of the major envelope proteins of the virus (Balasuriya et al., 1993, 1995, 1999).

Studies on the characterization of EAV antigenicity have determined that neutralization determinants of EAV are located on the GP5 (G_L) protein (Balasuriya et al., 1993, 1995; Deregt et al., 1994). Balasuriya et al. (1993, 1997, 2004) produced a large panel of neutralizing murine monoclonal antibodies (Mabs) to EAV and all bound to a GP5 major envelope glycoprotein indicating that it expresses the epitopes responsible for the neutralization of EAV. The authors showed that while the Mabs recognized

different epitopes, they are all located in the GP5 protein. The neutralizing domain within the GP5 protein is located between aa residues 75 and 104 (Glaser, de Vries, and Dubovi, 1995). Balasuriya *et al.* (Balasuriya et al., 1997) characterized at least four potential neutralization sites (site A [aa 49], site B [aa 61], site C [aa 67 to 90], and site D [aa 99 to 106]). With the exception of site A, these sites were all located in the V1 variable region (aa 61-121) within the second half of the N-terminal hydrophilic ectodomain of the GP5 protein. Studies using Mabs and reverse genetics have shown that substitution of individual amino acids within the GP5 ectodomain resulted in differences in neutralization phenotype of the recombinant viruses, analogous to differences in the neutralization phenotype of field strains of EAV and variants generated during persistent infection of EAV carrier stallions (Balasuriya et al., 1997, 2004). It has been demonstrated that heterodimerization of GP5 with M proteins is critical for the post-translational modification (glycosylation) and conformational maturation of neutralization determinants in the GP5 protein to form EAV epitopes that induce neutralizing antibodies in mice, and horses (Balasuriya et al., 2004; Zhang et al, 2008).

Characterization of the humoral immune response to EAV has been mainly focused on the structural proteins of the virus. Recently, Go *et al.* (2011) studied the equine humoral immune response to the nonstructural proteins (nsp) of EAV. The nsp are essential to EAV replication and are the first viral proteins to be synthesized in cells infected with the virus (Go et al., 2011). Data from this study showed that the equine humoral immune response to EAV nsps is directed against nsp2, nsp4, nsp5, and nsp12. None of the horse sera recognized nsp1, nsp3, and nsp6 through nsp11. Sera from horses vaccinated with the modified live virus (MLV) vaccine recognized the nsp2 and nsp12,

but not the nsp4 and nsp5. Therefore, nsp2 and nsp12 were suggested as a possible target for developing a serological diagnostic assay to differentiate seropositive vaccinated horses from naturally infected horses.

C. Cell mediated immune response

Cell-mediated immunity (CMI) involves, rather than antibodies, the action of the white blood immune cells (leukocytes) in response to an antigen; among these cells, lymphocytes play a crucial role in mediating cellular immune response (MacLachlan and Dubovi, 2011). The CMI response is well characterized in other arteriviruses such as PRRSV, and lactate dehydrogenase elevating virus (LDV) (Bautista and Molitor, 1997; Balasuriya and MacLachlan, 2004). However, little is known about CMI response to EAV. EAV-specific cytotoxic T-lymphocytes (CTL) were described using peripheral blood mononuclear cells (PBMCs) from convalescent ponies experimentally infected with EAV. The cytotoxicity induced by EAV-stimulated PBMCs was virus-specific and mediated by $CD8^+$ T cells; EAV-specific CTL precursors persisted for at least 1 year after infection in ponies (Castillo-Olivares, 2003). Other than this study, there are no comprehensive studies that describe the CMI response to EAV.

1.2.7. Diagnosis of equine viral arteriris

Diagnosis of EVA cannot be based solely on the clinical signs of the disease since a number of other diseases of the horse resemble equine viral arteritis (Timoney and McCollum, 1993a). These include other respiratory tract viral infections such as equine herpes virus (EHV-1 and EHV-4), equine influenza virus, equine infectious anemia,

equine rhinitis A and B viruses, equine adenovirus and other conditions which include toxicosis caused by hoary alyssum (*Berteroa* incana), and purpura hemorrhagica (Timoney and McCollum, 1993a; Balasuriya, 2012). Other two differential diagnosis of EVA are African Horse Sickness and Getah virus infection, which do not commonly occur in the Unites States (Timoney and McCollum, 1993a).

In the case of abortion, differential diagnosis of EVA include EHV-1 and rarely, EHV-4. However, abortion due to herpesvirus can frequently be distinguished from abortion caused by EAV, due the fact that in herpes viral infection, the fetuses are expelled fresh and characteristic gross lesions can frequently be observed; in contrast, EAV-infected fetuses are usually autolysed and pathognomonic lesions are rarely detected (Timoney and McCollum, 1993a; Balasuriya, 2012). Based on the difficulty of diagnosing EVA clinically, laboratory diagnosis of the disease currently relies on the combination of virus isolation and immunohistochemistry, viral nucleic or antigen detection (molecular diagnostics), and demonstration of virus specific antibody response (serologic) (OIE, 2004; Balasuriya, 2012).

A. Virus isolation and Immunohistochemistry

Virus isolation (VI) is the current test approved by the World Organization for Animal Health (OIE) for the detection of EAV in clinical specimens and from the semen of carrier stallions, and is the prescribed test for international trade (OIE, 2004). Propagation of EAV in cell culture can be done using a variety of continuous cell lines, such as baby hamster kidney (BHK-21), African green monkey kidney (Vero and BSC-1), rhesus monkey kidney (LLC-MK2; ATCC CCL-7), equine dermal cells (NBL-6;

CCL57), rabbit kidney (RK-13; ATCC CCL-37 and LLC-RK1), equine ovary cells (EO), hamster lung (HmLu), canine hepatitis virus-transformed hamster tumor cells (HS and HT-7), equine macrophages, and pulmonary equine endothelial cells (EEC) (Wilson et al., 1962; Hyllseth, 1969; Breese and McCollum, 1970; Radwan and Burger, 1973b; Konishi et al., 1975; Shinagawa et al., 1976; Glaser, de Vries, and Dubovi, 1995; Moore et al., 2002, 2003). However, high passage RK-13 cells (passage 399-409; KY RK-13 cells) are more sensitive than the low passage RK-13 (passage 194-204) and other cells lines for EAV infection and therefore, the best choice for primary isolation of virus from clinical specimens (OIE, 2004). The cytopathic effect (CPE) and virus titer can vary significantly among cell lines. The CPE in EAV infected cells is usually characterized by rounding, vacuolation, increased optical density, refractivity and detachment from culture vessels (McCollum et al., 1961; McCollum et al., 1962; Konishi et al., 1975).

The most appropriate specimens for virus isolation from live animals are nasopharyngeal and conjuctival swabs, and citrated or EDTA blood samples for separation of buffy coat cells (Timoney and McCollum, 1993a; OIE, 2004; Balasuriya, 2012). Heparin was shown to inhibit EAV in cell culture and so heparinized blood is not suitable for virus isolation (Asagoe et al., 1997). In the case of carrier stallions, the gel free sperm-rich fraction of the ejaculate, with which EAV is associated (Timoney et al., 1987), is optimal for virus isolation (Timoney and McCollum, 1993a; OIE, 2004; Balasuriya, 2012). To confirm EAV-induced abortion, placenta, fetal fluids, lung, spleen, and lymphoid tissues should be collected for virus isolation (Timoney and McCollum, 1993a; OIE, 2004; Balasuriya, 2012). Organs and lymph nodes associated with the alimentary and respiratory tracts should be collected for virus isolation in suspected cases

of “pneumoenteric” forms of EVA in young foals (Timoney and McCollum, 1993a; Balasuriya, 2012).

Immunoperoxidase histochemistry of skin biopsies is an auxiliary, reliable, and rapid assay for the clinical diagnosis of EVA in horses, especially when a cutaneous macropapular rash is evident or occasionally in skin biopsies (Lopez and Piero, 1996; MacLachlan et al., 1996; Del Piero et al., 1997). The identity of the isolates of EAV should be confirmed by RT-PCR, immunofluorescence or immunoperoxidase staining, or by microneutralization assay with EAV-specific antiserum (polyclonal) or monoclonal antibodies (OIE, 2004; Balasuriya, 2012).

B. Serological diagnostics

A variety of serological tests, including virus neutralization (VN), complement fixation (CF), indirect fluorescent antibody, agar gel immunodiffusion, the enzyme-linked immunosorbent assay (ELISA), and the fluorescent microsphere immunoassay assay (MIA) have been used for the detection of antibodies to EAV (OIE, 2004).

The virus neutralization test (VNT) is considered the “gold standard” for detection and determination of antibodies to EAV (Senne et al., 1985) and it is described in the OIE manual (OIE, 2004). Currently, the VNT is the only standardized test recognized by the OIE and it is the only validated test accepted for international trade. For diagnosis of acute EAV infection by the VNT, acute and convalescent sera should be collected at a 21- to 28-day interval, and a fourfold or greater increase in serum antibody titer is a confirmatory of recent infection (OIE, 2004; Balasuriya, 2012). Long-term carrier stallions usually have high serum neutralizing antibody titers despite the presence

of virus in their semen (Timoney, McCollum, Roberts, et al., 1986; Timoney et al., 1987; Neu and Timoney, 1988; Little et al., 1991). Although the VNT is very sensitive and highly specific, it has several disadvantages: it is expensive, labor-intensive and time-consuming to perform. In addition, the cytotoxicity caused by antibodies directed to the RK-13 cells can be confounded with viral CPE and so can be hard to interpret (OIE, 2004). Furthermore, the VNT test cannot discriminate between the serum antibody response of vaccinated from naturally infected horses (Balasuriya and MacLachlan, 2004).

In an attempt to overcome these disadvantages several enzyme-linked immunoassays (ELISA) have been developed to detect antibodies to EAV using whole virus, synthetic peptides, or recombinant viral proteins (GP5, M, N) expressed from bacterial or baculovirus systems, as antigens (Chirnside, Francis, and Mumford, 1995; Chirnside, Francis, de Vries, et al., 1995; Hedges et al., 1998; Kondo et al., 1998; Cho et al., 2000; Nugent et al., 2000; Starik et al., 2001; Wagner et al., 2003; Duthie et al., 2008). Recently, a MIA has been developed and shown to have several advantages over traditional ELISAs: accuracy, high sensitivity and specificity, reproducibility, high-throughput sample analysis and multiplex capability (Go et al., 2008). None of the existent ELISAs or described MIA offers equivalent specificity and sensitivity enough to replace the current VNT (OIE, 2004). The CF test, although of lower specificity and sensitivity than ELISA and VNT, can be used in the diagnosis of recent infection (OIE, 2004).

C. Molecular diagnosis

Molecular diagnostics have several potential advantages over the current virus isolation procedure approved by the OIE for the detection of EAV. They are often faster and less expensive (Balasuriya et al., 2002). Several standard RT-PCR (Chimside and Spaan, 1990; St-Laurent et al., 1994; Ramina et al., 1999; Westcott et al., 2003), RT-nested PCR (RT-nPCR) (Gilbert et al., 1997) and real-time RT-PCR (rRT-PCR) assays (Balasuriya et al., 2002; Lu et al., 2008; Miszczak et al., 2011) have been developed for the detection of EAV nucleic acid in tissue culture fluid, nasal secretions and semen. These assays target different genes (ORFs 1b, 3, 4, 5, 6, and 7) and have different sensitivities and specificities. Gilbert *et al*. (1997) were very successful in increasing the sensitivity and specificity of an RT-PCR based assay, when using a RT-nPCR that incorporates two primer pairs specific for ORF1b. Miszczak *et al.* (Miszczak et al., 2011) optimized a rRT-PCR assay developed by Balasuriya *et al*. (2002), which provided sensitivity equal to or slightly higher than that of virus isolation for the detection of equine arteritis virus in semen. Three different one-step real-time RT-PCR reagents in combination with two different magnetic bead-based RNA extraction methods were used to test 409 semen samples. This assay may be a faster, convenient and more economical alternative to VI, for detection of EAV nucleic acid in TCF and equine semen. In addition, the standardization of molecular diagnostics assays between laboratories may be easier in comparison with the standardization of the traditional diagnostics methods due to automation and use of commercial reagents kits that provide less variability to the assay (Miszczak et al., 2011).

1.2.8. Prevention and treatment to equine arteritis virus

As already discussed above, horses naturally or experimentally infected with virulent or avirulent EAV strains develop a long-lasting immunity against clinical disease (Doll et al., 1957b; McCollum, 1969; Fukunaga et al., 1992; Timoney and McCollum, 1993a; Balasuriya and MacLachlan, 2004). Therefore, vaccination is a logical and practical strategy for the control of EVA (Timoney and McCollum, 1993a; MacLachlan et al., 2008). Several studies were performed with the goal of developing a safe modified live virus (MLV) vaccine using the Bucyrus strain of EAV (Doll et al, 1968; McCollum, 1969, 1981, 1986; Timoney et al., 1988). In 1961, EAV was found to replicate in primary cell cultures of horse kidney (HK) (McCollum et al., 1961) and subsequently in other cell cultures (McCollum et al., 1962; Wilson et al., 1962). It was noted that viral virulence was reduced by continuous passage in cell culture but its immunogenicity was retained. Multiple passages of the highly virulent horse-adapted Bucyrus strain of EAV was performed in horse kidney (HK), rabbit kidney (RK) and equine dermal (ED) cells and viral attenuation was obtained (Doll et al., 1968; McCollum, 1969, 1986). This attenuated strain of virus did not show any reversion to virulence after five consecutive passages in horses (McCollum, 1969). The commercial MLV vaccine, ARVAC® was developed by further passage of the VB strain in cell culture (passage history HK-131, RK-111 and ED-24), and like the strains of earlier passage history, also shown to provide protection against the disease (Doll et al., 1968; McCollum, 1969, 1986; Timoney and McCollum, 1988; Fukunaka et al., 1982). The MLV vaccine induces the production of VN antibodies within 5 to 8 days post-vaccination (Timoney and McCollum, 1988). A single dose of the MLV vaccine was shown to stimulate only transient and low levels of VN antibodies and

thus, additional doses were necessary to boost antibody titers to levels able to be maintained for at least 9 to 12 months (Fukunaga et al., 1982; McKinnon et al., 1986; Timoney and McCollum, 1988). The MLV vaccine, ARVAC® is currently in use in North America, while the inactivated adjuvant virus vaccine, ARTERVAC®, is approved for use in certain countries in Europe (Balasuriya and MacLachlan, 2004; MacLachlan et al., 2008). The adjuvant-containing, inactivated EAV vaccine is also administered intramuscularly, and a booster immunization is recommended after 3 to 4 weeks and annually thereafter. This vaccine induces the production of high neutralizing antibody titers against EAV but its role in preventing persistent infection is less characterized than that of the MLV vaccine (MacLachlan et al., 2008).

The MLV vaccine have shown to be safe for use in mares and to provide protection against clinical signs of the disease, however, a transient mild fever response and a mild and transient lymphopenia were observed in a small number of animals (Doll et al., 1968; McCollum, 1969, 1986; Timoney et al., 1988). The MLV vaccine virus had been isolated from the nasopharynx, usually for up to approximately 7 days from a few of the vaccinated animals (Harry and McCollum, 1981; Fukunaga et al., 1982). Vaccinated mares inseminated with EAV-infective semen did not develop clinical disease, but virus was isolated for short period of times from the nasopharynx of these mares. Furthermore, a seronegative contact became infected in the same study (McCollum et al., 1988). Experimental vaccination studies have been also performed in pregnant mares (Doll et al., 1968; McCollum, 1969, 1981; Broaddus, Balasuriya, White, et al., 2011) and stallions (McKinnon et al., 1986; Timoney and McCollum, 1988), to ensure the safety of the vaccine for these categories of animals. Similarly to mares, the use of the MLV was

proved to be safe in stallions and protective against clinical signs of the disease, with the only clinical response observed being a mild fever of short duration and a slight leukopenia in some animals (Timoney and McCollum, 1988). In this study, horizontal transmission of vaccine virus from vaccinated to susceptible in-contact stallions was not demonstrated and in view of the very low titer of virus isolated from the nasopharyngeal secretions, was considered unlikely to occur. The vaccinated stallions did not shed virus in their semen or urine following vaccination with the MLV. In 2011, a study was undertaken to re-evaluate safety aspects of the commercial MLV vaccine against equine viral arteritis (ARVAC®) in stallions. Ten EAV seronegative stallions were vaccinated with one single dose of the vaccine and 1 stallion shed virus in the semen, on days 4 and 6 post-vaccination. Thus, first-time vaccinated stallions should not have their semen eligible for use for a minimum of 14 days to avoid the potential risk of virus transmission or having vaccine virus detected in semen frozen from a recently vaccinated stallion (Summers-Lawyer et al., 2011). Safety of the MLV was not proved for pregnant mares, especially in the last 2 months of pregnancy due the risk of abortion and because fetal infections with the MLV after vaccination of the pregnant mares have been documented (McCollum, 1969; Broaddus et al., 2011). Foals from immune dams will passively acquired antibodies against EAV from the colostrum and so, should be vaccinated after 6 months of age; however, vaccination can be done earlier than 6 months of age in the case of foals from non-immune dams or in high-risk situations (McCollum, 1976; Hullinger et al., 1998). Vaccination of pre-pubertal colts is central to control the spread of EAV infection, since vaccinated colts are resistant to the development of the persistently

infected carrier stallion after exposure to EAV (Timoney and McCollum, 1988; Balasuriya and MacLachlan, 2004).

Recently, a cDNA clone of the current MLV vaccine was generated. This infectious cDNA clone is identical to the wild type virus with the exception of a unique nucleotide mutation in ORF7 to distinguish recombinant vaccine virus from laboratory and field strains of EAV (Zhang et al., 2012). The safety and efficacy of this vaccine was evaluated following intranasally inoculation of the horses with the heterologous KY84 strain of EAV. The horses vaccinated with the recombinant MLV (rMLV) developed high neutralizing antibody titers against EAV similarly to studies with the MLV vaccine (Timoney et al., 1988; Summers-Lawyer et al., 2011), the animals shed reduced viral titers for a short period of time in nasal secretions and in the PBMCs within the first week of immunization. However, the level of protection provided by the rMLV vaccine did not completely fully protect the horses from developing clinical signs of EVA, even though these were of less severity. A formalin-inactivated virus vaccine without adjuvant was also shown to induce high titers of VN antibodies after repeated doses (Fukunaga et al, 1990, 1992).

Current serological tests cannot discriminate between naturally infected and vaccinated horses. Therefore, an effort to develop vaccines that make such discrimination and also that lack replication of the virus in the bodily fluids has been attempted. Examples include a GP5-specific oligopeptide and a bacterial fusion protein, both containing a key neutralizing domain of GP5 protein; a Venezuelan equine encephalitis virus replicon particle-based vaccine that co-expresses GP5 and M protein; a live marker vaccine that lack the major neutralization domain in GP5; and, a DNA vaccine expressing

ORF2b, -5, and -7 (Chirnside et al., 1995; Balasuriya et al., 2002; Castillo-Olivares et al., 2003; MacLachlan et al., 2008). The rMLV vaccine mentioned above, for instance, also has the advantage of providing a means of serologically differentiating EAV naturally infected from vaccinated animals.

As with other viral disease, there is no specific treatment for horses infected with EAV. There is currently no effective treatment to permanently eliminate virus from the semen of carrier stallions, other than castration. Suppression or inactivation of GnRH is responsible for decreasing the production and release of pituitary gonadotropins (LH and FSH) and consequently reduces the release of steroids, including testosterone. EAV is believed to be testosterone dependent (Little et al., 1992), and thus, some authors were able to temporarily remove the virus from the semen of persistently infected stallions with the use of a GnRH antagonist (Fortier et al., 2002) and a GnRH vaccine (Improvac® or Equity®, Pfizer Animal Health, Australia) (Burger et al., 2006). However, the treatment had the disadvantage of causing a transient decrease of circulating testosterone concentrations and quality of semen produced. Miszczak et al. (2012) also evaluated the efficacy of the Equity® anti-GnRH vaccine in persistently infected stallions by investigating clearance of the virus, systemic testosterone levels, and subsequent recover of fertility. This study was the first to use a considerable number of stallions (n=16) and to demonstrate the correlation between plasma testosterone and EAV viral load in semen. Plasma testosterone levels decreased during the first two months after vaccination and was followed by a decrease in virus load until 7-8 months and EAV clearance. Most of stallions cleared virus from semen between 3 and 10 months after treatment, and after 22 months EAV clearance was observed in all stallions. However, decreases in libido and

semen quality were reported after GnRH vaccination and more research in this area needs to be performed.

Potential antivirals against EAV, such as the phosphorodiamidate morpholino oligomer (PMO) have been tested *in vitro* (van den Born et al., 2005; Zhang et al., 2010). PMOs are single-stranded DNA analogues, usually 20-25 bases in length, water-soluble and nuclease-resistant that can base pair with a complementary RNA target sequence and form a steric blockade to interfere with gene expression, particularly with translation of the genome of EAV (Summerton, 1999). Peptide-conjugated PMO (PPMO) has been shown to be delivered more efficiently into cells and thus it was used for target of the 5' untranslated region (UTR) of the EAV genome (van den Born et al., 2005). Treatment with an antisense PPMO targeting the EAV 5'-terminus eliminated the virus from persistently infected HeLa cells (Zhang et al., 2010). Furthermore, horse cells transfected with small interfering RNA (siRNAs) targeting the ORF1ab prior to infection with EAV drastically reduced virus titers in cell culture (Heinrich et al., 2009).

II. THE STALLION REPRODUCTION

1.3. ANATOMY AND PHYSIOLOGY OF THE REPRODUCTIVE TRACT

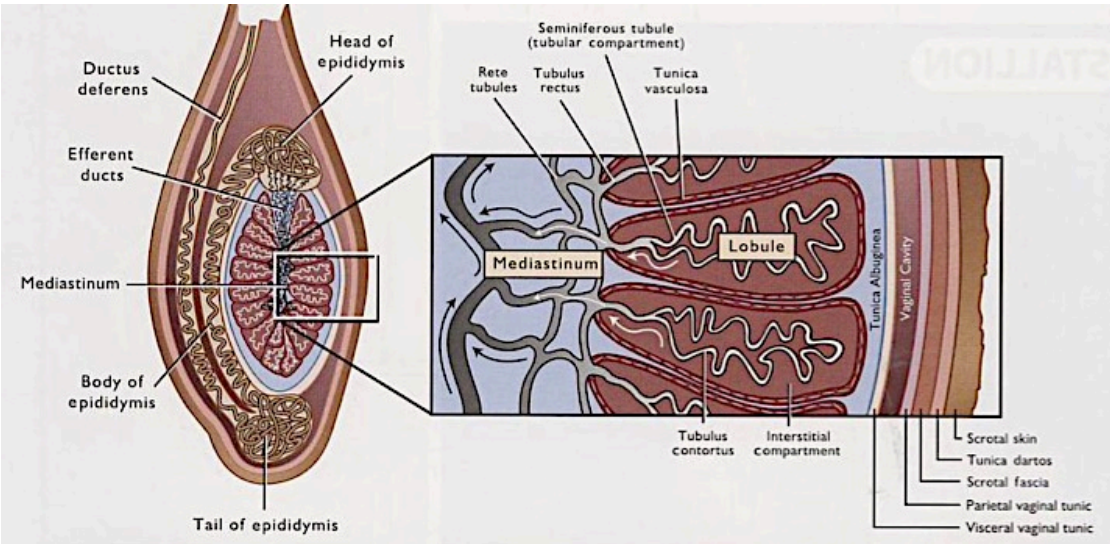
The reproductive system of the stallion encompasses the scrotum, testes, spermatic cord, excurrent duct system (efferent ducts, epididymides and deferent ducts), accessory sex glands, urethra and penis (Senger, 2003). The anatomical development and maturation of these tissues are essential for normal function of the reproductive tract and attainment of reproductive capability is dependent on each (Little and Holyoak, 1992).

Scrotum

The scrotum contains the two testes, epididymides, spermatic cords, and cremaster muscles. The scrotum plays a role in thermoregulation, support and protection of the testes (Little and Holyoak, 1992). It is a pouch of skin composed of two scrotal sacs located outside the body cavity into which the testes descend. The sacs lie on each side of the penis. The scrotum wall consists of 4 layers (Figure 1.3.1.), from the outermost to the innermost, they are: (1) skin: contains a large number of sweat glands and thermo sensitive nerves required for maintenance of adequate temperature; (2) tunica dartos: composed of smooth muscles fibers with connective tissue in between. It contracts, raising (during cold temperatures) or lowering the testis (during hot months), in response to changes in scrotal skin temperature. Development and maintenance of contractility of the tunica dartos is under androgen control; (3) loose connective tissue or scrotal fascia: allows for mobility of the testis vertically or horizontally, but normally prevents an 180^0 rotation, and; (4) parietal vaginal tunic (Senger, 2003; Amann, 2011b). This innermost

layer is closely apposed to the visceral vaginal tunic of the testis, but separated by a space termed the vaginal cavity. A thin layer of fluid lines the vaginal cavity to permit the testis to slide smoothly within the scrotum (Little and Holyoak, 1992).

Figure 1.3.1. Schematic illustration of the scrotal layers and the tubular pathway of the typical mammalian testis (Senger, 2003).



Testes

The testis is the male gonad and its primary functions are the production and secretion of spermatozoa and testosterone, the predominant male sex hormone. In addition, the testis produces inhibin, estrogens and a variety of proteins believed to be important to sperm function (Amann, 2011a). The testes have their long axis almost horizontal to the body, but when retracted (during cold temperatures) they assume a more vertical position, so the cauda of the epididymidis becomes ventrally rather than caudally located (Senger, 2003). Two connective tissue layers cover the testes: the tunica

albuginea and the visceral vaginal tunic, which together form what is called the testicular capsule. Supporting strands of connective tissue extends from the tunica albuginea closely associating the tunica with the testis parenchyma and dividing the testis into lobules (Figure 1.3.1.) (Senger, 2003; Chenier, 2007).

A cross section of the testis reveals the parenchyma, which consists of the seminiferous tubules (where spermatogenesis takes place) and an interstitial tissue made of Leydig cells that produce testosterone, and also of blood vessels, connective tissue, lymphatics and nerves (Senger, 2003).

i. Semineferous tubules and Sertolli cells

The seminiferous tubules are lined up by an epithelium that consists of developing germ cells (spermatogonia, spermatocytes and spermatids) and the somatic Sertolli cells. They are limited by a lamina propria made up of fibroblast, myoid somatic cells and laminin. The myoid cells are capable of rhythm contractions allowing for movement of spermatozoa and fluid from the seminiferous tubules towards the epididymis. The ends of the seminiferous tubules join to the rete tubules, which are housed by the mediastinum (the central connective core of the testis) (Figure 1.3.1.). The rete tubules terminate when they fuse with one of the efferent ducts that leads to the epididymal duct (Amann, 2011b). Although seminiferous tubules diameter, length and volume increase with age (Johnson and Neaves, 1981), the proportion of the testis they occupy (about 70%) is constant across the years (Johnson and Varner, 1991; Little and Holyoak, 1992).

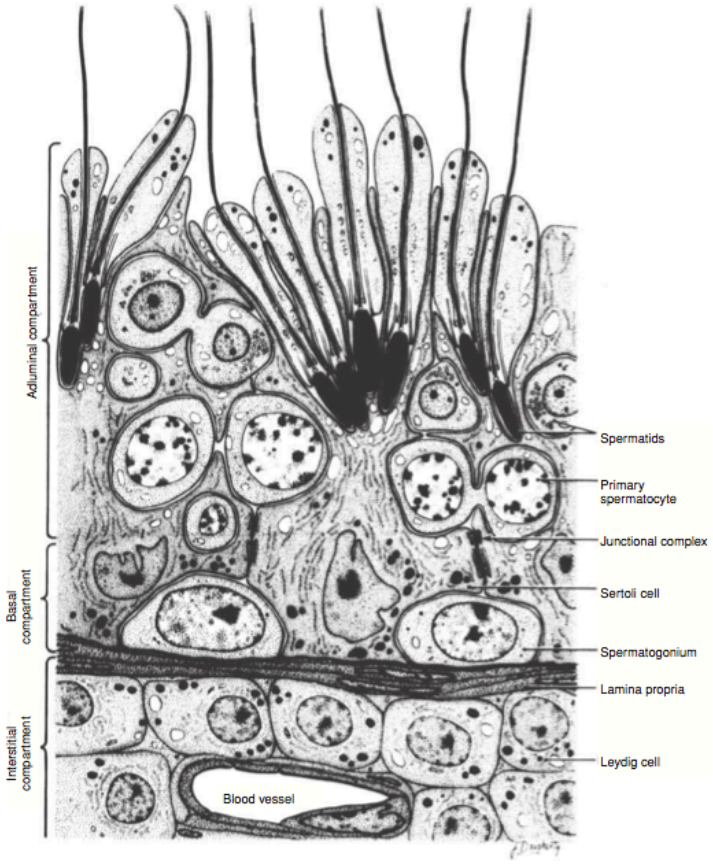
Sertolli cells are the somatic component of the seminiferous tubules and their role in spermatogenesis is not well elucidated (Johnson et al., 1997). The probable functions

of the Sertolli cells are: (1) to provide structural support to the developing germ cells and nutrients for their growth; (2) movement of developing germinal cells within the seminiferous epithelium; (3) release of mature spermatids in a process called spermiation; (4) removal of the waste by phagocytizing degenerating germ cells and residual bodies at the end of spermiation; (5) cell-to-cell communication with the developing germ cells, the myoid cell layer, and Leydig cells; (6) secretion of fluids, proteins and hormones, that have a role in spermatogenesis as well in the transport of the spermatozoa through the seminiferous tubules to the rete testis; and, (7) formation of the blood-testis barrier (Johnson et al., 2008). Adjacent Sertolli cells are tightly attached by junctional complexes, which form the major component of the blood-testis barrier and separate the seminal epithelium into two functional compartments: basal (houses spermatogonia and early primary spermatocytes) and adluminal (houses primary and secondary spermatocytes and spermatids) (Amann, 2011a) (Figure 1.3.2.). The blood-testis barrier prevents cells from the immune system to gain access to the adluminal compartment and destroy developing germ cells. Given that, spermatocytes and spermatids, which first appear at puberty and not prior to the development of the immune system, are protected and will not be recognized by the immune system as foreign cells. The immune system barrier continues within the epididymal ducts that transport and store spermatozoa (Johnson et al., 2008).

Some of the hormones produced by Sertolli cells are: androgen binding protein (ABP), transferrin, inhibin, anti-Mullerian hormone, activins and estradiol. The function of Sertolli cells is dependent on follicle-stimulating hormone (FSH) and testosterone (Amann, 2011a).

The young animal has a low number of Sertolli cells, which increases with age and stabilizes during sexual maturation until senescence, declining with advanced age (13-20 years old) (Johnson et al., 1997). The number of Sertoli cells in adult stallions varies according to the season of the year, being higher during the breeding season than in the non-breeding season (Johnson and Nguyen, 1986; Johnson and Varner, 1991). Even though stallions are seasonal breeders, they can produce spermatozoa through out the year. However, maximum testicular development and function occurs, in the Northern Hemisphere, during May, June and July (breeding season). The number of Sertolli cells in the testis is important to determine the production of spermatozoa. Higher the number of Sertolli cells, more spermatozoa the testis can produce (Johnson and Neaves, 1981; Johnson and Varner, 1991). During the non-breeding season stallions testis contain 31% fewer Sertolli cells and produce 40-50% fewer spermatozoa (Amann, 2011b). There is a positive correlation between the number of Sertolli cells and number of germ cells, and consequently daily spermatozoa production per testis (Johnson and Carter, 1994).

Figure 1.3.2. A section of the stallions seminiferous tubule showing the relationship of the germ cells to the adjacent Sertoli cells (*Equine Reproduction, 2nd edition*).



ii. Interstitial tissue of the testis – Leydig cells

Leydig cells are the most abundant component of the interstitial tissue. The primary role of Leydig cells is supporting spermatogenesis by the production and secretion of testosterone (Johnson et al., 1997). Leydig cells also produce other steroids hormones as, progesterone and estradiol, which aid in regulating function of the seminiferous epithelium, the hypothalamic-hypophiseal axis, and the accessory sex glands (Amann, 2011a). Equine Leydig cells have a considerable amount of smooth endoplasmic reticulum (SER) where pregnenolone is converted into testosterone (Johnson et al., 1997).

As a stallion matures, the total number and volume of Leydig cells per gram of parenchyma as well the individual size of those cells increase. Leydig cells augment in number over the years of a stallion's life and the ratio of Leydig cells to seminiferous tubules increases from 1:12 (2-3 years old stallions) to 1:4 (13-20 years old stallions). With increasing of age, postpubertal Leydig cells are replaced by adult Leydig cells, which are believed to produce more testosterone (Johnson and Neaves, 1981).

The number of Leydig cells per testis also is influenced by the season of the year and a greater amount of these cells are found in the testis of a stallion in the breeding season compared to the non-breeding season. During the non-breeding season, total number of Leydig cells is decreased (about 35%) which explains the seasonal variation of testosterone. Size of individual Leydig cells, though, appears to be constant through out the year without suffering any variation of the season (Thompson, 1986). The seasonal differences in Leydig cell smooth endoplasmic reticulum (SER) volume per testis was correlated to seasonal changes in Leydig cell number, and the increase in intracellular testosterone content during the breeding season was correlated to seasonal changes in Leydig cell number and volume of SER per testis (Johnson and Thompson, 1987).

Although testosterone is the steroid of greatest physiological importance, Leydig cells produce greater amounts of estradiol than testosterone (Amann, 2011a).

Leydig cells have luteinizing hormone (LH) receptors and increase production of testosterone in response to LH as well to human chorionic gonadotropin (hCG) stimulation.

Spermatic cord

The spermatic cord extends from the inguinal ring (passageway from the body cavity into the scrotum) to the dorsal pole of the testis. It suspends the testis in the scrotum (Little and Holyoak, 1992). The spermatic cord houses the testicular vasculature, lymphatics and nerves, as well as the duct deferens, the testicular artery and veins (Amann, 2011b). The testicular veins form an elaborated network called pampiniform plexus. The pampiniform plexus surrounds the highly convoluted testicular artery eventually forming a single vein, and serves as a countercurrent heat exchange system. Heat is transferred from the warm (39^0C) arterial blood from the body to the cooler (33^0C) venous blood leaving the surface of the testis. This venous blood has been cooled by heat loops from the testicular veins through the skin of the scrotum. Disruption or modification of the cooling system can compromises or completely suppress spermatogenesis (Senger, 2003).

The cremaster is a striated muscle located on the lateral to the spermatic cord and helps in testicular support and control of testicular temperature raising or lowering the testis for short period of times (Amann, 2011b).

Thermoregulation of testicular temperature

Maintenance of low testicular temperature (3 to 5^0C below than normal core body temperature), in most mammals, is necessary in order for spermatogenesis to occur (Setchell, 1998; Brito, 2007; Hansen, 2009). When testicular temperature is elevated due to conditions such as fever, testicular trauma, inflammation, edema and others, the thermoregulatory mechanism necessary to cool the testis and allow for normal

spermatogenesis is disrupted (Johnson et al., 1997). Metabolism and oxygen demand increase at a greater rate than blood flow goes from the testicular artery to the testes, and testicular hypoxia occurs with consequent detrimental effects on sperm production and quality (Setchell, 1998; Brito, 2007). Thermal regulation of the testis and epididymis depends on the combined action of the scrotum (sweat glands, thermosensitive nerves and tunica dartos muscle), pampiniform plexus, and the external cremaster muscle (Little and Holyoak, 1992).

Elevated temperature of the testis to body temperature or higher, for as little as 2 hours, can affect and cause the death of germinal developing cells within the semineferous tubules (Freidman et al., 1991), specially pachytene primary spermatocytes, but also B-spermatogonia and spherical spermatids (Setchell, 1998; Brito, 2007). Previous studies assessing the effects of increased testicular temperature in different species such as, mice (Pérez-Crespo et al., 2008), rams (Rasooli et al., 2010), boars (Wettemann et al., 1979), bulls (Karabinus et al., 1997) and stallions (Freidman et al., 1991; Love and Kenney, 1999) have shown that transient, as well as, chronic exposure of the testis to elevated temperatures can disrupt spermatogenesis.

In stallions, scrotal insulation for 24 and 48 hours resulted in a transitory decrease in the number and quality of spermatozoa in the ejaculate, mostly between 10 and 40 days after temperature increase, and a recovery of seminal characteristics was observed between 50 and 75 days after cessation of elevated testicular temperature (Freidman et al., 1991). Prolonged exposure of the testis to elevated temperatures has been shown to affect quality and functionality of spermatozoa within the epididymis in some species, but has not been tested in stallions (Amann, 2011b).

Excurrent duct system

The excurrent duct system allows for final maturation, storage and delivery of spermatozoa to the pelvic urethra. It consists of the epididymis, efferent and deferent ducts (Senger, 2003).

i. Epididymis

The epididymis is responsible for controlling the fertility acquisition of spermatozoa (maturation) and also their exit from the male reproductive tract (Little and Holyoak, 1992). The epididymis is divided into three anatomical regions: the caput (head), corpus (body) and, cauda (tail) (Burns, 2007). Exposition of the epididymis to testosterone, especially the caput and corpus, is essential for secretion of certain proteins by the epididymal epithelium (Amann, 2011b).

Within the caput of the epididymis, efferent ducts converge into a single duct, the epididymal duct. The function of the efferent ducts is to convey new spermatozoa and rete fluid to the epididymal duct. Peristaltic contractions of a smooth muscular layer surrounding the epididymal duct are responsible for spermatozoa movement along its course. The duct continues through the caput, corpus and cauda epididymidis and is continuous with the deferent duct (Senger, 2003). In the stallion, spermatozoa are retained in the caput for approximately one day, where the rete fluid is reabsorbed and replaced by secretions of the epididymal epithelium. Because of this reabsorption of fluid, spermatozoa is much more concentrated in the tail than in the head of the epididymis. Time required for spermatozoa to travel from the caput to the corpus of the

epididymis is not altered by the frequency of the ejaculation, and averages about 4.1 days (Little and Holyoak, 1992; Amann, 2011a).

Motility and fertility of spermatozoa begin to be evident as spermatozoa move into the corpus where reside for approximately two days. Stallion spermatozoa are stored in the cauda of the epididymis from 7-10 days where, eventually, reach full motility and fertility, and can maintain the ability to fertilize an oocyte for several weeks (Little and Holyoak, 1992). Spermatozoa stored in the cauda epididymis are transported by smooth muscle contractions of the duct deferens to the ampulla, which leads to the pelvic urethra (Senger, 2003). Spermatozoa entering the caput have a proximal cytoplasmic droplet located near the base of its head. As the spermatozoa move along the epididymis this droplet is translocated down their tail and it is lost in the distal tail or during ejaculation. Retention of the droplet indicates faulty epididymal maturation (Varner and Johnson, 2011).

Spermatozoa are produced continuously regarding of ejaculation frequency (Senger, 2003).

ii. Deferent ducts

The deferent duct is a continuation of the epididymal duct. It extends from the cauda of the epididymis through the spermatic cord to the pelvic urethra. Because of its thick smooth muscular wall the proximal deferent duct can be palpated through the scrotal skin. As it approaches the pelvic urethra it widens into a structure called ampulla. The ampulla is an enlargement of the deferens ducts that opens directly into the pelvic urethra. The ampullar region is enlarged due to the thick wall associated with the

presence of crypts and glands, and also presents a slightly increase of its luminal diameter compared with the deferent ducts. The ampulla is considered by some authors to be an accessory sex gland (Amann, 2011b).

Accesory sex glands

The accessory sex glands include the vesicular glands, prostate, and bulbourethral glands (Amann, 2011b). The vesicular gland produces the gel portion of the ejaculate whereas the bulbourethral glands produce the pre-ejaculatory fluid (Little and Holyoak, 1992). The secretion of prostate gland is thin and watery and it probably helps cleanse the urethra during ejaculation and constitutes a major portion of seminal plasma, especially if a second ejaculation occurs 1-3 hours after the first (Amann, 2011b).

Secretions from the accessory glands (as well as some from the ampulla of the vas deferens) provide fluid for spermatozoa transport and constitute the seminal plasma, the non-cellular part of semen. Additionally, these secretions provide nutrition and buffers to alleviate hostile acidic conditions found in the female reproductive tract (Senger, 2003). The sex glands products are secreted into the lumen of the pelvic urethra. Seminal plasma is needed for delivery of the sperm (Amann, 2011a). It has an important role in the modulation of breeding-induced endometritis through suppressive effects on complement activation, polymorphonuclear leukocytes (PMNL) chemotaxis, and phagocytosis (Troedsson et al., 2000). Fractionation of seminal plasma proteins suggested that some proteins have a protective effect on viable spermatozoa, whereas others promote phagocytosis and binding of dead spermatozoa and PMNL (Troedsson et al., 2005). Recently, it was reported for the first time in the horse, that a seminal plasma protein,

CRISP3 suppress PMNL/sperm binding in a dose-dependent manner (Doty et al., 2011).

The accessory sex glands, as well the ampulla, are dependent on testosterone in peripheral blood for full development and maintenance of their structure and function (Amann, 2011a).

Urethra

The urethra is a long tube that serves as an excretory canal for urine and semen and that extends from the bladder to the free end of the penis. Its pelvic region is overlaid by a thick, striated muscle that contracts vigorously during ejaculation. An erectile tissue called corpus cavernous surrounds the penile urethra. The urethra terminates in a free extension: the urethral process (Little and Holyoak, 1992).

Penis

The penis is the male copulatory organ. The stallion penis contains a large amount of erectile tissue (corpus carvenosus) enclosed in a connective capsule, the tunica albuginea. In addition, it contains a small amount of an erectile tissue surrounding the urethra, the corpus spongiosum, which is not enclosed by the tunica albuginea. Thus, the stallions' penis is muculocarvenosus, and upon sexual stimulation the spongy tissue is filled with blood increasing both the length and diameter of the penis. On the distal end of the penis is the bell-shaped glans (Amann, 2011b). The glans region is highly innervated and is responsible for inducing the ejaculatory process, which happens directly into the uterus (Senger, 2003).

1.4. STALLION REPRODUCTIVE ENDOCRINOLOGY

As in other mammalian species, normal testicular function in the horse is dependent upon a functional pineal gland that produces and release melatonin and on a hypothalamic-pituitary-testicular (HPT) axis (Roser, 2008). The major HPT hormones involved in the control of the stallions' reproductive functions are: the hypoyhalamic gonadotropin-releasing hormone (GnRH); the hypophyseal luteinizing hormone (LH) and follicle stimulating hormone (FSH); and, the testicular hormones such as testosterone, estrogens and, inhibin (Gerlach and Aurich, 2000; Roser, 2008).

Melatonin and GnRH

Melatonin, a photoinducible hormone, is a seasonal regulator of reproductive function secreted by the pineal gland. When released, during the non-breeding season, melatonin suppresses production and release of GnRH by the hypothalamus. During the breeding season melatonin production is low and consequently GnRH is high promoting testicular function and sperm production (Gerlach and Aurich, 2000; Malpaux et al., 2001; Sharp, 2011). It was shown, in stallions, that exogenous melatonin decreased plasma testosterone concentrations (Argo et al., 1991).

The hypothalamus releases GnRH in a pulsatile manner and stimulates pulsatile secretion of the pituitary hormones, LH and FSH, which act at the level of the testis. Inhibin, estrogen and testosterone are secreted by the testis into the blood and act as classical feedback regulators of hypothalamic and pituitary output (Roser, 2001, 2008).

Luteinizing hormone

Luteinizing hormone is essential for male reproduction. Knockout male mice for the LH ligand are infertile and show significant decreases in testes size, accessory gland size, intra-testicular testosterone level and Leydig cell number (Kumar, 2005).

Luteinizing hormone binds to its receptor on the testis interstitial Leydig cells and stimulates the secretion of testosterone and estrogens (Eisenhauer and Roser, 1995; Gerlach and Aurich, 2000; Roser, 2008). In stallions, LH release has shown to be positively correlated with day length. Even though pituitary LH concentrations are constant throughout the year, basal plasma LH concentrations during the breeding season are as high as twofold as LH concentrations during the winter months. Geldings do not show change in plasma LH concentrations according to the time of the year, indicating that the seasonal changes in LH release in horses require the presence of the gonads, indicating a steroid-dependent effects of photoperiod (Gerlach and Aurich, 2000).

Follicle stimulating hormone

Follicle stimulating hormone binds to Sertoli cells, which stimulate production of estrogens, inhibin, activin, androgen-binding protein (ABP), transferrin, insulin-like growth factor-1 (IGF-1), and other factors needed for spermatogenesis (Roser, 2011).

Inhibin inhibits synthesis of FSH at the level of the pituitary in the mare. A paracrine–autocrine role of inhibin, has not been well established in the stallion testes. In the stallion, inhibin has been localized in Leydig and Sertolli cells by immunocytochemistry. In addition, it is believed to be important for testicular recrudescence because peripheral FSH and inhibin exhibit similar seasonal changes:

increase in the spring and decrease in the fall (Nagata and Tsunoda, 1998). Inhibin may act in conjunction with testosterone and estrogen to control FSH secretion (Roser, 2011).

Activin has been shown to positively modulate the release of pituitary FSH in other species, but its action in the stallion is unknown (Roser, 2011).

Testosterone

Testosterone is essential for maintaining and restoring spermatogenesis in adults, affecting sexual drive (libido), stimulating metabolism, initiating and sustaining male secondary sex characteristics, and supporting organs of the male reproductive tract (Senger, 2003). Testosterone acts by binding to its receptors on Leydig and Sertolli cells and exerts a negative feedback on the hypothalamus via the peripheral circulation and inhibits synthesis and secretion of GnRH, but has no action on LH release at the level of the pituitary (Roser, 2011).

Estrogens

The stallion testes produce large amounts of estrogens. Estradiol 17-β has been shown to have an agonistic affect on the release of GnRH-induced LH secretion from the pituitary gland (Muyan et al., 1993). However, the role of estrogens in regulating pituitary FSH still remains unclear (Roser, 2011). Thompson and coworkers demonstrated that treatment with estradiol decreased circulating FSH levels, increased circulating LH levels, and decreased GnRH-induced FSH in geldings (Thompson et al., 1979).

1.5. SPERMATOZOA

The spermatozoon is a highly specified cell that has the abilities of active motility and fertilization of the ovum. This cell is composed of head and flagellum (or tail), both enclosed by a plasma membrane (Johnson et al., 1997)

The head can be subdivided into an acrosomal region, equatorial segment, post-acrosomal region, and posterior ring; the latter demarcates the junction between the head and tail. The two main structures of the sperm head are nucleus and acrosome. The nucleus contains the male genetic material (DNA) to be delivered to the ovum and it is surrounded by the nuclear envelope. Abnormal sperm chromatin structure may increase the susceptibility of the sperm cell to denaturation and may also interfere with decondensation, a prerequisite for male pronucleus formation during fertilization (Neild et al., 2005; Baumber-Skaife, 2011).

The acrosome is a bag of hydrolytic enzymes required for penetration of the egg during fertilization (acrosome reaction). Therefore, an integer acrosome is important to ensure fertilization occurs (Neild et al., 2005; Varner, 2008). The acrosome is located over the anterior surface of the nucleus and is surrounded by the inner and outer acrosomal membranes. The acrosome can be divided into the apical segment, principal segment, and equatorial segment (Johnson et al., 2011; Varner and Johnson, 2011).

The flagellum is anatomically divided into four parts: the neck region or connecting piece, the middle piece, the principal piece and, the end piece (Johnson et al., 2011; Varner and Johnson, 2011). Mitochondria, responsible for energy production, are located in the middle piece. Mitochondria function is required for spermatozoa motility (Varner, 2008). The principal piece contains the fibrous sheath with rib-like structures for

flexibility and is attached to the end piece. The tail is motile due to the presence of an axoneme (nine microtubular doublets and a central pair of microtubules) for all its lenght. The principal piece is the longest portion of the flagellum and contains a fibrous sheath, which allows for bending during movement. The end piece is composed of unsheathed, often single, microtubules (Johnson et al., 1997).

The spermatozoon is surrounded by a continuous and heterogeneous plasma membrane, with regional differences in composition reflecting the different physiological functions, such as sperm–oviductal adhesion, penetration of the cumulus–oophorus matrix, sperm–zona adhesion, the acrosome reaction, acquisition of activated motility and hyperactivated motility, and sperm–oocyte adhesion and fusion (Aurich, 2005; Varner and Johnson, 2011). Therefore, an intact and functional plasma membrane is vital to both sperm survival and fertilizing potential (Varner, 2008).

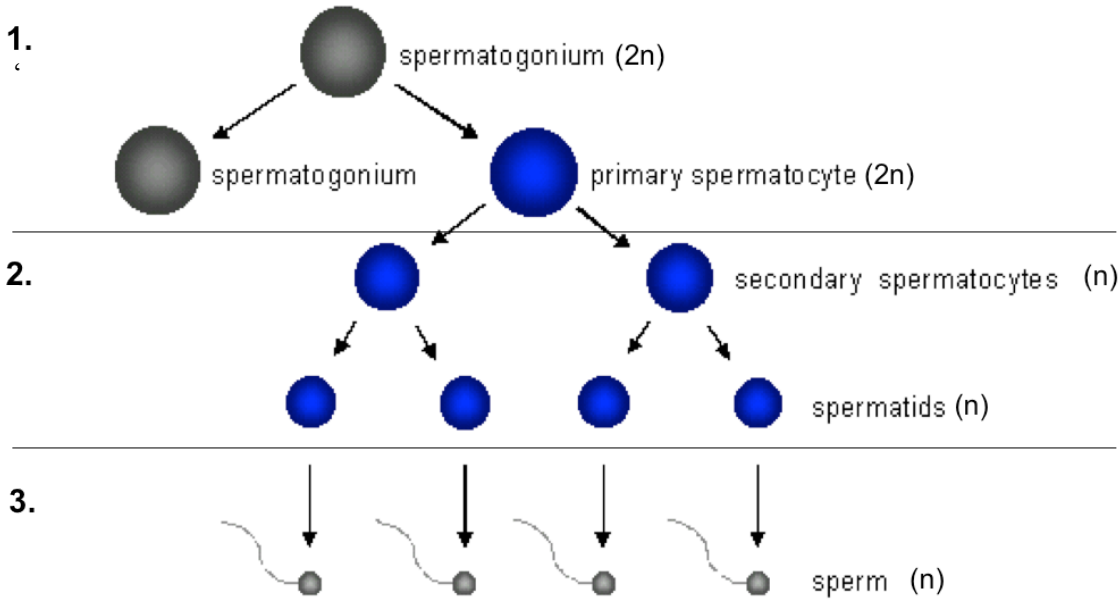
In the horse, spermatozoa are not mature when released from the seminiferous tubules. They have a cytoplasmic droplet on its middle piece and must undergo maturation in the epididymis to gain progressive motility, structural stability, and fertilizing ability (Johnson et al., 1980). In the cytosol of the mature spermatozoal head organelles are not found, other than the acrosome, and the mature spermatozoon is considered to be transcriptionally quiescent (Varner, 2008; Varner and Johnson, 2011).

1.6. SPERMATOGENESIS

Spermatogenesis is the process by which germ cells (spermatogonia, spermatocytes, and spermatids) in the seminiferous epithelium of the testes divide and differentiate to produce spermatozoa (Johnson et al., 1997; Brito, 2007). Spermatogenesis can be divided into three phases: (A) spermatocytogenesis (mitosis); (B) meiosis and, (C)

spermiogenesis (Figure 1.3.3.). The total duration of spermatogenesis in the horse is 57 days, in which spermatocytogenesis lasts 19.4 days, meiosis also lasts 19.4 days and spermiogenesis 18.6 days.

Figure 1.3.3. Spermatogenesis. Differentiation of a spermatogonium into a spermatozoon: (1) Spermatocytogenesis; (2) Meiosis; and, (3) Spermiogenesis. (Modified from: http://faculty.clintoncc.suny.edu/faculty/michael.gregory/files/Bio%20100/Bio%2010020 Lectures/Meiosis/meiosis.htm).



1.6.1. Spermatocytogenesis

Spermatocytogenesis involves spermatogonia, the most immature germ cell. Spermatogonia arise postnatally from gonocytes (positioned in precursos seminiferous tubules during the second month of gestation) and are located in the base of the seminiferous epithelium in adults.

There are two types of spermatogonia: the stem cell spermatogonia (uncommitted) and the committed spermatogonia. During spermatocytogenesis, the committed cell population of spermatogonia proliferates cyclically by mitosis to produce primary spermatocytes and at the same time the uncommitted spermatogonia renew their own number and continue the lineage of stem cells (Figure 1.3.3.). There are five subtypes of spermatogonia in the horse known as A1, A2, A3, B1, and B2 in the stallion. These cells grow, differentiate and divide by mitosis until ultimately the B1 spermatogonia divide producing preleptotene primary spermatocytes.

Spermatocytogenesis lasts 19.4 days in stallions.

1.6.2. Meiosis

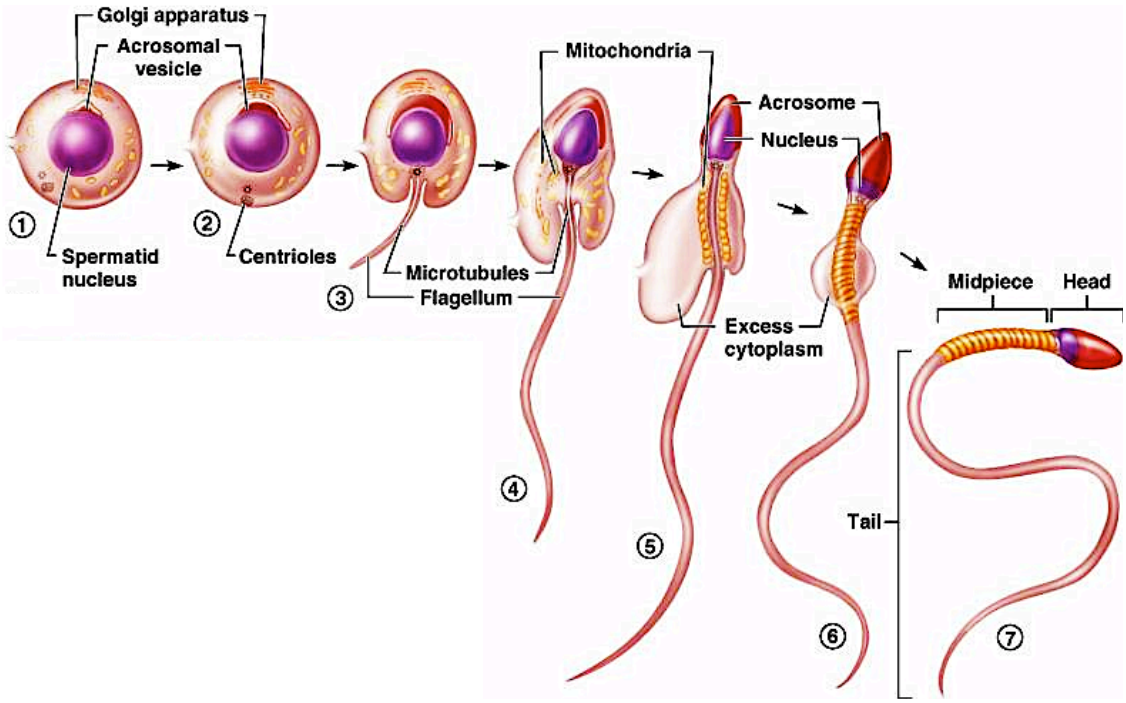
The meiotic phase includes the genetic recombination activity (crossing over) and concludes with the production of haploid spermatids. Meiosis occurs only in germ cells in the gonads (testes and ovaries) and in the male consists in the formation of haploid secondary spermatocytes and haploid spermatids from the diploid primary spermatocytes (Figure 1.3.3.). The preleptotene primary spermatocytes immediately begin meiosis I and as different phases of meiosis are completed, they develop into leptotene, zygotene (beginning of exchange of genetic material), pachytene, and diplotene primary spermatocyte (prophase I). Diplotene primary spermatocytes, after prophase I, rapidly undergo metaphase, anaphase, and telophase I. Secondary spermatocytes (n) result from the first meiotic division. Meiosis II resumes and round spermatids (n) are generated.

Meiosis lasts 19.4 days in the stallion.

1.6.3. Spermiogenesis

Spermiogenesis is a non-dividing period of spermatogenesis where round spermatids elongate into spermatozoa (Figure 1.3.3.). It does not involve any cellular divisions (DNA repair takes places and DNA synthesis is minimal) and rather it is the morphologic differentiation of the haploid round spermatids into the unique mature sperm cells. Spherical spermatids cells with spherical nuclei undergo metamorphosis and turn into spermatozoa: a cell with a streamlined head containing penetrative enzymes and a condensed nucleus carrying the male genome and a tail necessary for motility. The process of spermiogenesis in stallions is 18.6 days long and is divided into Golgi, cap, elongation, and maturation phases, depending on maturation changes within the spermatid noted by shape and predominant organelles (Figure 1.3.4.).

Figure 1.3.4. Spermiogenesis



Golgi phase spermatids have a prominent Golgi apparatus that gives rise to the acrosome of the mature spermatozoon. During the cap phase, the acrosomic vesicle flattens and begins to spread, forming a cap over the nucleus. The Golgi apparatus starts moving away from the acrosome of the cell and flagellar development becomes more obvious as it projects further from the cell's surface. The nucleus is still spherical at this time. As the cap extends over the nucleus, the nucleus begins to elongate and the elongation phase initiates. A unique and transient spermatid organelle called manchette elongates the nucleus and extends down to the developing flagellum, all within the cytoplasm, which is also elongated.

The final phase of spermatid development (maturation) is characterized by caudal migration and dissolution of the manchette. The manchette is not seen in a normal spermatozoon. During maturation, mitochondria moves to the mid piece region. Upon spermiation (release of fully developed spermatid into the lumen of seminiferous tubules) a large portion of cytoplasm found in the spermatid is left behind, creating the residual body, which is phagocytized and digested by its lysosome and by the Sertoli cells. Sertolli cells also phagocytose any other germ cells that die during spermatogenesis.

After spermatogenesis, spermatozoa leave the testis and are transported through the epididymis, where final maturation occurs and the spermatozoon gains ability of motility and fertilization of an oocyte in a process that lasts 8-11 days in the stallion. Therefore, sperm present in the ejaculated began being produced 65-68 days earlier and semen quality is a reflection of events that have occurred in the past 2 months and affected spermatogenesis and/or and final maturation of the sperm cell in the epididymis.

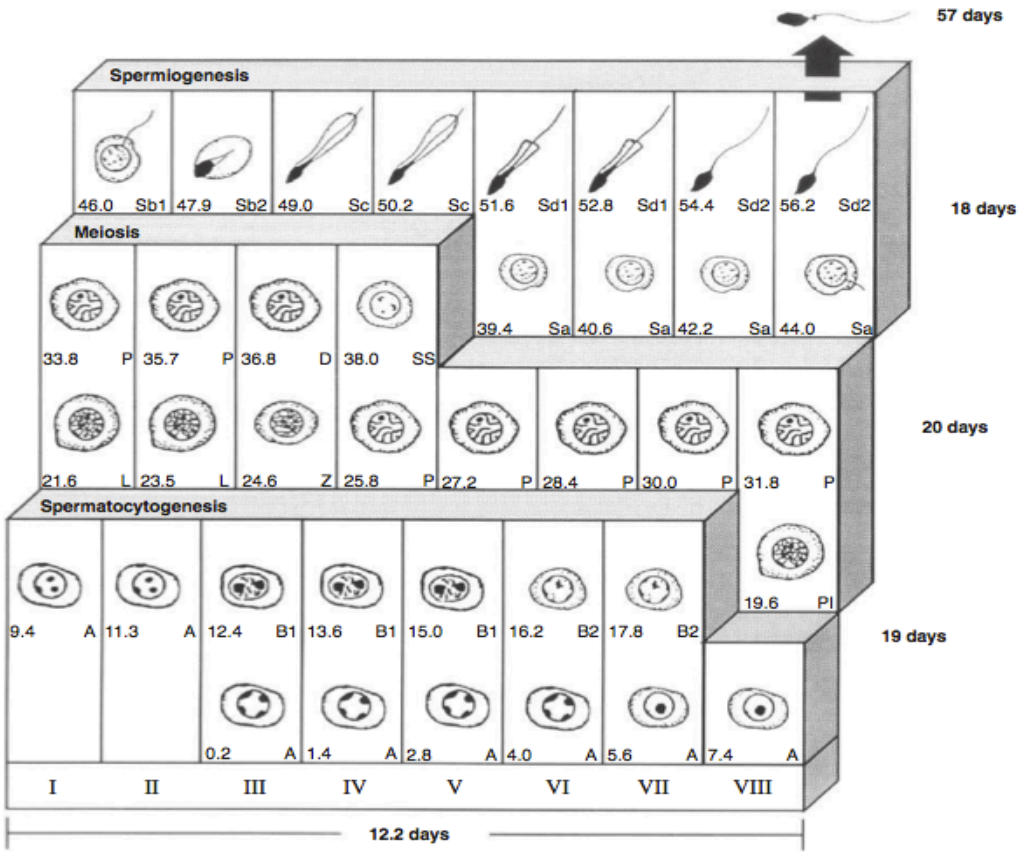
1.6.4. Spermatogenic Cycle

The spermatogenic cycle is defined as a series of changes that occur in a given region of the seminiferous epithelium between the appearances of the same developmental stage. A stage, in turn, refers to the vast number of cells at the same level of development (due to the existence of synchrony among germ cells) in a given region of the seminiferous tubules. If we use spermiation as a reference point, the cycle would be all the events that occur between two consecutive occasions of spermatozoa released from a given region of the seminiferous epithelium. Thus, even though the spermatogenesis is 57 days in the horse, spermatozoa are released every 12.2 days, which corresponds to the duration of the spermatogenic cycle (Figure 1.3.5.).

The stallion spermatogenic cycle was divided into 8 stages and first described by Swierstra *et al.* Classification of stages of the spermatogenic cycle has been based on the presence and location of specific germ cells, and on changes associated with spermiogenesis and spermiation (Swierstra et al., 1974).

While the spermatogenic cycle is the series of changes seen over time in a given region of the tubule, the distribution of consecutive stages along the length of the tubules at any given time is called spermatogenic wave.

Figure 1.3.5. Three major phases of spermatogenesis. Spermatocytogenesis, meiosis, and spermiogenesis are overlaid by the eight stages of the cycle of equine seminiferous epithelium. During the 19 days required for spermatocytogenesis, A-spermatogonia (A) enter cyclic activity during stage III and undergo division to produce B1 spermatogonia (B1), B2 spermatogonia (B2), and preleptotene primary spermatocytes (Pl). During the 20 days of meiosis, newly formed preleptotene primary spermatocytes (Pl) differentiate through leptotene (L), zygotene (Z), pachytene (P), and diplotene (D) before the first meiotic division to produce secondary spermatocytes (SS), and the second meiotic division to produce Sa spermatids (Sa). During the 18 days of spermiogenesis, Sa spermatids differentiate through Sb1 (Sb1), Sb2 (Sb2), Sc (Sc), Sd1 (Sd1), and Sd2 (Sd2) steps of development before their spermiation as spermatozoa. The letters indicate the cell type, while the numbers associated with the developmental step of each germ cell indicate the total time to formation of that cell type, calculated to the middle of that spermatogenic stage. In the horse, the cycle length is 12.2 days, and the duration of spermatogenesis is 57 days (Johnson et al., 2011).



III. JUSTIFICATION FOR THE THESIS RESEARCH PROJECT

The establishment of persistent infection in the reproductive tract of the infected stallion is a unique feature of EAV infection. Persistently infected stallions play a very important role in the dissemination and maintenance of EAV in horse populations worldwide (Timoney et al., 1986; Timoney and McCollum, 1993a). In addition, the long-term carrier stallion is responsible for generating the genetic heterogeneity that distinguishes individual field strains in contrast to the minimal virus diversity that is generated during EVA outbreaks when the virus is transmitted by respiratory and or venereal routes (Balasuriya, et al., 1999, 2004; Hedges et al., 1999).

Multiple factors may contribute to development of the carrier state in stallions. However, even though it is known that EAV persistence in the reproductive tract of stallions is testosterone dependent (Holyoak et al., 1993; Little et al., 1992; McCollum et al., 1994), the reason(s) why some stallions become long-term carriers and some do not, has never been elucidated. Using dual-color flow cytometry, previous studies in our laboratory demonstrated the existence of two groups of horses based on their $CD3^+$ T lymphocyte cells susceptibility to *in vitro* infection with the virulent Bucyrus strain of EAV (Go et al., 2010). Thus, the horse population can be divided into susceptible and resistant groups based on the susceptibility of their $CD3^+$ T cells to *in vitro* EAV. The differences in *in vitro* susceptibility to $CD3^+$ lymphocytes to EAV infection have a strong association to genetic markers on equine chromosome 11 (Go et al., 2011). Furthermore, carrier stallions with an *in vitro* $CD3^+$ T lymphocyte susceptible phenotype may be at a higher risk of becoming carriers following natural infection compared to those with the

resistant phenotype (Go et al., 2012). Based on these observations, in the second chapter of this thesis, we hypothesized that the establishment of persistent infection in stallions is associated with *in vitro* susceptibility of $CD3^+$ T lymphocytes to EAV infection. To test this hypothesis we investigated whether there is a relationship between *in vitro* susceptibility of $CD3^+$ T lymphocytes to EAV infection and the establishment of persistent infection in stallions following experimental inoculation with the KY84 strain of EAV.

Persistently infected stallions shed virus in semen for years following EAV infection, but not in other bodily tissues and fluids. Thus, they are able to transmit the virus only by venereal route. The long-term carrier stallion is clinically normal and has normal semen quality, although virus persists in the reproductive tract and is shed in semen. No long-term effects on stallions fertility have been observed on long-term carrier stallions (Timoney and McCollum, 1993a). Previously, it has been shown that the semen quality of acutely infected stallions was temporarily reduced, but it was not clear if this decrease was due to the direct effect of virus present in semen (Neu et al., 1992). Several viruses have been detected in the semen of a number of animal species and have been shown to be detrimental to the male reproductive tract and to the semen (Sur et al., 1997; Dejucq and Jégou, 2001). Therefore, in the third chapter of this thesis, we hypothesized that changes in semen quality are the results of fever and scrotal edema caused by EAV during acute phase of infection rather than the direct effects of the virus. To test this hypothesis, we investigated if the possible changes observed in semen quality following experimental EAV infection of stallions result either from the presence of virus in semen or from the fever and scrotal edema caused by the virus during acute phase of infection.

CHAPTER TWO

Association between *in vitro* CD3$^+$ T cell susceptibility to EAV infection and EAV persistence in stallions experimentally infected with the Kentucky 84 (KY84) strain of EAV

2.1. SUMMARY

Equine arteritis virus (EAV) is the causal agent of equine viral arteritis (EVA), a respiratory and reproductive disease of equids. Following EAV infection, a variable proportion of stallions (30-70%) can become carriers and continuously shed the virus in their semen for varying time periods. With the use of a dual-color flow cytometry it was demonstrated that the virulent Bucyrus strain of EAV (VBS) could infect, *in vitro*, a subpopulation of CD3$^+$ T lymphocytes of some but not all horses. Furthermore, a correlation between *in vitro* susceptibility of CD3$^+$ T lymphocytes to EAV infection and long-term persistent infection among stallions following natural infections has been shown. Here in this pilot study, we investigated whether the stallions with *in vitro* EAV susceptible CD3$^+$ T lymphocytes are at higher risk of becoming long-term carriers compared to those with the resistant phenotype following *in vivo* experimental infection with the KY84 strain of EAV. A group of eight stallions were divided into two groups (n=4) based on their susceptible or resistant CD3$^+$ T lymphocyte phenotypes and intranasally inoculated with the EAV KY84. Stallions were monitored for the development of clinical signs of EVA, number of lymphocytes, and shedding of virus into the nasal secretions and peripheral blood mononuclear cells (PBMCs) until 42 days post infection (dpi). Semen was collected and evaluated monthly for he presence of EAV until 198 dpi (6.5 months) to establish viral persistence. The data suggested that the

establishment of long-term carrier state seems to be associated with the *in vitro* $CD3^+$ T lymphocyte susceptible phenotypes.

2.2. INTRODUCTION

Equine arteritis virus (EAV) is a small enveloped virus with a single-stranded positive-sense RNA genome of 12.7 kb and is the prototype member of the family *Arteriviridae* (genus *Arterivirus*, order *Nidovirales* (Cavanagh, 1997; Snijder and Meulenberg, 1998; King et al., 2011). EAV is the causal agent of equine viral arteritis (EVA), a respiratory and reproductive disease of equids (McCollum et al., 1971; Timoney et al., 1987; Timoney and McCollum, 1993a). The virus was first isolated from a lung of an aborted fetus following an extensive outbreak of respiratory disease at Bucyrus, Ohio, U.S.A., in 1953 (Doll et al., 1957b; Doll, Bryans, and Knappenberger, 1957). There is only one known serotype of EAV and all strains evaluated thus far are neutralized by polyclonal antiserum raised against the virulent Bucyrus strain (Balasuriya et al., 1995, 1997, 2004; Chirnside, de Vries, et al., 1995; Balasuriya and MacLachlan, 2004; Zhang et al., 2012). However, field strains of EAV can often be distinguished on the basis of their neutralization phenotype with polyclonal antisera and MAbs. Likewise, geographically, and temporally distinct strains of EAV differ in the severity of the clinical disease they induce and in their abortigenic potential (Murphy et al., 1992; Timoney and McCollum, 1993a; Balasuriya et al., 1998, 1999; Patton et al., 1999; Vairo et al., 2012). While most EAV infections are asymptomatic, some infected horses can exhibit clinical manifestations such as fever, leukopenia, dependent edema, nasal and ocular discharge, conjunctivitis, and abortion (Doll, Bryans, and

Knappenberger, 1957; McCollum et al., 1971, 1995; Glaser et al., 1996). The vast majority of naturally infected adult horses recovers spontaneously and rarely dies from the disease. However, following natural infection neonate and young foals can develop severe interstitial pneumonia and pneumoenteritis and can die from EAV infection (Golnik et al., 1981; Vaala et al., 1992; Del Piero et al., 1997). The virus is transmitted mainly by respiratory (Doll, Bryans, and Knappenberger, 1957; McCollum et al., 1971) or venereal routes (Timoney, 1984; Timoney, McCollum, Roberts, et al., 1986). Following EAV infection, a variable proportion of stallions (30-70%) can become carriers and continuously shed the virus in their semen for varying time periods (Timoney, McCollum, Roberts, et al., 1986; Timoney et al., 1987; Neu and Timoney, 1988). The virus persists in the male reproductive tract, specifically in the ampulla of the vas deferens (Neu and Timoney, 1988), despite the frequent presence of high titer neutralizing antibodies in serum (Timoney, McCollum, Roberts, et al., 1986; Timoney et al., 1987; Neu and Timoney, 1988). The establishment and maintenance of EAV persistence infection in male reproductive tract is testosterone dependent (Little et al., 1992; Holyoak et al., 1993; McCollum et al., 1994).

In a recent study, using dual color flow cytometry we demonstrated that the virulent Bucyrus strain (VBS) of EAV could infect *in vitro* $CD3^+$ T lymphocytes from some but not all horses (Go et al., 2010). The data suggested that the $CD3^+$ T lymphocyte subpopulation of individual horses varied in their susceptibility to *in vitro* EAV infection. Furthermore, a genome wide association study (GWAS) identified a common, genetically dominant haplotype associated with the susceptible phenotype in the region of equine chromosome 11 (ECA11; 49572804-49643932) (Go et al., 2011).

As a result of this clearly defined genetic evidence, we hypothesized in a previous study that *in vitro* susceptibility of $CD3^+$ T lymphocytes to EAV infection may correlate with the clinical responsiveness of horses to challenge with virulent strains of EAV. We demonstrated that *in vitro* susceptibility/resistance of equine $CD3^+$ T lymphocytes to VBS correlated with the clinical responsiveness of horses experimentally inoculated with recombinant VBS (rVBS). It was found that clinical signs are greater in horses that had $CD3^+$ T lymphocytes resistant phenotype compared to those that had a susceptible phenotype when challenged with the rVBS of EAV (Go et al., 2012). In a parallel retrospective study we demonstrated that there is a correlation between the stallion carrier state of natural infected stallions and the *in vitro* $CD3^+$ T cell susceptible phenotype (Go et al., 2012). Based on these previous data, the specific aims of this study were: (1) to investigate, prospectively, whether there is an association between *in vitro* $CD3^+$ T lymphocyte resistance/susceptible phenotypes of stallions and the establishment of persistent infection of EAV in stallions following experimental inoculation with the KY84 strain of EAV; (2) to evaluate the clinical response to *in vivo* EAV infection among the $CD3^+$ T cell susceptible and resistant *in vitro* phenotype stallions. The EAV KY84 has been shown to establish persistent infection in the reproductive tract of stallions and to cause moderate to severe clinical signs of EVA infection in horses following natural and experimental infection (McCollum and Timoney, 1984; Timoney, 1984; Neu & Timoney, 1988; Zhang et al., 2012).

2.3. MATERIAL AND METHODS

Viruses

The virulent Bucyrus strain of EAV (ATCC VR-796, Manassas, VA) was used to identify the stallions with $CD3^+$ T lymphocyte susceptible/resistant phenotype in peripheral blood mononuclear cells as previously described (Go et al., 2011). The virulent KY84 strain of EAV (EAV KY84) was used as the challenge virus (McCollum and Timoney, 1984, 1999; Timoney, 1984; Cole et al., 1986). The MLV vaccine strain of EAV (ARVAC®, Pfizer Animal Health Inc., Kalamazoo, MI) was used as the reference virus in the microneutralization assay. Virus stocks were titrated by standard plaque assay in RK-13 cells, and titers were expressed as PFU/ml (McCollum et al., 1962).

Cells lines

The high passage rabbit kidney cell line (RK-13 KY; passage level 399-409) was maintained in Eagle's minimum essential medium (EMEM; Cellgro®, Mediatech, Inc., Manassas, VA) supplemented with 10% ferritin-supplemented bovine calf serum (Hyclone Laboratories, Inc., Logan, UT), 100 U/ml penicillin/streptomycin (Mediatech, Inc., Manassas, VA) and 1 μg/ml amphotericin B (Sigma-Aldrich, St. Louis, MO). Equine pulmonary artery endothelial cells (EECs; Hedges et al., 2001) were maintained in Dulbecco's modified essential medium (Cellgro®, Meditech, Inc., Manassas, VA) with sodium pyruvate, 10% fetal bovine serum (FBS; Hyclone Laboratories, Inc., Logan, UT), 100 U/ml penicillin/streptomycin (Mediatech, Inc., Manassas, VA), and 200 mM L-glutamine (Invitrogen™, Carlsbad, CA).

Antibodies

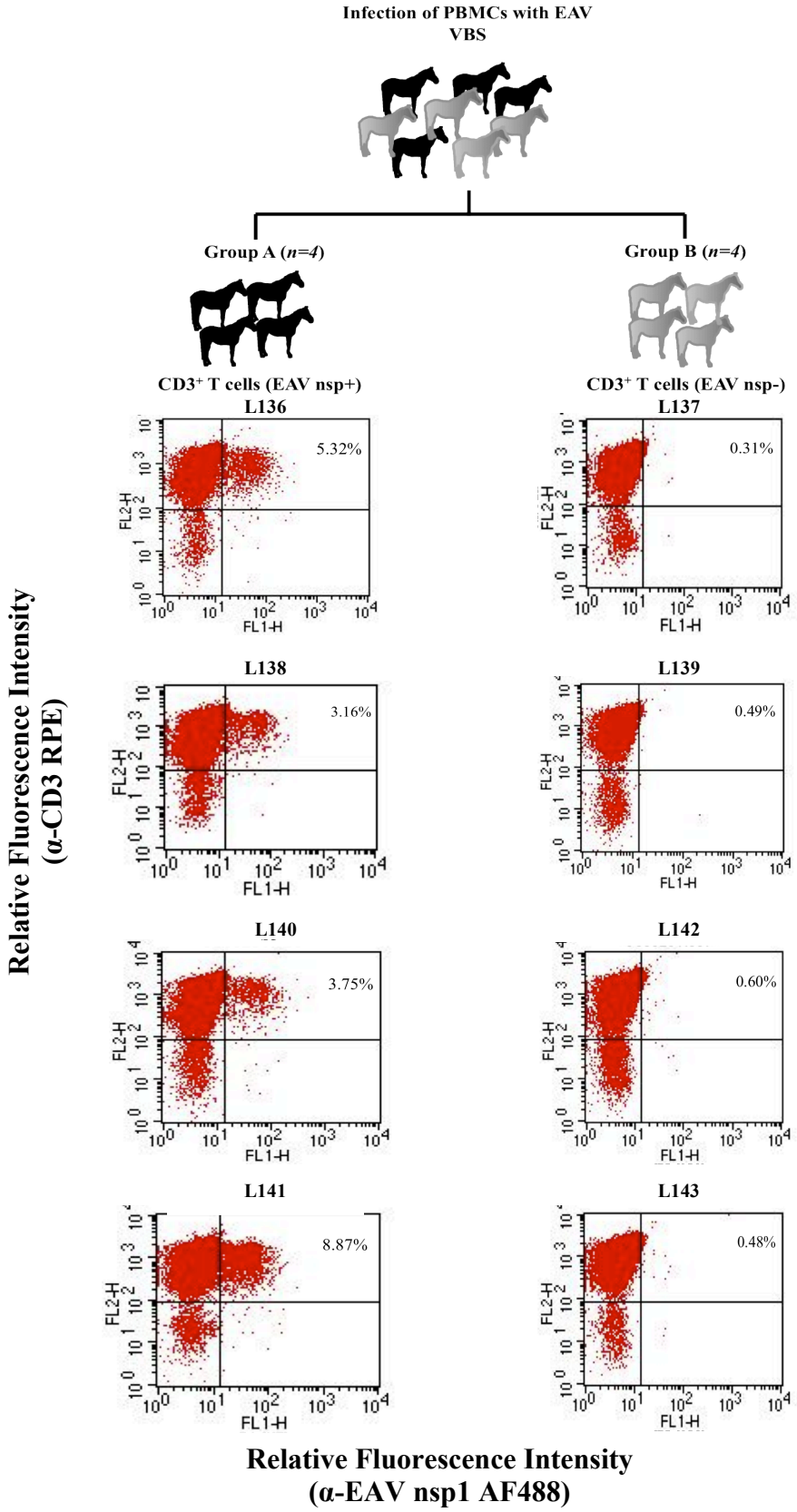
Monoclonal antibodies (MAbs) were used to identify the *in vitro* susceptible/resistant phenotype of the $CD3^+$ T lymphocytes of each of the stallions following *in vitro* infection with the EAV VBS. The anti-equine CD3 MAb UC F6G specific for $CD3^+$ T lymphocytes was kindly provided by Jeff Stott, University of California, Davis (Blanchard-Channell et al., 1994). The R-PE conjugated F(ab') fragment of goat anti-mouse IgG1 (Southern Biotech, Birmingham, AL) was used as the secondary antibody for the anti-equine CD3 surface marker. The EAV infected CD3+ T lymphocytes were detected with Alexa Fluor 488-labled MAb 12A4 specific for the nonstructural protein 1 (nsp1) of EAV (Wagner et al., 2003; Go et al., 2011).

Horses

Eight mature (4-16 years old) mixed breed stallions were included in the study (Stallions L136-L143). In order to divide stallions into two groups based on the *in vitro* susceptibility phenotype of their $CD3^+$ T lymphocytes to the EAV VBS, a significant number of stallions (n=15) were first screened by dual-color flow cytometry as previously described (Go et al., 2010). A total of 8 stallions were selected and divided into two groups according to their *in vitro* $CD3^+$ T lymphocytes susceptible/resistant phenotype to EAV VBS strain for use in this study: group A (susceptible [n=4; L136, L138, L140, and L141]) and group B (resistant [n=4; L137, L139, L142, and L143]) (Figure 2.3.1.). Horses were obtained from a local commercial vendor and acclimated to the new farm environment for approximately 2 months before the beginning of the study. During this period animals were located in individual paddocks and trained to mount a

mare or a phantom and to collect semen in an artificial vagina (Botucatu model, Botupharma, Botucatu, SP, Brazil). All horses were confirmed seronegative for EAV neutralizing antibodies several times before intranasal inoculation with the virus, using a previously described protocol (Senne et al., 1985; OIE, 2004). The animals were then housed in an isolation facility at the Department of Veterinary Science's Maine Chance Farm, Lexington, KY. This study was carried out in accordance with an Institutional Animal Care and Use Committee (IACUC) approved protocol at the University of Kentucky, Lexington, KY (protocol number 2011-0888).

Figure 2.3.1. CD3^{+} T lymphocyte susceptible and resistant stallions' phenotypes.



Preparation of peripheral blood mononuclear cells

Equine peripheral blood mononuclear cells (PBMCs) from each of 8 stallions were isolated as previously described (Go et al., 2010). Briefly, blood was collected aseptically from each stallion in Vacutainer tubes containing 15% EDTA solution (Kendall Healthcare, Masnfield, MA). PBMCs were isolated from the buffy-coat fraction by centrifugation through Ficoll-Plaque™ PLUS (Amersham Biosciences, Piscataway, NJ) at 500 × g for 30 minutes at 4^0C. The PBMCs layer was collected and washed twice with PBS (pH 7.4) by centrifugation at 100 × g for 10 min to eliminate the platelets. The remaining cells were resuspended in complete RPMI (cRPMI) 1640 medium (Gibco®, Carlsbad, CA) supplemented with 10% heat-inactivated FBS (HyClone Laboratories, Logan, UT), 2mM glutamine, 100U/ml penicillin/streptomycin, and 55 μM 2-mercaptoethanol. PBMCs were counted using a Vicell Counter-XR (Beckman Coulter, Miami, FL) and inoculated with EAV VBS at a multiplicity of infection (MOI) of 2.

***In vitro* infection of peripheral blood mononuclear cells**

PBMC cultures, in 6-well plates (USA® Scientific Inc., Orlando, FL), were infected with EAV VBS at an MOI of 2 as previously described (Go et al., 2010). Infected cells were incubated at 37^0C in a CO_2 incubator for 36h. As negative controls, mock-infected PBMCs were cultured under identical conditions.

Dual-color immunofluorescence staining and flow cytometry analysis

Dual-color flow cytometry was used to analyze the *in vitro* susceptibility/resistance of each stallion's $CD3^+$ T lymphocytes to EAV VB strain as

previously described (Go et al., 2010). Briefly, EAV infected and mock infected PBMC cultures (approximately 1×10^6 cells) were incubated on ice for 30 minutes with the equine $CD3^+$ specific MAb UC F6G. After washing, cells were incubated with secondary R-PE-conjugated goat anti-mouse IgG1 for 30 min on ice. After washing to remove unbound secondary antibody, cells were fixed with 4% paraformaldehyde and then washed once in PBS-saponin buffer (PBS [pH 7.4] supplemented with 1% FBS, 0.1% saponin and 0.1% sodium azide). Intracellular staining for EAV antigen was performed using an Alexa Fluor® 488 conjugated anti-EAV nsp1 MAb 12A4 in PBS-saponin buffer and incubated on ice for 30 min. After incubation, washed cells were resuspended in PBS containing 0.5% paraformaldehyde for two-color cytometric acquisition using a FACSCalibur (Becton Dickinson, San Jose, CA). Lymphocytes were gated and selected based on forward and side-scatter parameters of analysis. Cells were evaluated by a two-color plot of anti-EAV antigen (FL-1) versus cell surface antigen (FL-2), and the percentage of $CD3^+$ T lymphocytes and EAV antigen positive cells was determined by CellQuest™ quadrant statistics. Results were expressed as the percentage of lymphocytes infected with EAV, after subtraction of the non-specific staining of mock-infected cells.

Experimental infection of stallions and clinical evaluations

The stallions in group A (susceptible: L136, L138, L140, and L141; n=4) and group B (resistant: L137, L139, L142, and L143; n=4) were randomly assigned to individual stalls in an isolation barn located at the University of Kentucky's Maine Chance Farm. All horses were clinically evaluated and several (6) blood samples for

blood cell counts were collected prior to experimental infection (see below). Pre-inoculation (7, 5 and 2 days before experimental infection) clinical examinations were performed once daily to determine base-line values for body temperature and to ensure the stallions were clinically normal before inoculation with the virus. Edema was classified on a scale from 0 to 5 and recorded as absent (0), mild (1-2), moderate (3) and severe (4-5). The stallions were inoculated intranasally with 3.75×10^5 plaque forming units (PFU) of the KY84 strain of EAV in 5.0 ml of EMEM using a fenestrated catheter passed via the posterior nares into the nasopharynx as previously described (Balasuriya et al., 2007; Zhang et al., 2008). The animals were monitored for the appearance of clinical signs of EVA twice daily (every 12 hours) for the first 2 weeks after infection. The highest level of body temperature and the more severe clinical signs observed each day were recorded. Clinical signs continued to be monitored once a week (one time a day) for an additional 4 weeks of the experiment (at 21, 28, 35 and 42 dpi). All clinical parameters were recorded by the same investigator who was blinded to the status of the group each stallion belonged to. Blood samples were collected at 0 (before infection), 2, 4, 6, 8, 10, 12, 14, 21, 28, 35 and 42 dpi in order to determine each stallion's serum neutralizing antibody response to EAV.

Collection and process of clinical samples for virus isolation

Shedding of EAV in the semen, circulation and in the respiratory tract was monitored following experimental EAV KY84 infection. Specifically, ejaculates were collected from each horse at 2 days before infection, every other day for the first 15 days of the experiment (1, 3, 5, 7, 9, 11, 13, and 15 dpi), and approximately once a month until

6.5 months post-infection (23, 44, 65, 86, 107, 128, 149, 170, and 198 dpi). The raw gel-filtered semen samples were aliquoted and stored at -80^{0}C until virus isolation was attempted (see below). Blood samples were collected at the day of infection, d 0 (before infection), every other day for the first 15 days of the experiment (2, 4, 6, 8, 10, 12, and 14 dpi), and once a week until 42 dpi (21, 28, 35, and 42 dpi) for complete blood cell counts and virus isolation from PBMCs. Nasal swabs were collected on same dates as blood samples using sterile rayon swabs (1/2" × 1") with plastic shafts (16"). After collection, the tip of each swab was placed into 7ml of virus transport medium (VTM) and kept on ice until transported to the laboratory. In the laboratory, each nasal swab sample was squeezed and the VTM was placed in a sterile syringe and filtered through a 0.45μm filter. The filtrates were aliquoted and stored at -80^{0}C until subsequent attempted virus isolation. All virus isolations were performed by the same investigator who was blinded to the status of the group each stallion belonged to.

Clinical laboratory assays

Hematological analyses were performed at the Hagyard Equine Medical Institute (4250 Iron Works Pike, Lexington, Kentucky) using an electronic cell counter (Coulter Electronic Inc.). Differential counts were performed manually.

Virus Isolation – PBMCs and Nasal Swab

Virus isolation from PBMCs and nasal swabs was attempted on confluent monolayers of RK-13 KY cells as previously described (Balasuriya et al., 2007; Zhang et al., 2008). Briefly, confluent RK-13 KY cells monolayers in 6-well plates (USA®

Scientific Inc., Orlando, FL) were inoculated with 10-fold dilutions (10^0-10^{-5}) in duplicate and overlaid with supplemented EMEM containing 0.75% carboxymethycellulose (CMC; Sigma-Aldrich, St. Louis, MO). Inoculated cells were then incubated for 4 days at 37^0C in a CO_2 (5%) incubator. At day 4 post-inoculation the plates were checked under a light microscope for the presence of cythopatic effect (CPE). If CPE was not visualized a second passage was performed on the 4th day. All first passage 6-well plates were stained with a 1% crystal violet solution containing 1% formaldehyde on day 4 post-inoculation for counting of plaques to determine the virus titer (PFU/ml). The tissue culture fluid (TCF) samples from first and second cell culture passages were harvested and stored at -80C, at day 4 post-inoculation.

Virus Isolation – Semen

Virus isolation from raw gel-free semen samples was attempted in RK-13 KY cells according to a standard protocol used by the OIE Reference Laboratory at the Maxwell H. Gluck Equine Research Center (OIE, 2004). Briefly, semen samples were sonicated for 45 seconds (3 × 15 sec) at 4^0C and sperm and cellular debris were sedimented by centrifugation (2,800 × g, 10 min). Serial decimal dilutions (10^{-1} to 10^{-4}) of each sample supernatant were prepared in minimum essential medium (MEM; Cellgro®, Mediatech, Inc, Manassas, VA). One ml of each dilution was inoculated into a 25-cm^2 flask containing confluent monolayers of RK-13 KY cells. Flasks were incubated at 37^0C for 1 hour. Following incubation, flasks were overlaid with the supplemented EMEM containing 0.75% carboxymethylcellulose (CMC; Sigma-Aldrich®, St. Louis, MO). The flasks were incubated at 37^0C for 4 days and checked under a light

microscope for the appearance of CPE. If CPE was not detected, a second passage was performed on that day. All first passage flasks were stained with a 1% crystal violet solution containing 1% formaldehyde on day 4 post-inoculation. The plaques were counted and virus titer was expressed as PFU/ml. Second passage flasks were also stained with crystal violet 4 days after inoculation. The TCF samples from first and second passages were harvested and stored at -80C.

Microneutralization assay

The neutralizing antibody titers of the test sera were determined as described by Senne *et al.* (Senne et al., 1985; OIE, 2004). Briefly, duplicate serial two-fold dilutions of each serum sample from 1:4 to 1:512 were made in supplemented EMEM containing 10% guinea pig complement (Rockland Immunochemicals, Gilbertsville, PA) and tested in 96-well plates (Corning®, Corning Inc., NY). An equal volume of 200 $TCID_{50}$ of the modified live vaccine strain of EAV (ARVAC®, Pfizer Animal Health, New York, NY) was added to each well. Plates were incubated at 37^0C in 5% CO_2 for 1 hour. After incubation, a suspension of RK-13 KY cells was added to each well and the plates were incubated for 72 h, at 37°C, in a CO2 (5%) incubator, until viral cytopathic effect had fully developed in the virus control wells. The titer of a sample was recorded as the reciprocal of the highest serum dilution that provided at least 50% neutralization of the reference virus.

Real-time RT-PCR

Virus isolates were confirmed by EAV specific TaqMan® real-time RT-PCR assay as previously described (Balasuriya, Leutenegger, et al., 2002; Miszczak et al., 2011). Briefly, viral RNA was directly isolated from 50 µl tissue culture fluid (TCF) using a commercial RNA isolation kit (MagMAX™ -96 Viral RNA Isolation Kit, Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The viral RNA was eluted in 50 µl of nuclease free water and stored at −80 °C. RNA extracted from TCF from PBMCs, nasal swab, and semen samples that were negative for EAV, as well as from nuclease free water were included as negative controls. Viral RNA extracted from a KY84 virus stock sample was used as a positive control. A one-tube TaqMan® real-time RT-PCR assay was performed as previously described (Miszczak et al., 2011).

2.4. STATISTICAL ANALYSIS

Locally weighted polynomials (LOWESS) were fit to the raw data for each horse to produce smooth curves (Cleveland, 1979). The curves were then averaged for each group separately, with standard errors calculated accordingly and used to construct pointwise confidence intervals. A t-test was performed at the nadir or zenith of the curve to compare possible differences between the groups. Significance was set as $p < 0.05$. All analyses were carried out using the R software for statistical computing and graphics (www.r-project.org).

2.5. RESULTS

Clinical Signs

All eight stallions developed moderate to severe clinical signs of EVA following experimental infection with the KY84 strain of EAV, including any combination of fever (> 38.6^{0}C), moderate to severe lymphopenia, dependent edema of the limbs, scrotum and prepuce, periorbital edema, nasal and ocular discharge, photophobia, dyspnea, tacycardia, anorexia, decreased libido and congestion, petechiae and ecchymosis of the oral mucous membranes (Table 2.5.1.).

Table 2.5.1. Clinical findings in stallions following inoculation of stallions with the KY84 strain of equine arteritis virus.

Stallions Group	Clinical Findings							
	Fever	Ocular edema	Front limb edema	Hind limb edema	Scrotal and preputial edema	Nasal discharge	Congestion oral mucosa	Low lymphocyte counts
A	4/4 (100%)	3/4 (75%)	3/4 (75%)	3/4 (75%)	3/4 (75%)	3/4 (75%)	4/4 (100%)	3/4 (75%)
B	4/4 (100%)	1/4 (25%)	4/4 (100%)	4/4 (100%)	2/4 (50%)	3/4 (75%)	2/4 (50%)	4/4 (100%)

All stallions had fever ranging from 38.7 to 40.8^{0}C for 4-8 days (Table 2.5.2.). Fevers (38.7-40.8^{0}C) were seen in group A stallions starting from 2-4 to 8-9 dpi and between 1-3 and 8 dpi in group B stallions. Highest body temperatures were observed among group A and group B stallions between 5-7 dpi and 6-7 dpi, respectively. The highest body temperature (40.8^{0}C) was observed in group B, stallion L138 at 7 dpi, and group A stallion L142 at 6 dpi.

Table 2.5.2. Body temperature (^{0}C) of group A and group B stallions before and after inoculation with the KY84 strain of equine arteritis virus.

Days Post	Group A Stallion's IDs				Group B Stallion's IDs			
Inoculation (dpi)	L136	L138	L140	L141	L137	L139	L142	L143
-7	37.3	38.2	37.4	37.0	37.1	37.2	36.4	36.7
-5	37.5	36.9	37.7	36.9	36.8	37.7	37.7	37.4
-2	37.9	37.7	37.7	37.3	37.3	37.8	37.8	37.7
0	37.3	37.4	38.0	38.0	37.4	38.1	38.4	37.7
1	38.0	37.6	38.1	37.5	37.3	38.2	39.6*	37.2
2	38.8*	38.6	38.7*	37.6	37.5	38.2	39.2*	39.3*
3	38.1	38.9*	38.9*	38.0	38.8*	39.1*	39.8*	37.9
4	38.4	39.0*	38.9*	39.3*	38.8*	39.6*	39.6*	38.4
5	39.4*	39.6*	39.0*	39.7***	39.6*	39.7*	40.2*	39.6*
6	40.0***	40.4*	39.6*	39.3	39.8*	40.1*	40.4***	39.7***
7	39.9*	40.8***	39.7*	38.9*	39.9***	40.2***	40.2*	39.3*
8	38.8*	39.7*	39.6*	39*	39.4*	39.6*	38.7*	38.6*
9	36.8	39.1*	38.0	37.4	38.2	37.5	37.6	37.9
10	37.1	37.8	37.1	37.1	37.1	37.7	37.7	37.0
11	37.1	37.2	37.4	37.2	37.1	37.7	37.6	37.3
12	37.4	37.7	37.7	37.1	37.3	37.8	37.8	37.3
13	37.3	37.5	37.9	37.0	37.2	37.9	37.8	37.4
14	37.4	37.4	37.4	37.3	37.3	38.1	37.6	37.3
15	37.3	37.2	37.7	37.3	37.1	37.9	37.4	37.1
21	37.2	37.3	37.6	36.8	37.3	37.7	37.6	37.2
28	36.8	36.9	37.4	36.9	37.0	37.8	37.7	37.4
35	37.1	36.8	37.3	36.9	36.9	37.7	37.8	36.8
42	37.5	36.9	37.2	37.1	37.1	37.8	37.6	37.4

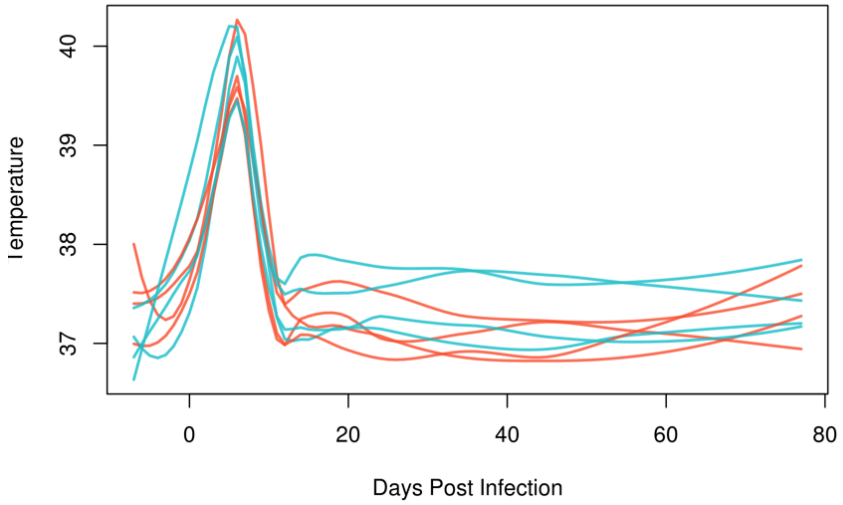
Yellow box * = fever (> 38.6^{0}C)

Orange box *** = highest body temperature for each stallion during acute phase of infection

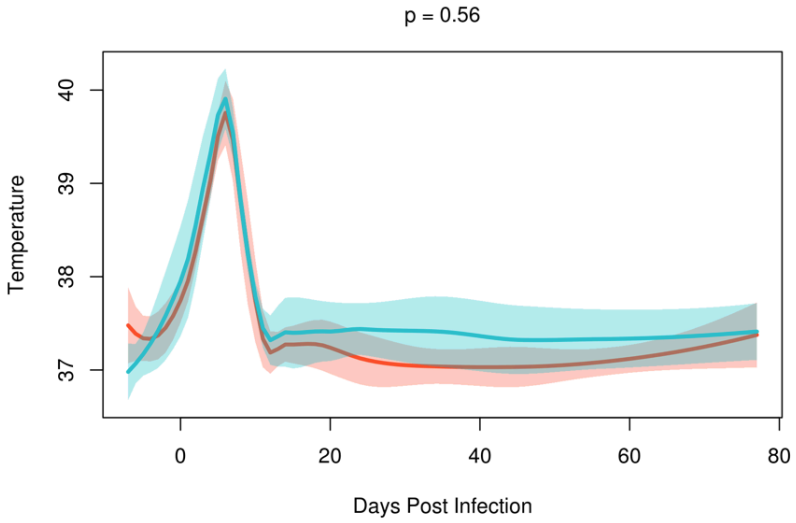
The body temperature across all stallions within a group was averaged for each time point. The highest mean value of fever for group A stallions was observed at 7 dpi while for group B stallions was at 8 dpi. On average, body temperature was not statistically different between the two groups of horses (Figure 2.5.1.).

Figure 2.5.1. Body temperature (^{0}C) following inoculation of the stallions with the KY84 strain of equine arteritis virus. (A) Individual body temperature values from group A (red), and group B (blue) stallions. (B) Mean body temperature of stallions from group A (red) and B (blue).

A.

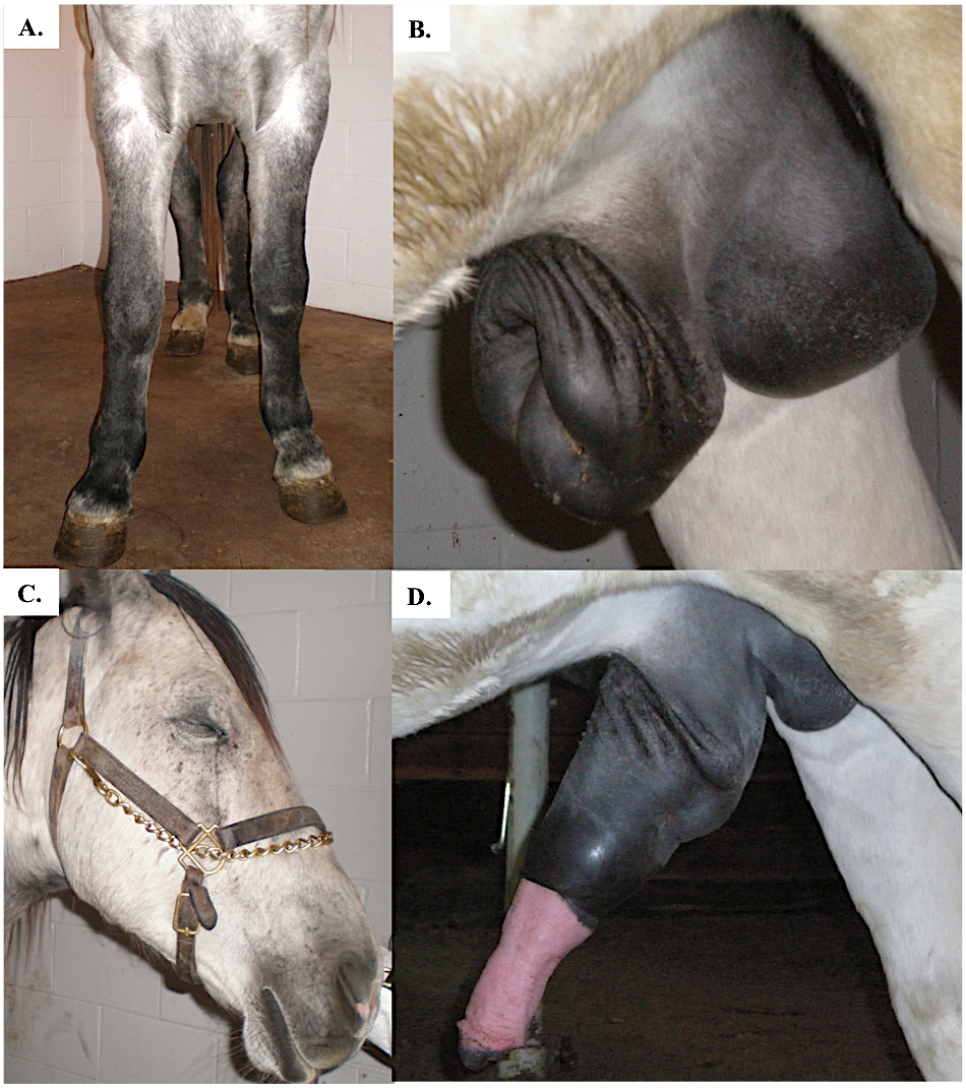


B.



The severity and duration of eyes, limbs, prepucial and scrotal edema varied between stallions (Figure 2.5.2.).

Figure 2.5.2. Representative pictures of edema following experimental inoculation of stallions with the KY84 strain of equine arteritis virus. (A) edema of limbs (stallion L138; 8 dpi); (B) scrotal and preputial edema (stallion L140; 7 dpi); (C) periobirtal edema with the presence of lacrimal discharge (stallion L138; 8 dpi); and, (D) erect penis with preputial edema (stallion L140; 7 dpi).



Periobirtal edema was seen in 3 out of 4 stallions belonging to group A (L138, L140, and L141; 75%), starting from 2-4 dpi and lasting until 4-8 dpi (2-5 days), and in only 1 group B stallion (L143; 25%) out of 4 between 1-6 dpi (Table 2.5.3.). Stallion L138 (group A) was the only stallion in which periobirtal edema was severe on days 4 and 5 post infection.

Table 2.5.3. Periobirtal edema of group A and group B stallions before and after inoculation with the KY84 strain of equine arteritis virus.

Days Post Inoculation (dpi)	Group A Stallion's IDs				Group B Stallion's IDs			
	L136	L138	L140	L141	L137	L139	L142	L143
-7	0	0	0	0	0	0	0	0
-5	0	0	0	0	0	0	0	0
-2	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	2 +
2	0	0	2 ++	0	0	0	0	2 +
3	0	0	2 ++	1 ++	0	0	0	1 +
4	0	2 +++	2 +	1 +	0	0	0	0
5	0	2 +++	2 ++	0	0	0	0	2 +
6	0	2 ++	2 +	0	0	0	0	2 ++
7	0	2 ++	0	0	0	0	0	0
8	0	2 +	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0
28	0	0	0	0	0	0	0	0
35	0	0	0	0	0	0	0	0
42	0	0	0	0	0	0	0	0

0 = edema absent

+ = edema is present

1 = unilateral

2 = bilateral

Green box + = mild edema

Blue box ++ = moderate edema

Red box +++ = severe edema

Front and hind limb edema were observed in 3 of 4 stallions from group A (L138, L140, and L141; 75%) and in all (L137, L139, L142, and L143; 100%) group B stallions (Table 2.5.4.). The stallion belonging to group A (L136) who did not develop any periobirtal edema also did not develop any edema of the front and hind limbs. Group A and group B stallions developed front limb edema starting from 7-8 dpi and lasting until 13-15 dpi, and from 1-8 dpi to 11-14 dpi, respectively. Hind limb edema was seen in stallions from group A starting from 0-7 dpi and lasting to at least 15-42 dpi.

Table 2.5.4. Edema of front (A) and hind (B) limbs of group A and group B stallions before and after inoculation with the KY84 strain of equine arteritis virus.

A.

Days Post Inoculation (dpi)	Group A Stallion's IDs				Group B Stallion's IDs			
	L136	L138	L140	L141	L137	L139	L142	L143
-7	0	0	0	0	0	0	0	0
-5	0	0	0	0	0	0	0	0
-2	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0
2	0	0	0	0	2 +	0	0	0
3	0	0	0	0	2 +	0	0	0
4	0	0	0	0	2 +	0	0	0
5	0	0	0	0	2 +	0	0	0
6	0	0	0	0	2 ++	0	0	1 +
7	0	2 +	2 ++	0	2 ++	0	0	1 ++
8	0	2 +++	2 ++	2 ++	2 +++	1 ++	1 ++	2 +
9	0	2 +++	2 ++	2 ++	2 +++	1 ++	1 +	1 +
10	0	2 ++	1 ++	2 ++	2 ++	2 ++	0	2 +
11	0	2 ++	1 +	2 ++	2 ++	2 ++	0	1 +
12	0	2 +	1 +	2 ++	2 ++	0	0	0
13	0	2 +	1 +	2 ++	2 ++	0	0	0
14	0	2 +	0	2 +	2 ++	0	0	1 +
15	0	2 +	0	1 +	2 ++	0	0	0
21	0	0	0	0	0	0	0	0
28	0	0	0	0	0	0	0	0
35	0	0	0	0	0	0	0	0
42	0	0	0	0	0	0	0	0

Table 2.5.4. (continued)

B.

Days Post Inoculation (dpi)	Group A Stallion's IDs				Group B Stallion's IDs			
	L136	L138	L140	L141	L137	L139	L142	L143
-7	0	0	0	0	0	0	0	0
-5	0	0	0	0	0	0	0	0
-2	0	0	0	0	0	0	0	0
0	0	0	0	1 +	0	2 +	0	0
1	0	0	0	1 +	0	2 +	0	0
2	0	0	2 ++	1 +	0	2 +	0	0
3	0	0	1 +	1 +	0	2 +	0	0
4	0	0	2 ++	1 ++	0	2 +	0	2 ++
5	0	0	2 ++	1 ++	0	2 ++	0	1 ++
6	0	0	2 ++	1 ++	2 +	2 ++	0	1 ++
7	0	1 ++	2 ++	1 ++	2 ++	2 ++	2 +	2 ++
8	0	2 ++	2 +++	2 ++	2 +++	2 ++	2 ++	2 ++
9	0	2 ++	2 +++	2 ++	2 +++	2 ++	2 ++	2 ++
10	0	1 +	2 +++	2 ++	2	2 ++	2 +	2 ++
11	0	1 +	2 ++	2 ++	2	2 ++	2 +	2 ++
12	0	1 +	2 ++	1 +	2	2 ++	0	2 ++
13	0	2 +	2 ++	1 +	2	2 ++	0	2 ++
14	0	1 +	2 ++	2 ++	2	2 ++	0	2 +
15	0	2 +	2 ++	1 +	2 +	2 ++	0	2 +
21	0	0	2 +	1 ++	2 +	2 +	0	1 +
28	0	0	0	1 ++	0	0	0	0
35	0	0	0	1 ++	0	0	0	0
42	0	0	0	1 ++	0	0	0	0

0 = edema absent

+ = edema is present

1 = unilateral

2 = bilateral

Green box + = mild edema

Blue box ++ = moderate edema

Red box +++ = severe edema

Five out of 8 stallions (group A: L138, L140, and L141; and group B: L137 and L139) developed moderate to severe scrotal and preputial edema. Three of the group A stallions (L136, L138, and L140; 75%) had edema of the scrotum/prepuce starting from 4-6 dpi to 13-15 dpi (3-9 days). Two stallions belonging to group A (L138 and L140) had severe scrotal/preputial edema for 4-6 days (starting from 7-11 and lasting until 11-12 dpi). Stallion L141 did not develop any dependent edema of the prepuce or scrotum.

In stallions from group B, preputial and scrotal edema were observe only in 2 out of 4 animals (L137 and L139; 50%) starting from 4-6 dpi and lasting until 13-15 dpi (10 days). In this group of stallions severe preputial and scrotal edema was developed only in stallion L137 and lasted for 5 days (Table 2.5.5.).

Table 2.5.5. Scrotal and preputial edema of group A and group B stallions before and after inoculation with the KY84 strain of equine arteritis virus.

Days Post	Group A Stallion's IDs				Group B Stallion's IDs			
Inoculation (dpi)	L136	L138	L140	L141	L137	L139	L142	L143
-7	0	0	0	0	0	0	0	0
-5	0	0	0	0	0	0	0	0
-2	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0
4	0	0	0	0	0	+	0	0
5	0	0	0	0	0	+	0	0
6	0	0	++	0	++	+	0	0
7	++	+	+++	0	++	++	0	0
8	++	+++	+++	0	+++	++	0	0
9	++	+++	+++	0	+++	++	0	0
10	0	+++	+++	0	+++	++	0	0
11	0	+++	+++	0	+++	++	0	0
12	0	++	+++	0	+++	+	0	0
13	0	++	++	0	++	+	0	0
14	0	++	+	0	++	0	0	0
15	0	++	0	0	++	0	0	0
21	0	0	0	0	0	0	0	0
28	0	0	0	0	0	0	0	0
35	0	0	0	0	0	0	0	0
42	0	0	0	0	0	0	0	0

+ = edema is present

0 = edema absent

Green box + = mild edema

Blue box ++ = moderate edema

Red box +++ = severe edema

Stallions belonging to group A and group B developed a mucopurulent nasal discharge during the acute phase of EAV infection (Figure 2.5.3.).

Figure 2.5.3. Representative picture of mucopurulent nasal discharge following experimental inoculation of stallions with equine arteritis virus. Stallion L142, at 3 dpi.

Three out of 4 stallions from group A (L136, L138, and L140; 75%) had a mild mucopurulent nasal discharge starting from 5-6 dpi until 5-10 dpi (1 to 3 days) (Table 2.5.6.). Group B stallions also had 3 stallions (L139, L142, and L143; 75%) with mild to moderate mucopurulent nasal discharge for 2-10 days (from 3-7 dpi to 8-13 dpi).

Table 2.5.6. Mucopurulent nasal discharge in group A and group B stallions before and after inoculation with the KY84 strain of equine arteritis virus.

Days Post Inoculation (dpi)	Group A Stallion's IDs				Group B Stallion's IDs			
	L136	L138	L140	L141	L137	L139	L142	L143
-7	0	0	0	0	0	0	0	0
-5	0	0	0	0	0	0	0	0
-2	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	1 ++	0
4	0	0	0	0	0	0	2 +	0
5	0	1 +	0	0	0	0	2 ++	0
6	0	0	2 +	0	0	2 ++	2 ++	0
7	2 +	0	1 +	0	0	0	2 +	2 +
8	2 +	0	2 +	0	0	2 +	2 ++	2 +
9	0	0	0	0	0	1 +	2 +	0
10	1 +	0	0	0	0	0	0	0
11	0	0	0	0	0	2 +	2 +	0
12	0	0	0	0	0	1 +	1	0
13	0	0	0	0	0	1 +	1 +	0
14	0	0	0	0	0	1 +	0	0
15	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0
28	0	0	0	0	0	0	0	0
35	0	0	0	0	0	0	0	0
42	0	0	0	0	0	0	0	0

0 = mucopurulent nasal discharge is absent

+ = mucopurulent nasal discharge is present

1 = unilateral

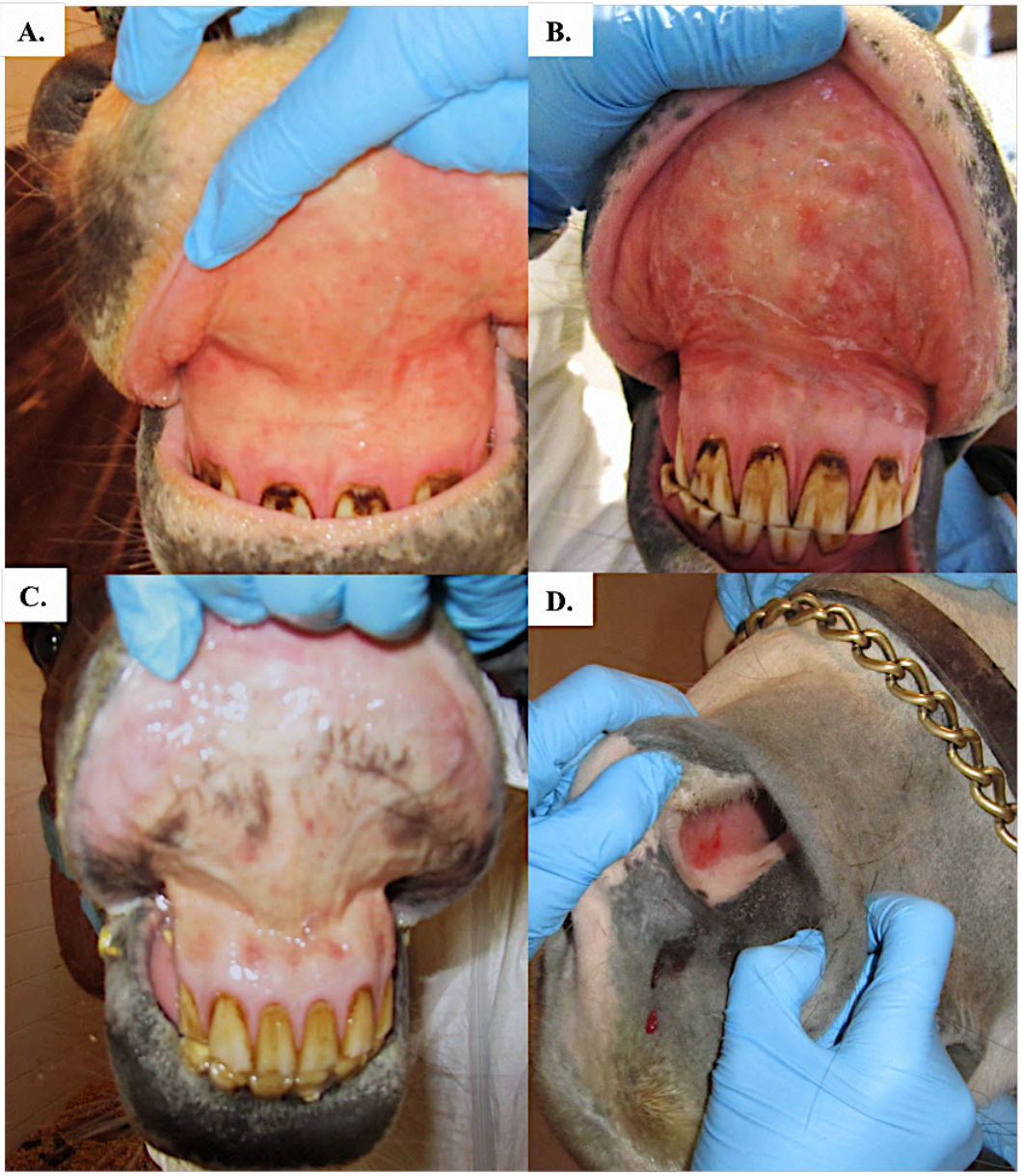
2 = bilateral

Green box + = mild mucopurulent nasal discharge

Blue box ++ = moderate mucopurulent nasal discharge

Inoculated stallions showed varying degrees of alteration of the oral mucosa during the acute phase of EAV infection (Figure 2.5.4.).

Figure 2.5.4. Representative pictures of changes in oral mucosa following experimental inoculation of stallions with the KY84 strain of equine arteritis virus. (A) congested oral mucosa (stallion L138; 6 dpi); (B) petechiae (stallion L140; 5 dpi); (C) ecchymosis (stallion L141; 5 dpi); and, (D) nasal hemorrhage (stallion L140; 5 dpi).



The most accentuated changes were seen in stallions from group A (Table 2.5.7.). In this group, 2 stallions (L136 and L140; 50%) had petechiae in their oral mucosa from 5-6 dpi to 6-7 dpi (2 days); 1 stallion (L141; 25%) developed ecchymosis for 7 days (4-10 dpi); and, another stallion (L138; 25%) had congested oral mucosa for 2 days (6-7 dpi). Interestingly, stallion (L140) had a mild nasal hemorrhage at 5, 6, and 7 dpi. In group B stallions such alterations were not observed, and only 2 out of 4 animals (L139 and L143; 50%) had oral mucosal congestion for 1-2 days (from 6-7 dpi).

Table 2.5.7. Appearance of oral mucosa in group A and group B stallions before and after inoculation with the KY84 strain of equine arteritis virus.

Days Post	Group A Stallion's IDs				Group B Stallion's IDs			
Inoculation (dpi)	L136	L138	L140	L141	L137	L139	L142	L143
-7	0	0	0	0	0	0	0	0
-5	0	0	0	0	0	0	0	0
-2	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0
4	0	0	0	Ecchymosis	0	0	0	0
5	0	0	Petechiae	Ecchymosis	0	0	0	0
6	Petechiae	Congested	Petechiae	Ecchymosis	0	Congested	0	Congested
7	Petechiae	Congested	0	Ecchymosis	0	0	0	Congested
8	0	0	0	Ecchymosis	0	0	0	0
9	0	0	0	Ecchymosis	0	0	0	0
10	0	0	0	Ecchymosis	0	0	0	0
11	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0
28	0	0	0	0	0	0	0	0
35	0	0	0	0	0	0	0	0
42	0	0	0	0	0	0	0	0

0 = mucosa pigmentation within normal limits

All stallions from both group showed a decrease in libido during the acute phase of infection. The time to drop the penis as well as the number of times each stallion had to jump the mare or the phantom for ejaculation increased. All the clinical signs along

with the moderate to severe edema in hind limbs of the stallions would have made it difficult for them to mount, which contributed to the increased time to obtain the ejaculate. However, none of seven stallions failed to mount the mare or the phantom. One stallion from group B (L142) failed to ejaculate and instead, provided only the pre-ejaculatory fluid at 1 dpi. One stallion from group B (L143) always had very low libido and was never able to ejaculate, even though serum testosterone concentrations were within normal limits (data not showed). All stallions seroconverted to EAV starting from 8 dpi and maintained high neutralizing antibody titers in serum until at least 42 dpi (1:64 to > 1:512).

Lymphocyte count

The number of lymphocytes across all stallions from each group was averaged for each blood sample collection and compared to the baseline average before inoculation. All stallions from both groups had on average, a baseline value within normal limits for the number of lymphocytes (1.8-5.7 K/µL), with the exception of animal L143 from group B, which showed a slightly low lymphocyte baseline mean of 1.3 K/µL previous to experimental inoculation with the KY84 EAV (Table 2.5.8.) All stallions had a significant decrease ($p < 0.05$) in the number of lymphocytes following inoculation of the animals with the virus. However, one stallion (L136) belonging to group A, had lymphocyte counts within normal limits throughout the experiment.

Table 2.5.8. Lymphocyte counts (K/µL) in group A and group B stallions before and after inoculation with the KY84 strain of equine arteritis virus.

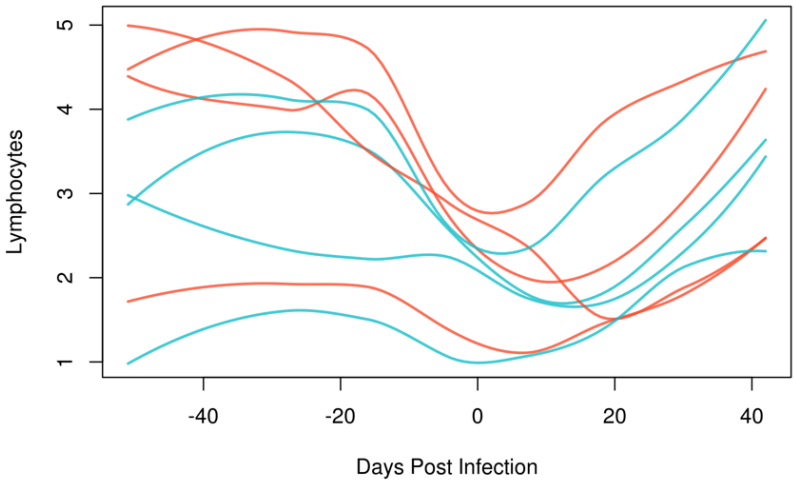
Days Post Inoculation (dpi)	Group A Stallion's IDs				Group B Stallion's IDs			
	L136	L138	L140	L141	L137	L139	L142	L143
-51	4.6	5	5.2	1.6*	2.9	3	3.6	1.1*
-44	4.7	3.3	4.5	2	3.2	2.6	4.8	1.0*
-36	4.5	3.7	4.8	2.1	3.8	2.8	3.6	1.7*
-29	5	4	4.5	1.6*	3.6	2.1	4.1	1.4*
-15	4.7	4.2	4.3	1.9	3.5	2.3	3.8	2
-8	4.2	3.7	1.7*	1.8	3	2.1	3.8	0.7*
0	2.7	2.6	3.2	1.3*	2.8	1.9	2.2	1.4*
2	2.4	1.7*	3.3	1.6*	2.2	3.1	2.2	1.3*
4	2.5	1.9	2.9	0.8*	1.7*	2	1.8	0.8*
6	3	1.9	2	0.9*	1.3*	1.3*	2.6	0.8*
8	2.1	1.9	1.3*	0.7*	1.4*	1.2*	1.3*	0.6*
10	4.2	2.3	2.2	0.7*	1.5*	1.5*	3.7	1.2*
12	2.6	1.8	2.2	1.9	2.9	1.5*	2.6	1.5*
14	4.5	2.7	1.9	1.8	1.5*	2.2	3.3	1.3*
21	3.2	1.6*	1.5*	1.2*	1.4*	1.8	2.9	1.3*
28	4.9	2.8	1.3*	1.7*	2.8	2.1	3.9	2.1
35	4.1	3.9	3	2	3.2	2.6	4.2	2.7
42	4.8	4	2.1	2.5	3.5	3.5	5.1	2.1

Green box * = low lymphocyte counts (normal range: 1.8-5.7 K/µL)

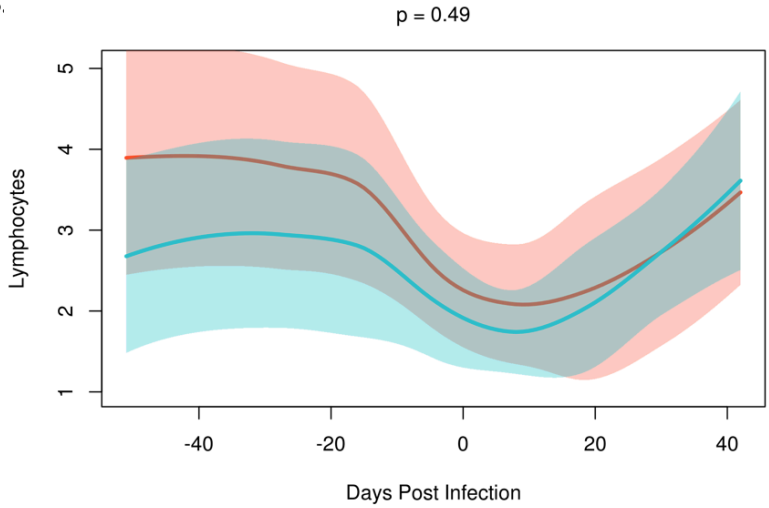
On average, a decrease in the number of lymphocytes following experimental infection of the stallions with the KY84 strain of EAV was observed between 8 and 28 dpi for group A and group B experimentally infected stallions ($p<0.05$) and no significant differences were seen between the groups (Figure 2.5.5.).

Figure 2.5.5. Lymphocytes count before and after inoculation of the stallions with the KY84 strain of equine arteritis virus. (A) Individual lymphocyte values from group A (red) and, group B (blue) stallions. (B) Mean lymphocyte counts from group A (red) and B (blue) stallions. The shaded area corresponds to the 95% C.I.

A.



B.



Virus Isolation

All eight stallions inoculated with EAV KY84 shed virus in nasal secretions, PBMCs, and semen (with the exception of stallion L143 from who semen assessment was not possible, as mentioned above) during the acute phase of EAV infection. Virus titers in nasal swab secretions, PBMCs and semen, across all stallions within each group were averaged for each time point. Virus titers varying between 1 and 5.7×10^4 PFU/mL in group A nasal swab secretions from 2 to 10-21 dpi (9-20 days), and in group B stallions titers between 1 and 4.2×10^4 PFU/mL from 2 to 10-14 dpi (9-13 days) (Table 2.5.9.).

Table 2.5.9. Results of attempted virus isolation from nasal swabs collected from group A and group B stallions before and after experimental inoculation with the KY84 strain of equine arteritis virus.

Days Post	Group A Stallion's IDs				Group B Stallion's IDs			
Inoculation (dpi)	L136	L138	L140	L141	L137	L139	L142	L143
0	0	0	0	0	0	0	0	0
2	++	++	++	++	++	++	++	++
4	++	++	++	++	++	++	++	++
6	++	++	++	++	++	++	++	++
8	++	++	++	++	++	++	++	++
10	++	++	++	++	++	++	++	++
12	++	++	0	++	§	++	++	++
14	0	++	0	++	0	++	0	0
21	0	0	0	++	0	0	0	0
28	0	0	0	0	0	0	0	0
35	0	0	0	0	0	0	0	0
42	0	0	0	0	0	0	0	0

Days Post	Group A Stallion's IDs				Group B Stallion's IDs			
Inoculation (dpi)	L136	L138	L140	L141	L137	L139	L142	L143
0	0	0	0	0	0	0	0	0
2	4.20E+02	4.90E+02	4.20E+02	1.52E+02	6.90E+01	2.00E+00	2.10E+01	1.64E+02
4	1.33E+03	1.25E+04	4.10E+03	1.74E+03	7.60E+03	1.40E+02	5.80E+03	1.78E+03
6	8.50E+03	2.23E+03	1.42E+04	5.70E+04	1.58E+04	1.28E+04	8.20E+03	4.20E+04
8	1.21E+02	1.30E+03	1.07E+03	1.06E+04	5.10E+03	1.52E+03	1.57E+02	1.53E+02
10	4.40E+01	6.50E+01	8.00E+00	1.79E+02	3.70E+01	1.63E+02	4.00E+00	3.10E+01
12	4.00E+00	5.00E+00	0	6.00E+00	§	7.20E+01	1.00E+00	6.00E+00
14	0	1.00E+00	0	1.00E+00	0	2.00E+00	0	0
21	0	0	0	1.00E+00	0	0	0	0
28	0	0	0	0	0	0	0	0
35	0	0	0	0	0	0	0	0
42	0	0	0	0	0	0	0	0

0 Virus isolation negative in cell culture

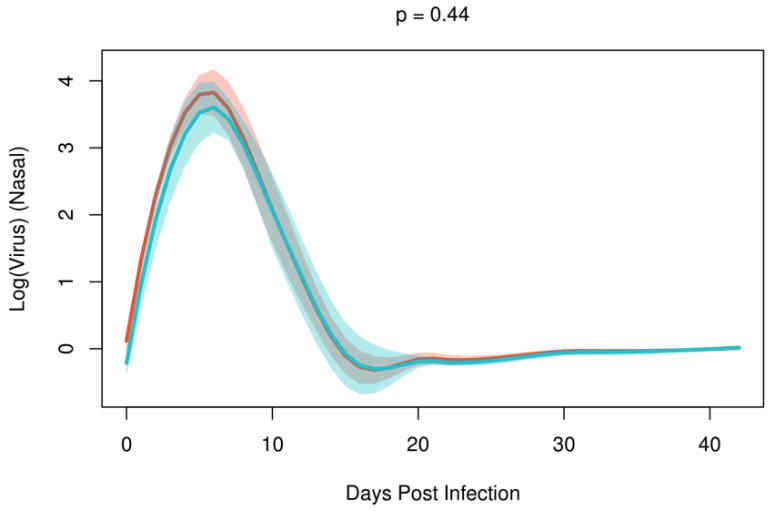
++ Virus isolation positive on first passage in cell culture

§ Virus isolation positive only after second passage in cell culture

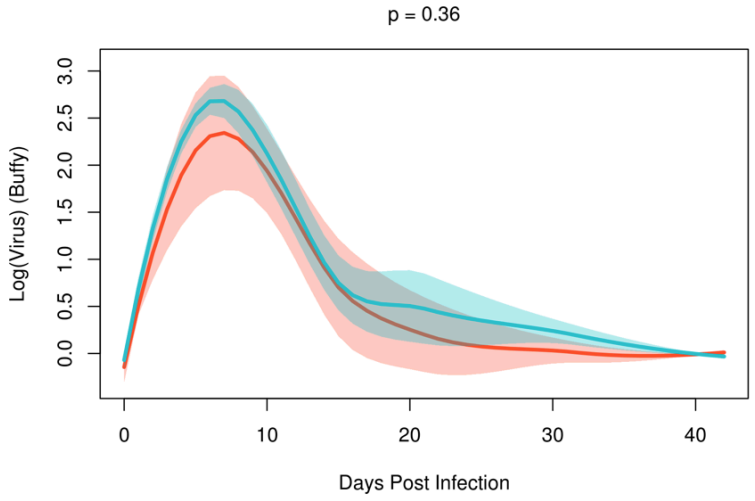
On average, the highest virus titer in nasal swab was seen at 6 dpi in group A (2.05×10^4 PFU/mL) and group B (1.97×10^4 PFU/mL) stallions, and no statistical differences in nasal swab viral shedding were found between the two groups at any time point (Figure 2.5.6.).

Figure 2.5.6. Mean viral titers (PFU/mL) in collected samples following inoculation of the stallions with the KY84 strain of equine arteritis virus. (A) Nasal swab secretions (NS); (B) PBMCs; and, (C) Semen samples (SS). The shaded area corresponds to the 95% C.I.

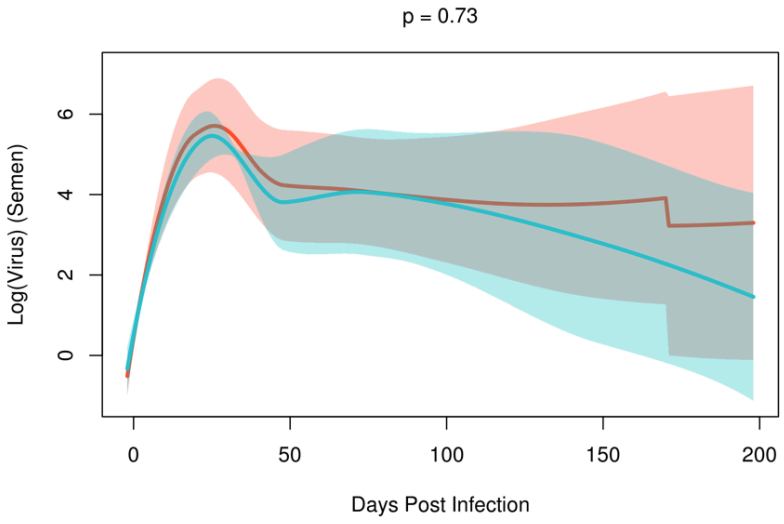
A. NS



B. PBMCs



C. SS



Similarly, on average, no significant differences were seen in virus titers detected in PBMCs, with highest titers (group A: 1.49×10^3 PFU/mL and group B: 1.10×10^3 PFU/mL) being observed in both groups at 6 dpi (Figure 2.5.6.). Group A stallions circulated virus (1 to 5.1×10^3 PFU/mL) in PBMCs from 13-27 days (2 to 14-28 dpi) and all group B stallions circulated virus in PBMCs for 27 days (2-28 dpi) and titer varied from 1 to 1.45×10^3 PFU/mL (Table 2.5.10.).

Table 2.5.10. Results of attempted virus isolation from PBMC samples from group A and group B stallions before and after experimental inoculation with the KY84 strain of equine arteritis virus.

Days Post Inoculation (dpi)	Group A Stallion's IDs				Group B Stallion's IDs			
	L136	L138	L140	L141	L137	L139	L142	L143
0	0	0	0	0	0	0	0	0
2	++	++	++	++	++	++	++	++
4	++	++	++	++	++	++	++	++
6	++	++	++	++	++	++	++	++
8	++	++	++	++	++	++	++	++
10	++	++	++	++	++	++	++	++
12	++	++	++	++	++	++	++	++
14	++	++	++	++	++	++	++	++
21	0	§	++	++	++	++	§	++
28	0	§	++	++	++	++	++	++
35	0	§	0	§	§	§	0	0
42	0	0	0	0	§	0	0	0

Days Post Inoculation (dpi)	Group A Stallion's IDs				Group B Stallion's IDs			
	L136	L138	L140	L141	L137	L139	L142	L143
0	0	0	0	0	0	0	0	0
2	1.40E+01	4.00E+00	3.00E+00	7.00E+00	4.10E+01	8.00E+00	2.50E+01	1.20E+01
4	2.50E+00	5.10E+02	2.90E+01	9.50E+01	1.38E+02	5.30E+01	1.24E+02	7.70E+01
6	1.33E+02	5.10E+03	1.39E+02	5.70E+02	8.80E+02	1.45E+03	1.27E+03	8.00E+02
8	8.70E+01	3.90E+02	9.00E+01	6.00E+02	7.20E+02	1.33E+03	1.28E+02	2.15E+02
10	1.40E+01	6.30E+01	1.40E+01	1.15E+02	1.11E+02	1.50E+02	2.80E+01	6.20E+01
12	3.60E+01	2.10E+01	3.00E+00	7.60E+01	1.00E+01	2.60E+01	1.00E+01	1.40E+01
14	1.00E+00	1.00E+00	4.00E+00	4.60E+01	6.00E+00	1.70E+01	2.00E+00	1.00E+01
21	0	§	1.00E+00	4.00E+00	5.00E+00	1.00E+01	§	2.00E+00
28	0	§	1.00E+00	1.00E+00	3.00E+00	1.00E+00	1.00E+00	2.00E+00
35	0	§	0	§	§	§	0	0
42	0	0	0	0	§	0	0	0

0 Virus isolation negative in cell culture

++ Virus isolation positive on first passage in cell culture

§ Virus isolation positive only after second passage in cell culture

On average, no significant differences were seen in virus titers shed in semen from group A and group B stallions at different time points (Figure 2.5.6.); the highest virus titers detected in semen within group A animals was 5.35×10^6 PFU/mL at 9 dpi and within group B animals was 1.05×10^6 PFU/mL at 7 dpi. Stallions from both groups started shedding virus in semen at 5 dpi; an exception was one group A stallion (L141)

which shed detectable virus as early as 3 dpi. Seminal virus titers from group A stallions varied from 1 to 1.88×10^7 PFU/mL and for group B stallions from 1 to 3.05×10^6 PFU/mL (Table 2.5.11.). All stallions from group A and B shed virus in semen until at least 107 dpi (3.5 months). One stallion belonging to group A (L138; 25%) stopped shedding virus somewhere between 107-128 dpi (3.5 and 4 months). Stallions L136, L140, and L141 remained carriers of EAV until the end of the experiment (198 dpi; 6.5 months). Two out of three stallions from group B (L137 and L142; 66.6%) stopped shedding virus in their semen sometime between 128-149 dpi and 170-198 dpi, respectively. Stallion L139 (33.3%) remained infected until the end of the study, at 198 dpi (6.5 months). Interestingly, this stallion had a serious hind limb injury and started being treated with a non-sterirodal anti-inflammatory (phenylbutazone) from 76 until 198 dpi. Stallion L143, as mentioned before, never ejaculated and unfortunately no semen could be obtained to check the status of virus shedding in his semen.

Table 2.5.11. Results of attempted virus isolation from gel-free semen samples of group A and group B stallions before and after experimental inoculation with the KY84 strain of equine arteritis virus.

Days Post Inoculation (dpi)	Group A Stallion's IDs				Group B Stallion's IDs			
	L136	L138	L140	L141	L137	L139	L142	L143
-2	0	0	0	0	0	0	0	-
1	0	0	0	0	0	0	0	-
3	0	0	0	§	0	0	0	-
5	++	++	++	++	++	++	++	-
7	++	++	++	++	++	++	++	-
9	++	++	++	++	++	++	++	-
11	++	++	++	++	++	++	++	-
13	++	++	++	++	++	++	++	-
15	++	++	++	++	++	++	++	-
23	++	++	++	++	++	++	++	-
44	++	++	++	++	++	++	++	-
65	++	++	++	++	++	++	++	-
86	++	++	++	++	++	++	++	-
107	++	++	++	++	++	++	++	-
128	++	0	++	++	§	++	++	-
149	++	0	++	++	0	++	++	-
170	++	0	++	++	0	++	++	-
198	***	0	++	++	0	++	0	-

Days Post Inoculation (dpi)	Group A Stallion's IDs				Group B Stallion's IDs			
	L136	L138	L140	L141	L137	L139	L142	L143
-2	0	0	0	0	0	0	0	-
1	0	0	0	0	0	0	0	-
3	0	0	0	§	0	0	0	-
5	2.50E+01	1.20E+01	1.00E+01	3.60E+02	1.00E+01	4.00E+01	4.50E+01	-
7	5.20E+06	4.60E+05	2.30E+05	1.21E+05	1.99E+03	9.30E+04	3.05E+06	-
9	3.35E+05	1.88E+07	1.71E+05	2.09E+06	4.10E+05	1.88E+06	6.90E+05	-
11	6.10E+04	5.90E+04	5.60E+02	2.88E+05	1.77E+05	1.33E+06	8.90E+03	-
13	1.72E+05	2.70E+03	1.50E+03	2.80E+05	3.20E+04	2.04E+05	9.50E+02	-
15	1.81E+05	6.45E+02	4.00E+03	9.40E+05	5.50E+03	2.27E+04	8.00E+02	-
23	8.80E+05	6.95E+02	1.07E+04	2.18E+05	2.45E+04	7.60E+03	2.14E+03	-
44	1.84E+05	6.00E+02	4.80E+03	2.18E+05	7.00E+02	1.20E+03	1.90E+05	-
65	3.52E+05	5.00E+02	1.20E+03	1.04E+05	8.00E+03	2.50E+03	2.37E+05	-
86	4.10E+05	8.00E+01	1.60E+04	5.20E+04	1.60E+03	1.20E+03	5.50E+05	-
107	2.90E+04	6.00E+01	1.14E+04	7.20E+04	8.00E+01	2.07E+04	9.10E+04	-
128	9.40E+04	0.00E+00	3.40E+04	5.90E+04	§	1.96E+04	4.70E+04	-
149	5.10E+05	0.00E+00	3.60E+04	6.10E+04	0	4.10E+03	6.70E+03	-
170	8.00E+05	0.00E+00	1.26E+05	9.60E+04	0	6.20E+04	3.20E+03	-
198	***	0.00E+00	9.50E+03	6.70E+05	0	8.20E+03	0	-

0 Virus isolation negative in cell culture

++ Virus isolation positive on first passage in cell culture

§ Virus isolation positive only after second passage in cell culture

- Stallion failed to ejaculate

*** Missing sample - stallion was confirmed to be a carrier at 254 dpi

2.6. DISCUSSION

The objective of the present study was to investigate whether there is a possible association between susceptibility of $CD3^+$ T lymphocytes and the establishment of virus persistence in experimentally EAV infected stallions. Eight seronegative EAV stallions were divided into two groups according to their susceptible/resistant phenotype (group A = susceptible [n=4], and group B = resistant [n=4]) and intranasally inoculated with the KY84 strain of EAV. Following KY84 EAV infection, the animals were monitored for the development of clinical signs of EVA, circulation of the virus in PBMCs, and shedding of the virus in nasal secretions and semen samples. The stallions were also monitored for the establishment of the carrier state for a period of 6.5 months. In contrast to the results of Go et al. (Go et al., 2012), in which clinical response and viremia burdens were more pronounced in the group of stallion with the $CD3^+$ T lymphocytes resistant phenotype, in the present study, no differences in clinical response, shedding of virus in nasal secretions and viremia were observed between the groups. All the stallions in both groups developed clinical signs characteristic of EVA and the severity of these changes did not vary much between the two groups. It is possible that the conflicting results regarding development of clinical signs are the result of using different virus strains for the experimental infection of the animals. Also, the viral titer in the semen of the experimentally infected stallions during the acute phase of the infection was not significantly different between group A (susceptible) and group B (resistant) stallions. Establishment of the carrier state seems to be associated with the susceptibility of $CD3^+$ T lymphocytes to *in vitro* EAV infection. Three (L136, L140, L141) out of 4 stallions (75%) belonging to group A (susceptible) became carriers of EAV and were still

shedders of high titers of the virus, at least until 6.5 months following infection. However, only one stallion out of the 3 (L139; 33%) that had a $CD3^+$ T lymphocyte resistant phenotype was still a carrier of EAV at 6.5 months after infection. Even though a complete association between the $CD3^+$ T lymphocyte resistant/susceptible phenotype and the establishment of the carrier state in stallion was not conclusively established as indicated in the retrospective study performed by Go et al., the results of both studies suggest that stallions that have a susceptible $CD3^+$ T cell phenotype are at higher risk of becoming persistently infected than the stallions with $CD3^+$ T cells resistant to *in vitro* EAV infection. The flow cytometric analysis findings in this study did not provide a perfect prediction of whether stallions exposed to EAV will or will not become a carrier and shedder of the virus in semen. Interestingly, the only stallion that had a $CD3^+$ T cell resistant phenotype (L139) and was a carrier at 6.5 months post-infection (198 dpi), developed an injury in one of his hind limbs and was treated with a non-steroidal anti-inflammatory drug (phenylbutazone) from 76 until 198 dpi. Perhaps, the treatment had an influence in the EAV persistence in the reproductive tract of this stallion, and therefore, he shed virus in semen for longer compared to the other stallions belonging to the same group (group B). Another possible reason for this stallion to keep shedding virus in semen could be that 198 days was not enough time for him to clear the virus from the reproductive tract, and maybe further semen collections (> 6.5 months after EAV infection) would result in a negative virus isolation test and clearance of the carrier state. The partial association between $CD3^+$ T cell phenotype and the establishment of viral persistence in the reproductive tract of stallion observed in this study could also be explained by the fact that the stallions were experimentally infected with EAV rather than

naturally exposed to the virus. The percentage of the $CD3^+$ T lymphocyte population that was susceptible to EAV infection in group A stallions ranged from 3.15% to 8.9%. Interestingly, the only stallion that belonged to group A ($CD3^+$ T lymphocyte susceptible phenotype) and cleared virus from the semen, had only 3.15% of the lymphocyte cells infected *in vitro*. The carrier stallions evaluated by Go et al. (2012) were all $CD3^+$ T cell susceptible, but all had at least 4% of the $CD3^+$ T lymphocytes *in vitro* infected by EAV VBS. Perhaps, there is a minimal amount of CD^+ T lymphocytes that need to be infected *in vitro* for a stallion to become a long-term carrier of EAV.

Recently, it was demonstrated in a genome-wide association study (GWAS) that differences in the $CD3^+$ T cells susceptibility are under genetic control since genes located within the region of equine chromosome 11 (ECA11) positions 49572804 to 49643932 are associated with the *in vitro* susceptible $CD3^+$ T cell phenotype (Go et al., 2011). The authors focused on Thoroughbred horses since they have less diversity for genetic markers than most other breeds of horses. In the present study, horses of mixed breeds were used, and a possible explanation for the fact that a $CD3^+$ T cell resistant stallion was still a shedder at 6.5 months after infection could be that the genes of this stallion that are responsible for the trait underwent mutation during evolution and so he lacks susceptibility to *in vitro* EAV infection.

It is possible to conclude form this pilot study, that horses with a CD^+ lymphocyte susceptible phenotype seem to be at higher risk of becoming carrier stallions upon infection with EAV. However, further studies to elucidate persistence of EAV infection in the stallion are needed.

CHAPTER THREE

Evaluation of semen quality of stallions challenged with the Kentucky 84 (KY84) strain of EAV

3.1. SUMMARY

Equine arteritis virus (EAV) is the causal agent of equine viral arteritis (EVA), a respiratory and reproductive disease of equids. Some strains of EAV cause fever and scrotal and preputial edema during acute phase of infection. We hypothesized that fever and scrotal edema observed during the acute phase of the infection will have a significant effect on semen quality. A group of seven stallions were intranasally inoculated with the KY84 strain of EAV. Stallions were monitored for clinical signs of EVA until 42 days post infection (dpi). Semen was collected every other day for the first 15 days and 2 times a week up to 79 dpi. Three additional samples were collected at 147, 149 and 151 dpi. Semen from each stallion was evaluated on the basis of motility, total number of spermatozoa, curvilinear velocity, membrane integrity and morphology. EAV titers in semen samples were determined in RK-13 cells. There were significant decreases in sperm quality from 11 to 76 dpi. Loess curves for each horse were fit and integrated to quantify fever, virus and edema exposure over the 67 days period prior to each ejaculation. Linear mixed models were then fit to isolate the effects of each factor on semen quality. These models demonstrated strong evidence ($p \leq 0.002$) that edema and fever exert independent effects on all the semen quality parameters, but virus seems to exert little to no direct effect, as virus titers remained high long after semen quality returned to baseline.

3.2. INTRODUCTION

Equine arteritis virus (EAV) is the causal agent of equine viral arteritis (EVA), a respiratory and reproductive disease of equids. EAV was first isolated from a lung of an aborted fetus in 1953 following an extensive outbreak of respiratory disease and abortion in a Standardbred breeding farm at Bucyrus, Ohio (Doll et al., 1957a; Doll et al., 1957b). The virus is transmitted mainly by respiratory (Doll et al., 1957b; McCollum et al., 1971) and venereal routes (Timoney, McCollum, Roberts, et al., 1986). Most EAV strains cause subclinical infection following infection of horses but some strains can cause moderate to severe clinical signs (Prickett et al., 1972; Timoney and McCollum, 1993a; McCollum et al., 1995; MacLachlan et al., 1996; Balasuriya et al., 1998, 2007; Patton et al., 1999; Balasuriya, 2012; Zhang et al., 2012). The clinical signs of EVA are characterized by fever, nasal and ocular discharge, conjunctivitis, depended edema, leukopenia and abortion (Doll, et al. 1957b; McCollum et al., 1971, 1995). The 1984 EVA outbreak in Thoroughbred breeding farms generated widespread interest and major concern to the equine industry (Timoney and McCollum, 1993a). Epidemiological data collected from that outbreak strongly suggested the establishment of a long-term persistence of EAV in stallions and the importance of the carrier stallion in the dissemination and perpetuation of the virus within the horse population (Timoney and McCollum, 1985). Following EAV infection, a variable proportion of stallions (30-70%) can become carriers and continuously shed the virus in their semen for varying time periods (Neu and Timoney, 1988; Timoney et al., 1986, 1987). The virus persists in the male reproductive tract, specifically in the accessory glands with highest titers being found in the ampulla of the vas deferens (Neu and Timoney, 1988), despite the presence

of high titer neutralizing antibodies in serum (Neu and Timoney, 1988; Timoney et al., 1986, 1987). The mechanism of EAV persistence in the reproductive tract of stallions is unclear, however it has been shown that establishment and maintenance of infection is testosterone dependent (Little et al., 1992; Holyoak et al., 1993; McCollum et al., 1994). Isolation of EAV from the pre-ejaculatory fluid from the semen of persistently infected stallions was not successful and thus, virus is believed to be associated with the sperm-rich fraction of the ejaculate (Timoney et al., 1987). Persistently infected stallions play a major role in the transmission of EVA to mares during natural breeding and artificial insemination with fresh, cooled and frozen semen (Balasuriya et al., 1998, 1999; Timoney et al., 1986, 1987). Recently, it has been demonstrated that under experimental conditions, EAV can be transmitted to naïve recipient mares via embryo transfer from a donor mare inseminated with EAV-infective semen (Broaddus et al., 2011).

Several viruses have been detected in the semen of a number of animal species, some of which can exert direct or indirect detrimental consequences to the male reproductive tract as well as to the semen (Sur et al., 1997; Dejucq and Jégou, 2001). Little is known about the effects of EAV on the semen quality of stallions during acute and persistent infection. Semen quality of stallions experimentally infected with EAV was decreased for a period of time after inoculation with the virus but it was not clear if the negative effect on semen parameters was due to the direct effect of the presence of the virus in semen (Neu et al., 1992). Thus, the objectives of this study were to determine: 1) if there is a significant effect on semen parameters of stallions experimentally infected with the KY84 strain of EAV; 2) if the possible changes observed in the semen quality result from direct effects of the virus present in semen or from the indirect effects of

clinical signs (fever and scrotal edema) during the acute phase of infection with EAV. The KY84 strain of EAV has been shown to establish persistent infection in the reproductive tract of stallions and to cause moderate clinical signs of EVA infection in horses (Neu and Timoney, 1988).

3.3. MATERIAL AND METHODS

Cells and viruses

The high passage rabbit kidney cell line (RK-13 KY; passage level 399-409) was maintained in Eagle's minimum essential medium (EMEM; Mediatech, Manassas, VA) supplemented with 10% ferritin-supplemented bovine calf serum (Hyclone Laboratories, Logan, UT), 100 U/ml penicillin/streptomycin (Mediatech, Manassas, VA) and 1 μg/ml amphotericin B (Sigma-Aldrich, St. Louis, MO). The virulent KY84 strain of EAV (EAV KY84) was used as the challenge virus (McCollum and Timoney, 1984, 1999; Cole et al., 1986; McCollum et al., 1995). The modified live virus (MLV) vaccine strain of EAV (ARVAC®, Pfizer Animal Health Inc., Kalamazoo, MI) was used as the reference virus in the microneutralization assay.

Stallions

Seven sexually mature (4-16 years old) stallions of mixed breed were included in the study (stallions IDs: L136 to L142). Horses were obtained from a local commercial vendor and acclimated to the new farm environment for approximately 2 months before the beginning of the study. During this period, animals were located in individual paddocks and trained to mount a mare or a phantom and to collect semen in an artificial

vagina. All stallions showed good libido and normal sexual behavior. All horses were confirmed seronegative for EAV neutralizing antibodies several times before intranasal inoculation with the virus using a previously described protocol (Senne et al., 1985; OIE, 2004). The animals were then housed in an isolation facility at the Department of Veterinary Science's Maine Chance Farm, Lexington, KY. The experiment described in this manuscript was carried out in accordance with the Institutional Animal Care and Use Committee (IACUC) approved protocol at the University of Kentucky, Lexington, KY (protocol number 2011-0888).

Experimental infection of stallions and clinical examinations

The stallions were inoculated intranasally with 3.75×10^5 plaque forming units (PFU) of the KY84 strain of EAV in 5.0 ml of EMEM using a fenestrated catheter passed via the posterior nares into the nasopharynx as previously described (Balasuriya et al., 2007; Zhang et al., 2008). The animals were monitored for the appearance of clinical signs of EVA and shedding of the virus in the semen, in addition to semen quality evaluation following experimental infection (see below). All clinical parameters were taken by the same investigator. Scrotal edema was classified on a scale from 0 to 5 and recorded as absent (0), mild (1-2), moderate (3) and severe (4-5). Pre-inoculation (7, 5 and 2 days before experimental infection) clinical examinations were performed once daily to determine baseline values for body temperature and also to certify the parameters were within normal limits before experimental infection of the stallions. Specifically, fever and edema were monitored for the first 15 days after infection, twice daily (every 12 hours) and the highest value of the day was recorded. Clinical signs continued to be

monitored once a week on the next 4 weeks of the experiment (at 21, 28, 35 and 42 dpi). Blood samples were collected at 0, 2, 4, 6, 8, 10, 12, 14, 21, 28, 35 and 42 dpi in order to determine each stallion's serum neutralizing antibody response to EAV.

Semen collection

Two ovariectomized mares in behavioral estrus and previously vaccinated with the commercial MLV vaccine (ARVAC®, Pfizer Animal Health, Inc., New York, NY) (antibody titer = 1:256 and 1:512) against EVA were used to tease the stallions. Each stallion was mounted either on the mare or on a breeding phantom for semen collection in an artificial vagina (Botucatu model, Botupharma, Botucatu, SP, Brazil). The artificial vagina was disinfected with an antiviral disinfectant (Roccal®-D Plus, Pfizer Inc., New York, NY, USA) between collections and a disposable liner lubricated with sterile lubricant (Priority Care, First Priority Inc., Elgin, IL, USA) was used for semen collection of each stallion to avoid cross contamination between ejaculate samples. Semen was collected from each stallion 3 times a week for a period of 3 weeks, and discarded, before evaluation of samples started being performed.

Semen was then collected and evaluated 7, 5 and 2 days before infection as well as every other day during the first 15 days after infection and twice weekly until 79 dpi. Three additional samples were collected and evaluated at 147, 149 and 152 dpi. Pre-inoculation semen samples were obtained to determine base-line values for percentage of total and progressively motile sperm cells (TMOT and PMOT), total number of spermatozoa cells (TNS), curvilinear velocity (VCL), percentage of live spermatozoa (LS), and percentage of morphologically normal spermatozoa (MNS). With the purpose

of following virus persistence in the reproductive tract of the stallions, semen was collected for virus isolation approximately once monthly until 198 dpi (6.5 months).

Semen processing and computer-assisted sperm analysis

Following collection, the total sperm number in gel-free semen was estimated by measuring the volume with a graduated cylinder and the initial sperm concentration with a spectrophotometer-based instrument (SpermaCue; Minitube of America Inc., Verona, WI, USA). An aliquot of each ejaculate was fixed in 10% buffered formalin for analysis of sperm morphology. The gel-free semen was extended to approximately $25x10^6$ spermatozoa/ml with a warm (37^0C) commercial skim milk-based extender containing gentamicin (EQUIPRO®; Minitube of America Inc., Verona, WI, USA) and evaluated using a computer-assisted sperm analysis (CASA) instrument (Sperm Vision® 3.5 Software; Minitube of America Inc., Verona, WI, USA) to determine the percent of TMOT, PMOT and VCL. A 10 μL aliquot of extended semen was placed on a warmed (37^0C) glass slide and overlaid with a warmed (37^0C) 22 × 22 mm cover slip. The slide was then placed in a warmed stage microscope connected to a computer and spermatozoa motion characteristics were analyzed. A total of 6 microscopic fields were examined per sample by the same examiner each time.

Membrane Integrity

The integrity of the spermatozoa plasmatic membrane was determined using eosin-nigrosin staining (Hancock Stain; Animal Reproduction Systems, Dupree Inc., Chino, CA, USA). Duplicate smears stained with the Hancock stain were prepared for

spermatozoa membrane integrity evaluation (Hancock, 1951). Briefly, 10 µl of the Hancock stain solution was placed on the end of a warmed (37^0C) glass slide. A 5µl drop of semen was placed next to the stain and the drops were mixed gently with the tip of a pipette. With the use of another slide a smear of the mixture was created. The smear was air-dried and observed under a microscope for analysis of the plasma membrane integrity. The percentage of live spermatozoa (LS) was determined by counting 200 cells throughout the slide for each of the two slides and the average number of live (unstained) versus dead (stained) cells was recorded. When the number of sperm cells was low due to low concentration of the sample, less than 200 spermatozoa were counted and the percentage number was recorded. All slides were prepared and counted by the same individual who was blind to the samples that were being analyzed.

Sperm Morphology

Sperm morphology (normal morphology, head, acrosome, mid-piece and tail abnormalities, detached heads and proximal and distal droplets) was assessed by differential-interference contrast microscopy (Axio Imager Upright Microscope). For analysis, 1.5µL aliquots of fixed semen were applied to a microscope slide and then overlaid with a cover glass. Individual spermatozoa were assigned to only one morphological category even if they exhibited several abnormalities. When more than one defect was seen in the same sperm cell the more proximal defect was recorded. A total of 100 sperm cells per sample were examined. All slides were counted by the same individual who was blind to the samples that were being analyzed.

Virus Isolation

Virus isolation from raw gel-free semen samples was attempted in RK-13 KY cell lines according to the gold standard protocol used by the OIE Reference Laboratory (OIE, 2004). Briefly, semen samples were sonicated for 45 seconds (3 × 15 sec) at 4^0C and sperm and cellular debris were sedimented by centrifugation (2,800 3 × g, 10 min). Serial decimal dilutions (10^{-1} to 10^{-4}) of each sample supernatant were prepared in minimum essential medium (MEM; Cellgro®, Mediatech, Inc, Manassas, VA). One ml of each dilution was inoculated into a 25-cm^2 flask containing confluent monolayers of RK-13 KY cells grown in supplemented EMEM. Flasks were incubated at 37^0C for 1 hour. Following incubation, flasks were overlaid with the supplemented EMEM containing 0.75% carboxymethylcellulose (CMC; Sigma-Aldrich, St. Louis, MO). The flasks were incubated at 37^0C for 4 days and checked on a microscope for the appearance of cytopathic effect (CPE). If CPE was not detected, a second passage was performed on day 4. All first passage flasks were stained with a 1% crystal violet solution containing 1% formaldehyde on day 4 post-inoculation for plaques counting and consequently virus titration. Second passage flasks were also stained 4 days after inoculation for detection of CPE. The same individual performed all virus isolations.

Microneutralization assay

The neutralizing antibody titers of the test sera were determined as described by Senne *et al*. (1985). Briefly, duplicate serial two-fold dilutions of each serum sample from 1:4 to 1:512 were made in supplemented EMEM containing 10% guinea pig complement (Rockland Immunochemicals, Gilbertsville, PA) and tested in 96-well

plates. An equal volume of 200 $TCID_{50}$ of the modified live vaccine strain of EAV (ARVAC®, Pfizer Animal Health, New York, NY) was added to each well. Plates were incubated at 37^0C in 5% CO_2 for 1 hour. After incubation, a suspension of RK-13 KY cells was added to each well and the plates were incubated for 72 h at 37°C, until viral cytopathic effect had fully developed in the virus control wells. The titer of a sample was recorded as the reciprocal of the highest serum dilution that provided at least 50% neutralization of the reference virus.

Real-time RT-PCR

Virus isolates were confirmed by EAV specific TaqMan® real-time RT-PCR assay as previously described (Balasuriya et al., 2002; Miszczak et al., 2011). Briefly, viral RNA was directly isolated from 50µl tissue culture fluid (TCF) using a commercial RNA isolation kit (MagMAX™ -96 Viral RNA Isolation Kit, Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The viral RNA was eluted in 50 µl of nuclease free water and stored at −80 °C. RNA extracted from TCF from semen samples that were negative for EAV as well as from nuclease free water were included as negative controls. Viral RNA extracted from a KY84 virus stock sample was used as a positive control. A one-tube TaqMan® real-time RT-PCR assay was performed as described in Miszczak et al., 2011 (Miszczak et al., 2011).

3.4. STATISTICAL ANALYSIS

Locally weighted polynomials (LOWESS) were fit to the raw data for each horse to produce smooth curves (Cleveland, 1979). To quantify spermatozoa exposure

to each potentially causative factor (fever, edema, virus concentration), these curves were then integrated over the 67-day period prior to each sperm quality measurement. Regression models were then fit for each semen quality outcome to determine the effects of each explanatory factor while adjusting for the other factors. To account for within-stallion correlation among the measurements, linear mixed effect models were used. All analyses were carried out using R software for statistical computing and graphics (www.r-project.org). A two-sample t-test was used to compare the differences between pre-virus-inoculation and post-virus-inoculation semen quality parameters. Significance was set as $p < 0.05$.

3.5. RESULTS

Clinical signs

All seven stallions developed moderate to severe clinical signs of EVA following experimental infection with the KY84 strain of EAV which included any combination of fever (> 38.6^{0}C), moderate to severe lymphopenia, dependent edema of the limbs, scrotum and prepuce, periorbital edema, nasal and ocular discharge, photophobia, dyspnea, anorexia, decreased libido and, congestion, petechiae and ecchymosis of the oral mucous membranes. All stallions had fever ranging from 38.7^{0}C to 40.8^{0}C starting from 1-2 dpi and lasting until 8-9 dpi (5-8 days) (Table 3.5.1.). Highest body temperatures were observed among the stallions between 5 to 7 dpi and the highest body temperature (40.8^{0}C) was observed in stallion L138 at 7 dpi. On average, fever was observed from 3 to 8 dpi (Figure 3.5.1.).

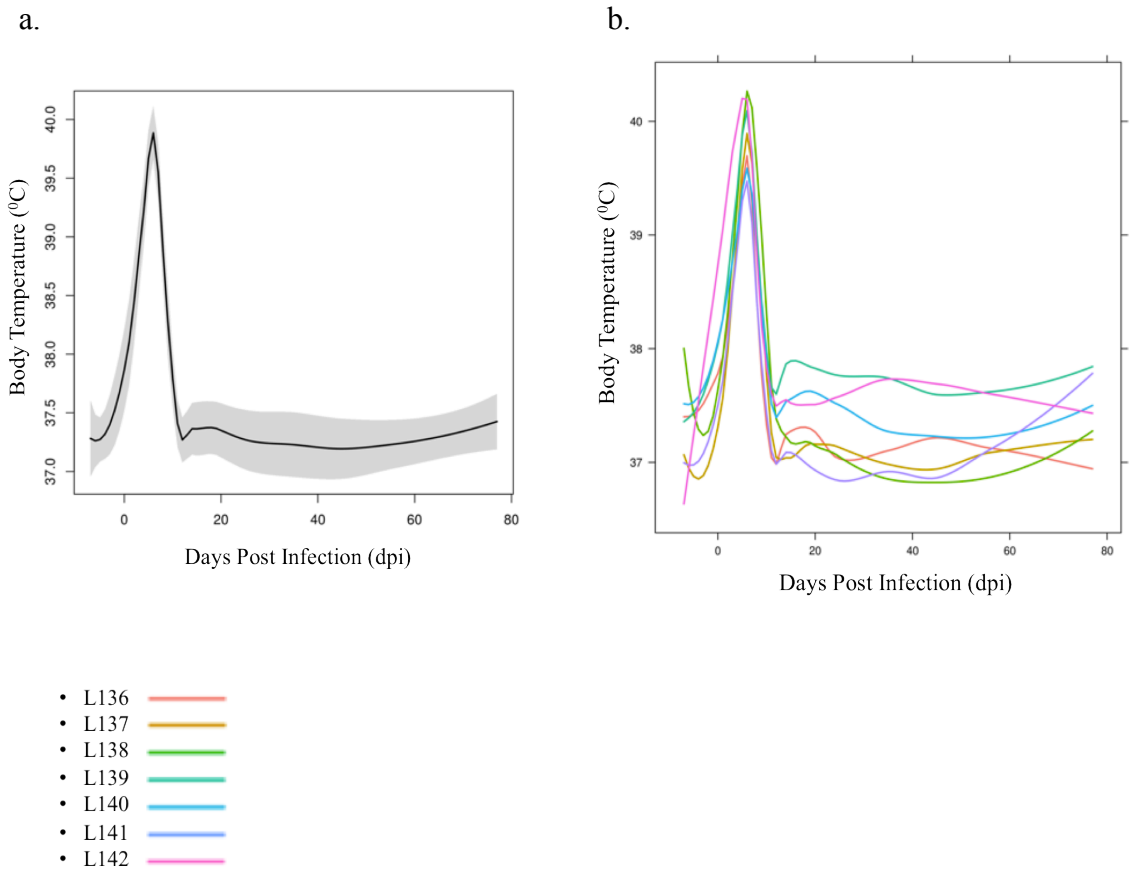
Table 3.5.1. Body temperature of stallions (^{0}C) before and after inoculation with the KY84 strain of equine arteritis virus.

Days Post Inoculation (dpi)	Stallions IDs						
	L136	L137	L138	L139	L140	L141	L142
-7	37.3	37.1	38.2	37.2	37.4	37.0	36.4
-5	37.5	36.8	36.9	37.7	37.7	36.9	37.7
-2	37.9	37.3	37.7	37.8	37.7	37.3	37.8
0	37.3	37.4	37.4	38.1	38.0	38.0	38.4
1	38.0	37.3	37.6	38.2	38.1	37.5	39.6*
2	38.8*	37.5	38.6	38.2	38.7*	37.6	39.2*
3	38.1	38.8*	38.9*	39.1*	38.9*	38.0	39.8*
4	38.4	38.8*	39.0*	39.6*	38.9*	39.3*	39.6*
5	39.4*	39.6*	39.6*	39.7*	39.0*	39.7***	40.2*
6	40.0***	39.8*	40.4*	40.1*	39.6*	39.3	40.4***
7	39.9*	39.9***	40.8***	40.2***	39.7*	38.9*	40.2*
8	38.8*	39.4*	39.7*	39.6*	39.6*	39*	38.7*
9	36.8	38.2	39.1*	37.5	38.0	37.4	37.6
10	37.1	37.1	37.8	37.7	37.1	37.1	37.7
11	37.1	37.1	37.2	37.7	37.4	37.2	37.6
12	37.4	37.3	37.7	37.8	37.7	37.1	37.8
13	37.3	37.2	37.5	37.9	37.9	37.0	37.8
14	37.4	37.3	37.4	38.1	37.4	37.3	37.6
15	37.3	37.1	37.2	37.9	37.7	37.3	37.4
21	37.2	37.3	37.3	37.7	37.6	36.8	37.6
28	36.8	37.0	36.9	37.8	37.4	36.9	37.7
35	37.1	36.9	36.8	37.7	37.3	36.9	37.8
42	37.5	37.1	36.9	37.8	37.2	37.1	37.6
49	37.1	36.7	36.9	37.3	37.4	36.8	37.7
56	37.1	37.1	36.7	37.7	37.2	36.8	37.5
63	36.9	37.7	37.1	37.8	37.1	37.6	37.7
70	37.0	36.8	36.9	37.7	37.6	37.8	37.3
77	37.0	37.3	37.3	37.8	37.4	37.6	37.5

Yellow box * = fever (> 38.6^{0}C)

Orange box *** = highest body temperature for that stallion during acute phase of infection

Figure 3.5.1. Body temperature of stallions (^{0}C) following experimental inoculation with the KY84 strain of equine arteritis virus. (a) Average smoothed curve of body temperature of stallions. The shaded area represents the 95% confidence interval. (b) Smoothed curve of stallions' individual body temperature.



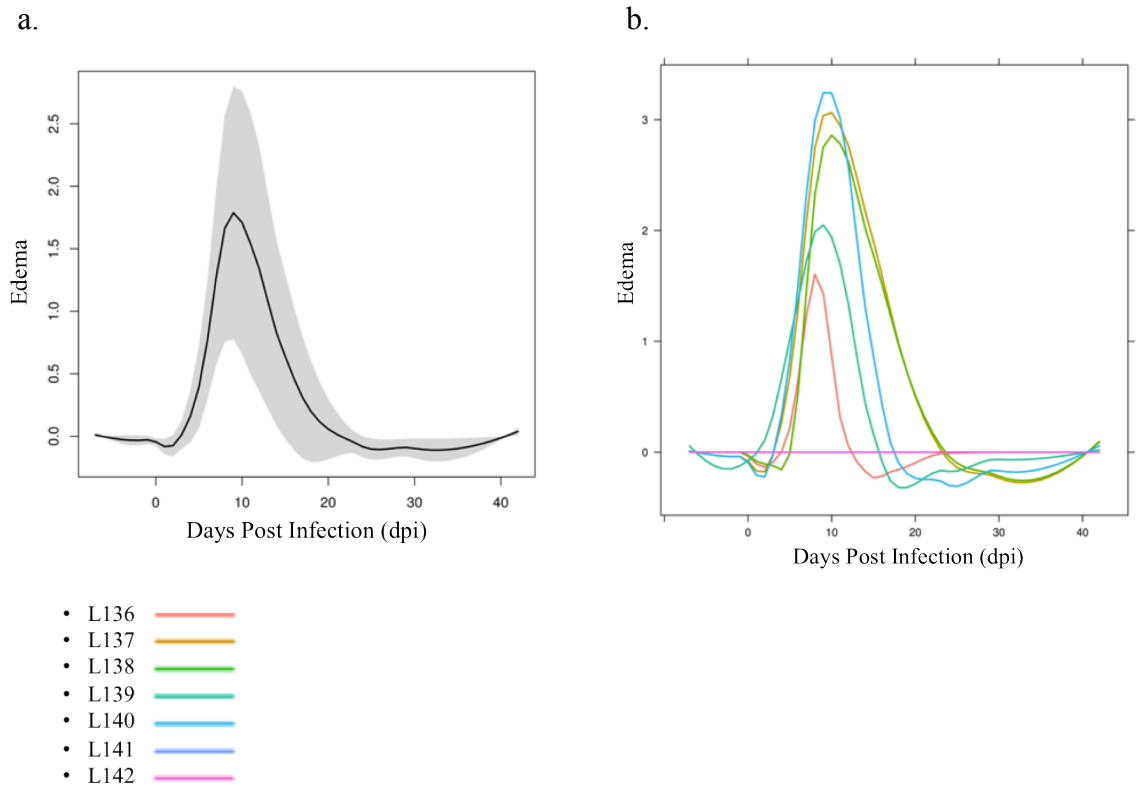
The severity and duration of edema varied between stallions. Five out of 7 stallions (L136-L140; 71.43%) developed moderate to severe scrotal edema starting from 4-7 dpi and persisting until 9-15 dpi (Table 3.5.2.). Scrotal edema was observed, on average, from 4 dpi until 15 dpi (Figure 2.5.2.).

Table 3.5.2. Scrotal edema of stallions before and after inoculation with the KY84 strain of equine arteritis virus.

Days Post Inoculation (dpi)	Stallions IDs						
	L136	L137	L138	L139	L140	L141	L142
-7	0	0	0	0	0	0	0
-5	0	0	0	0	0	0	0
-2	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0
4	0	0	0	+	0	0	0
5	0	0	0	+	0	0	0
6	0	++	0	+	++	0	0
7	++	++	+	++	+++	0	0
8	++	+++	+++	++	+++	0	0
9	++	+++	+++	++	+++	0	0
10	0	+++	+++	++	+++	0	0
11	0	+++	+++	++	+++	0	0
12	0	+++	++	+	+++	0	0
13	0	++	++	+	++	0	0
14	0	++	++	0	+	0	0
15	0	++	++	0	0	0	0
21	0	0	0	0	0	0	0
28	0	0	0	0	0	0	0
35	0	0	0	0	0	0	0
42	0	0	0	0	0	0	0

+ = edema is present
0 = edema absent
Green box + = mild edema
Blue box ++ = moderate edema

Figure 3.5.2. Scrotal edema following inoculation of stallions with the KY84 strain of equine arteritis virus. (a) Average smoothed curve of scrotal edema exposure. The shaded area represents the 95% confidence interval. (b) smoothed curve of individual stallions scrotal and preputial edema. For the purpose of this graphic: 0 = no edema; 1 = mild edema; 2 = moderate edema; and, 3 = severe edema. Note that edema was not seen in all stallions, and thus the average is an oversimplification of the data.



Two of the five stallions (L136 and L139) had moderate edema for 3-5 days (7-11 dpi); three of the five stallions (L137, L138 and L140) had severe edema for 4-6 days (7-12 dpi) (Figure 3.5.3.).

Figure 3.5.3. Representative example of severe scrotal and preputial edema following experimental inoculation of stallions with the KY84 strain of equine arteritis virus. Stallion L140 at 7 days post infection (dpi).

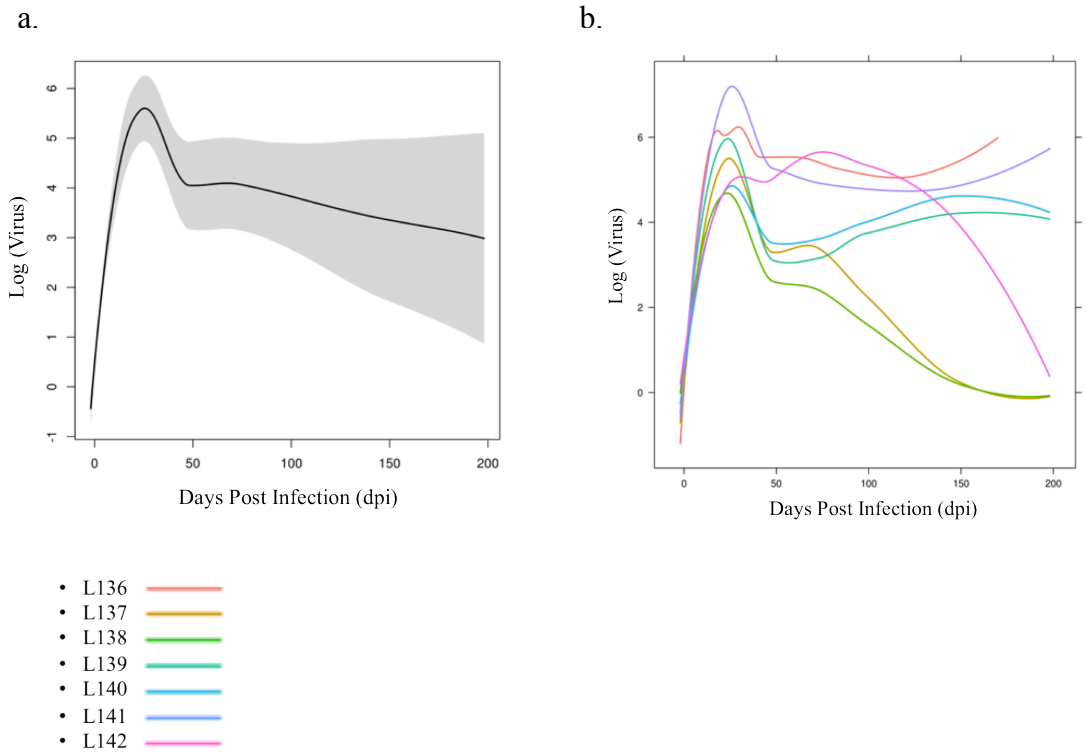
Two stallions (L141 and L142) did not develop any edema of prepuce or scrotum. Stallion L141 had hemospermia following EAV inoculation, specifically at 11, 13 and 15 dpi. This stallion had a small wound in the gland of the penis. Even though the wound persisted when blood was still observed in the semen, it is not possible to be sure if hemospermia was associated with EAV infection. All stallions showed decreased libido during the acute phase of infection. The time to drop the penis as well the number of times each stallion had to jump the mare or the phantom for ejaculation increased. All the clinical signs along with the moderate to severe edema in hind limbs of the stallions observed during the acute phase of infection would have made it difficult for them to mount, which contributed to the increased time to obtain the ejaculate. However, none of the seven stallions failed to mount the mare or the phantom. One stallion (L142) failed to ejaculate and instead, provided only the pre-ejaculatory fluid at 1 and 30 dpi. All

stallions seroconverted to EAV starting from 8 dpi and maintained high neutralizing antibody titers in serum until at least 42 dpi (1:64 to > 1:512).

Evaluation of semen for virus shedding

Following experimental inoculation of the stallions with EAV KY84, semen samples from each stallion were tested for EAV shedding by virus isolation (VI) in cell culture until 198 dpi. All stallions shed EAV in their semen starting at 3 dpi and continued to shed at least until 107 dpi (3.5 months) (Figure 3.5.4.).

Figure 3.5.4. Virus titer in semen following inoculation of stallions with the KY84 strain of equine arteritis virus. (a) Average smoothed curve of virus titer in semen; and, (b) smoothed curve of virus titer in semen of individual stallions.



The virus titers in their semen varied from 1×10^1 to 1.88×10^7 PFU/ml during the acute phase of infection. Following the acute phase of infection, all seven stallions continued to shed very high titers of virus in their semen until 107 dpi, with the exception of stallions L138 and L137, who shed low titer virus equivalent to 6×10^1 PFU/ml and 8×10^1 PFU/ml, respectively, at 107 dpi. Those stallions (L138 and L137) were no longer carriers at 128 and 149 dpi, respectively. Stallion L142 stopped shedding virus sometime between 170 and 198 dpi. Four out of 7 stallions (57%) kept shedding high titer virus at least until 198 dpi (L136, L139, L140 and L41).

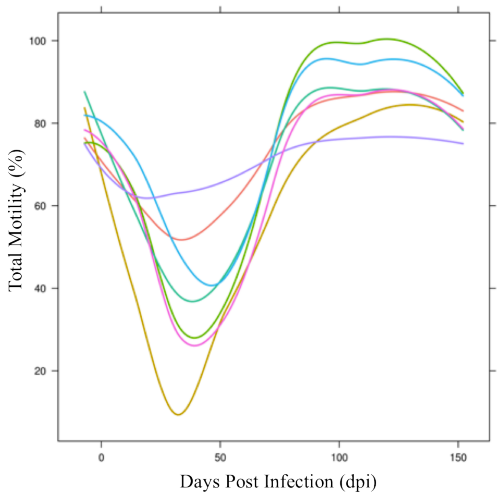
Evaluation of semen quality parameters

A total of 201 sequential semen samples (29 samples per stallion from stallions L136 to L141, and 27 samples from stallion L142) from all seven stallions were collected over a period of 12 weeks and these included 21 samples (3 per stallion) prior to the experimental inoculation of stallions with the KY84 strain of EAV. Only one stallion (L142) failed to ejaculate at 1 and 30 dpi. Three semen samples from each stallion collected prior to experimental infection (-7, -5 and -2 days) were used to establish the normal semen characteristics (baseline values) for total number of spermatozoa (TNS) per ejaculate, total and progressive sperm motility (TMOT and PMOT, respectively), curvilinear velocity (VCL), percentage of live sperm cells (LS), and percentage of morphologically normal spermatozoa (MNS). Before inoculation the mean baseline values for TNS, TMOT, PMOT, VCL, LS, and MNS were 5.87×10^9 spermatozoa $\pm$ 0.05 $\times 10^9$; 75.63% $\pm$ 2.24; 66.14% $\pm$ 3.62; 141.61 μm/s $\pm$ 9.43, 90% $\pm$ 0.01; and, 53.52% $\pm$ 3.59, respectively. Deterioration of at least one of the parameters analyzed for semen

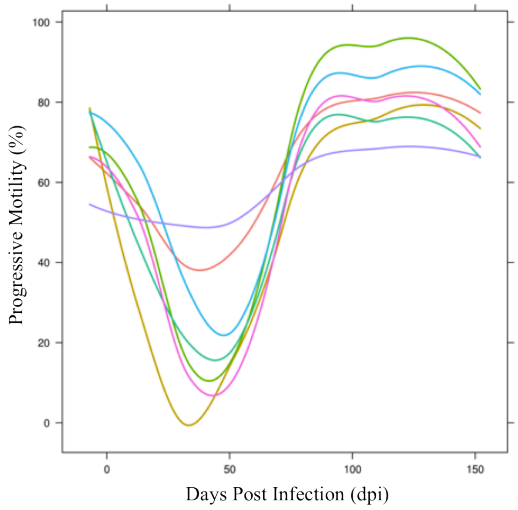
quality was observed in all stallions after inoculation with the KY84 strain of EAV (Figure 3.5.5.).

Figure 3.5.5. Smoothed curve of the changes seen in semen quality parameters of individual stallions following experimental inoculation with the KY84 strain of equine arteritis virus. (a) total motile spermatozoa (% TMOT); (b) progressive motile spermatozoa (% PMOT); (c) total number of spermatozoa (TNS); (d) curvilinear velocity (VCL); (e) percentage of live spermatozoa (%LS); and, (f) morphologically normal spermatozoa (% MNS).

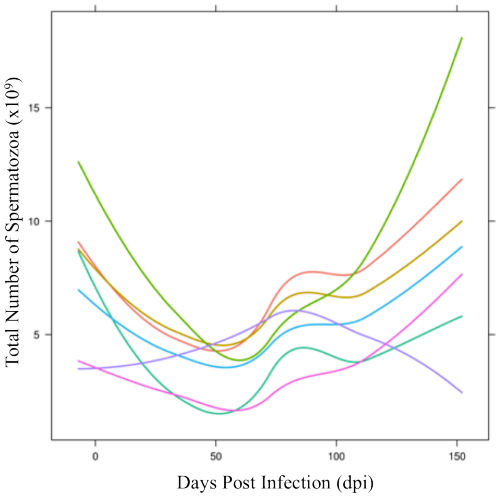
a. %TMOT



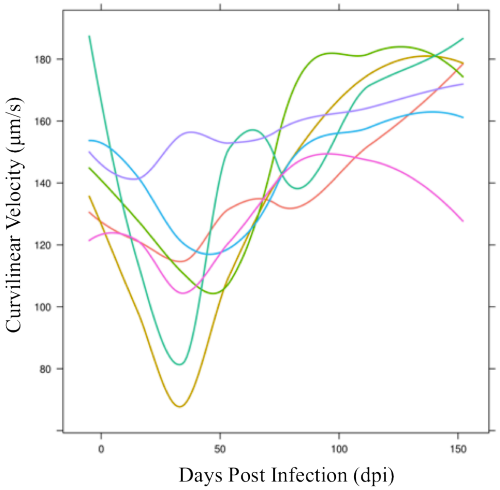
b. %PMOT



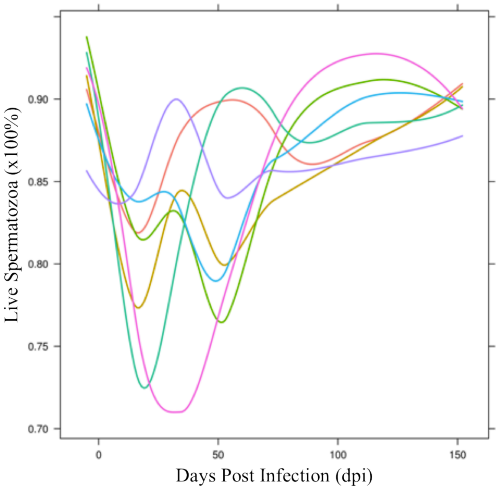
c. TNS



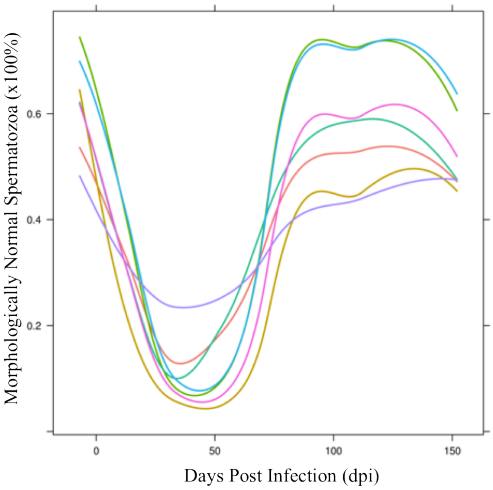
d. VCL



e. %LS



f. %MNS



- L136
- L137
- L138
- L139
- L140
- L141
- L142

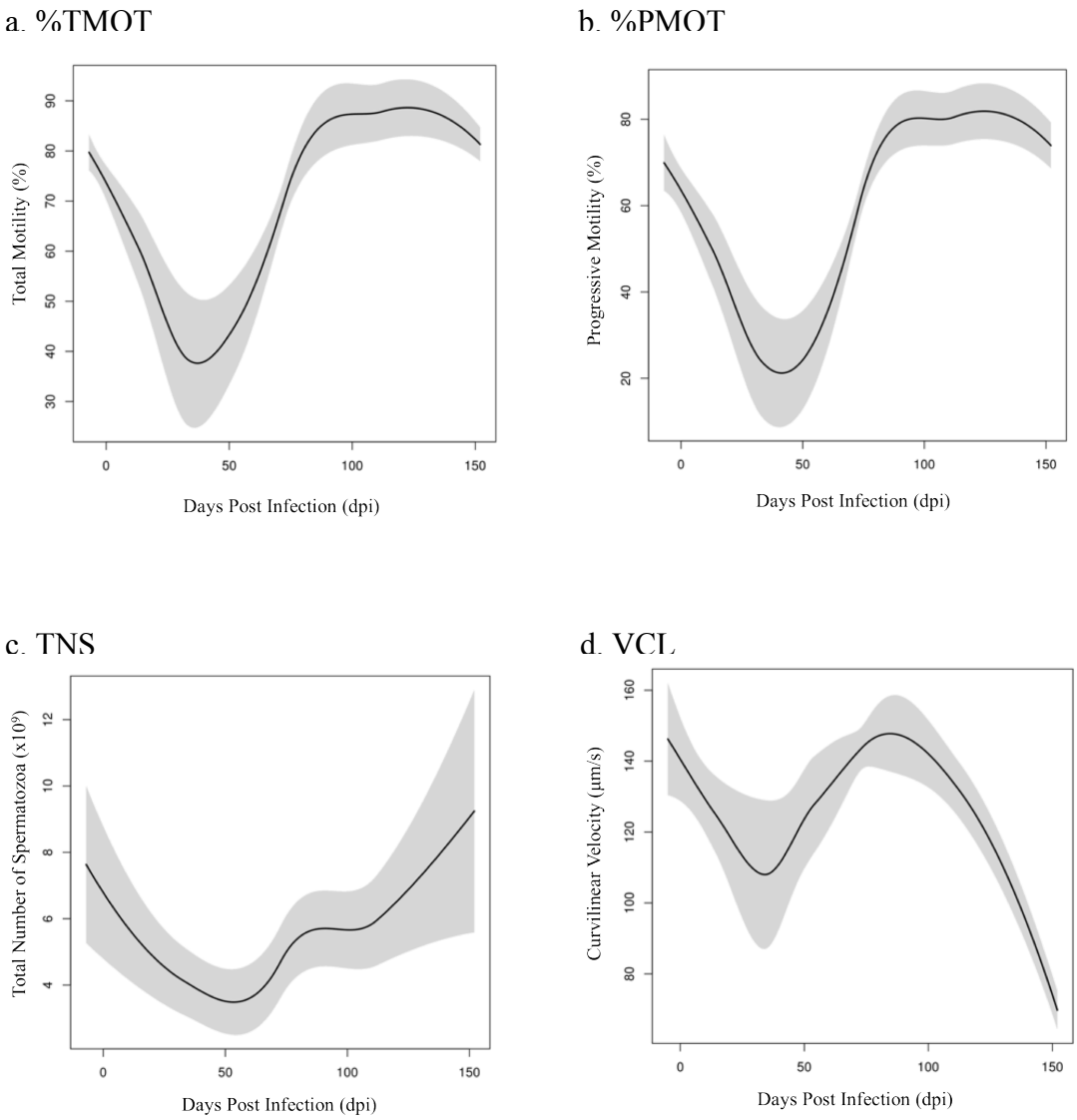
With the exception of one stallion (L141), TMOT, PMOT, VCL, TNS and percentage of LS followed a similar trend in all other 6 stallions. Stallion L141, showed a significant drop only in MNS following experimental inoculation with the EAV KY84, but never showed a significant decrease in the other semen parameters (TMOT, PMOT, TNS and percentage of LS). The alteration in the number of morphological normal spermatozoa followed a similar trend in all 7 stallions, including L141.

Computer-assisted sperm analysis and evaluation of total number of spermatozoa

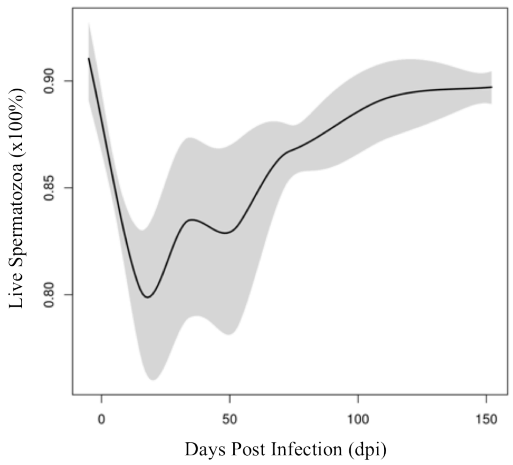
The percentage of TMOT, PMOT, VCL, and TNS across all stallions were averaged for each collection and compared to the baseline average before inoculation with the EAV KY84.

On average, semen motility decreased (TMOT and PMOT) between 20 and 65 dpi ($p < 0.05$) (Figure 3.5.6.). There was more than 50% reduction in TMOT and PMOT of sperm between 30-51 dpi. Sperm motility (TMOT and PMOT) returned to pre-inoculation values around 65 dpi (Table 3.5.3.). Stallion L141 never showed a significant drop in total or progressive motility following experimental infection with the virus. EAV infection of the stallions also had an effect on VCL of sperm cells, with mean value decreases being observed between 23 and 30 dpi ($p < 0.05$) (Figure 3.5.6.). TNS reduced, on average, between 44-69 dpi ($p < 0.05$). Two stallions, L139 and L142, had azoospermia at 34, 37, 41, 48, 51, 55 dpi, and 44, 48, 51 dpi respectively (Figure 3.5.5.). The TNS count returned to baseline values at around 69 dpi (Table 3.5.3.).

Figure 3.5.6. Averaged smoothed curve of the changes seen in semen quality parameters following experimental inoculation of stallions with the KY84 strain of equine arteritis virus. (a) total motile spermatozoa (% TMOT); (b) progressive motile spermatozoa (% PMOT); (c) total number of spermatozoa (TNS); (d) curvilinear velocity (VCL); (e) percentage of live spermatozoa (%LS); and, (f) morphologically normal spermatozoa (% MNS). The shaded area represents the 95% confidence interval.



e. %LS



f. %MNS

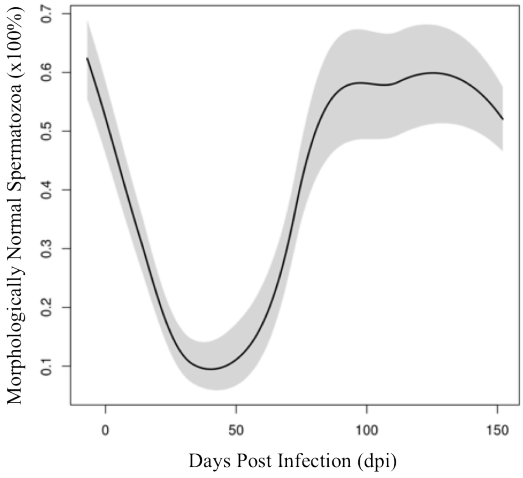


Table 3.5.3. Period of reduced semen quality in stallions following infection with the KY84 strain of equine arteritis virus (a) Decrease in semen parameters; (b) Increase in spermatozoa morphological abnormalities. Note that this is an oversimplification table based on a two-sample t-test statistical analysis and not all stallions had necessarily a change in all parameters evaluated.

a. Semen parameters

Semen Quality Parameters	Total Motility	Progressive Motility	Total Number of Spermatozoa	Percentage of Morphological Normal Spermatozoa
Onset (dpi)	20	20	44	11
Recovery (dpi)	65	65	69	76

b. Spermatozoa morphological abnormalities

Spermatozoa defects	Head	Detached Head	Acrosome	Mid-Piece	Tail	Proximal Droplet	Distal Droplet
Onset (dpi)	23	15	15	62	48	13	9
Recovery (dpi)	69	72	23	65	58	27	11

Membrane Integrity

The percentage of spermatozoa with intact plasmatic membrane (live spermatozoa; LS) across all stallions were averaged for each collection and compared to the baseline average before inoculation with the EAV KY84. Before inoculation of the stallions with the virus, all had at least 81% of LS and the mean baseline value for LS was 90% ± 0.01 (Figure 3.5.5.). Changes in membrane integrity observed following experimental infection of the stallions with EAV KY84 followed a similar trend in all stallions, with the exception of stallion L141 as mentioned above. After inoculation, damage of the plasmatic membrane was observed in the spermatozoa of all stallions starting at 13 dpi. On average, decreases in the number of LS were significantly different from baseline between 13-23 dpi ($p < 0.05$) (Figure 6). Recovery of membrane integrity occurred at around 23 dpi (Table 3.5.3.).

Evaluation of sperm morphology

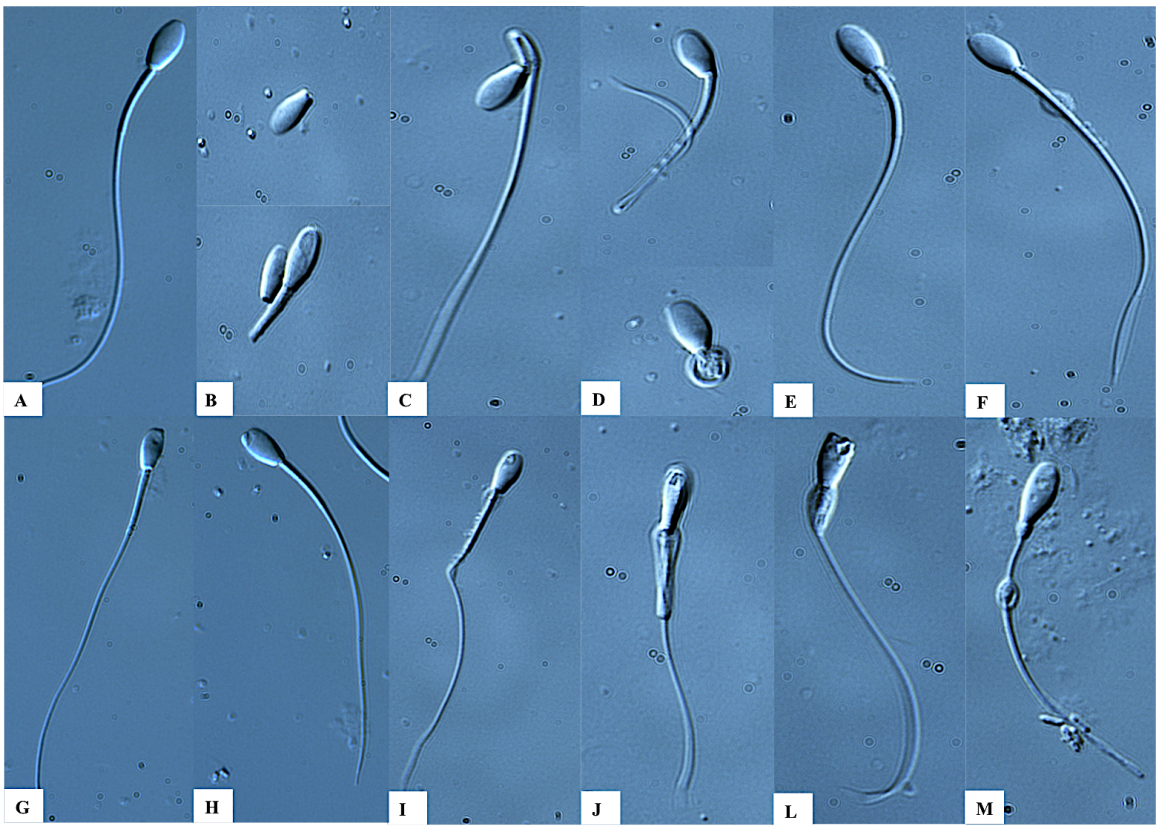
The percentage of MNS across all stallions were averaged for each collection and compared to the baseline average before inoculation with the EAV KY84.

With the exception of two stallions (L136 and L141), others had at least 50% MNS prior to experimental inoculation. Stallion L136 had 48% of MNS while stallion L141 had only 41% MNS. All stallions showed reduction in the percentage of MNS starting from 9-15 dpi. Percentage of MNS returned to normal values in all animals ranging from 73-79 dpi with the exception of one stallion (L137). Stallion L137 showed improvement in the number of MNS starting from 62 dpi and increasing until 79 dpi. However, the percentage of MNS for this stallion did not return to baseline values during

the time of the study (Figure 3.5.5.). On average, MNS was decreased as early as 11 until 76 dpi ($p < 0.05$) (Figure 3.5.6.) and recovered to normality around 76 dpi (Table 3.5.3.). Spermatozoa morphological abnormalities followed a similar trend in all 7 stallions, including L141.

The formalin fixed ejaculate aliquot was examined for the presence of spermatozoa abnormalities as: head, acrosome, mid-piece and tail defects, as well as for the presence of detached spermatozoa and proximal and distal droplets (Figure 3.5.7.).

Figure 3.5.7. Representative picture of morphological abnormalities observed in spermatozoa following experimental inoculation of stallions with KY84 strain of equine arteritis virus. (a) Normal spermatozoa; (b) detached spermatozoa; (c) mid-piece abnormality (bent); (d) tail abnormalities (bent and coiled); (e) proximal droplet; (f) distal droplet; (g) head abnormality; (h) acrosome abnormality; and, (i-m) multiple abnormalities on the same spermatozoon. 10% formalin fixed non-stained semen samples viewed by differential interference contrast (DIC) microscopy.

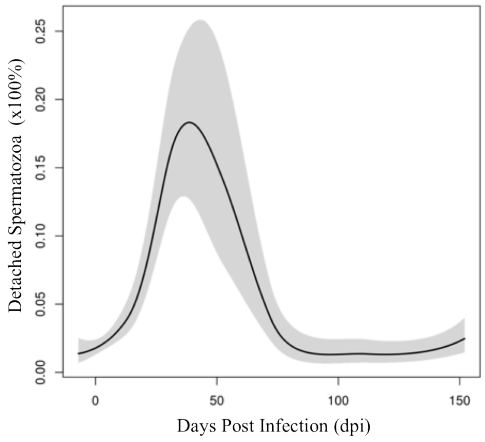


Spermatozoa abnormalities across all stallions were averaged for each collection and compared to the baseline average before inoculation with the EAV KY84. Before inoculation of the stallions with the KY84 strain of EAV baseline values for all sperm

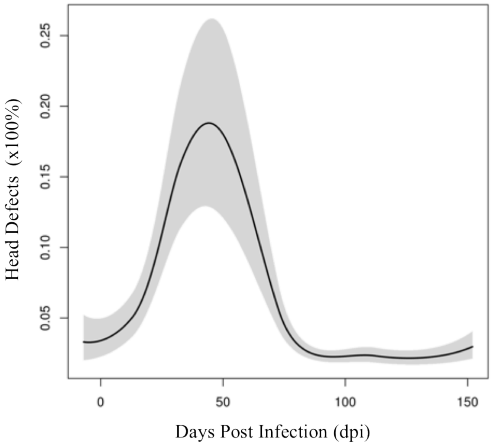
cell abnormalities were seen on a frequency lower than 10%, with the exception of mid-piece defects, which were observed on a high frequency (25.10%). An increase in a wide variety of spermatozoa morphologic alterations was observed after inoculation of the stallions with EAV KY84 and individual abnormalities were often, higher than 10%. The abnormalities that mostly increased after the infection of the stallions were detached heads, followed by head and proximal droplet defects, tail, distal droplets, mid-piece and finally, acrosome (Figure 3.5.8.).

Figure 3.5.8. Averaged smoothed curve of the spermatozoa abnormalities (%) following experimental inoculation of stallions with the KY84 strain of equine arteritis virus. (a) Detached spermatozoa; (b) head abnormalities; (c) proximal droplets; (d) distal droplets; (e) mid-piece abnormalities; (f) acrosome abnormalities; and, (g) tail abnormalities.

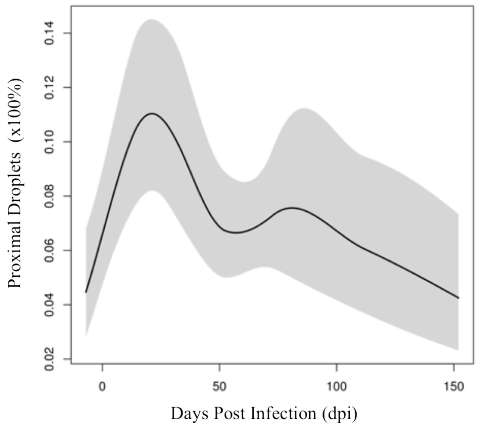
a. Detached spermatozoa



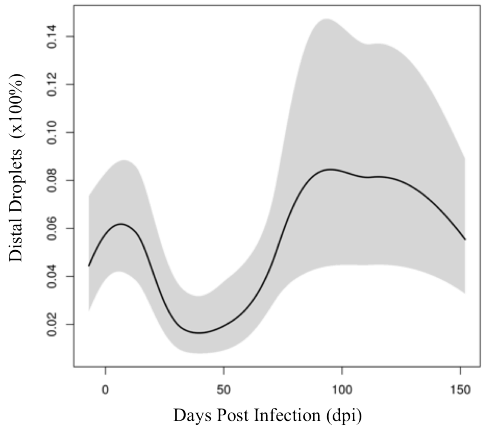
b. Head abnormalities



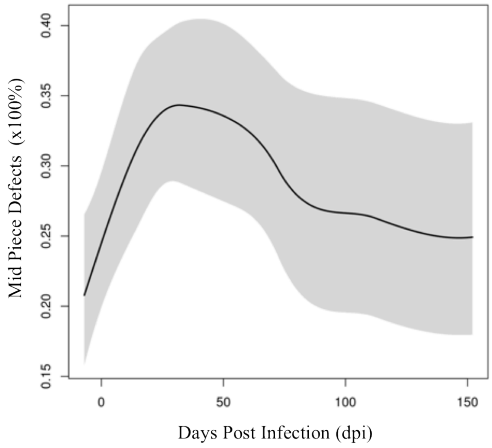
c. Proximal droplets



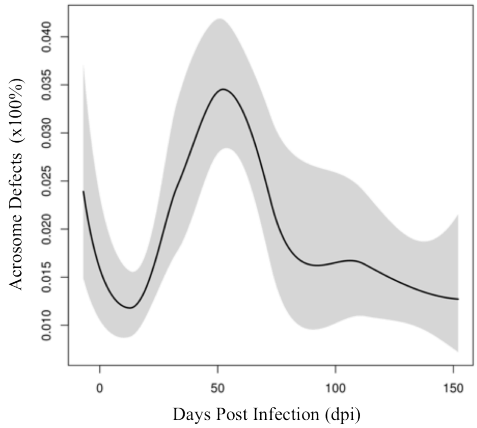
d. Distal droplets



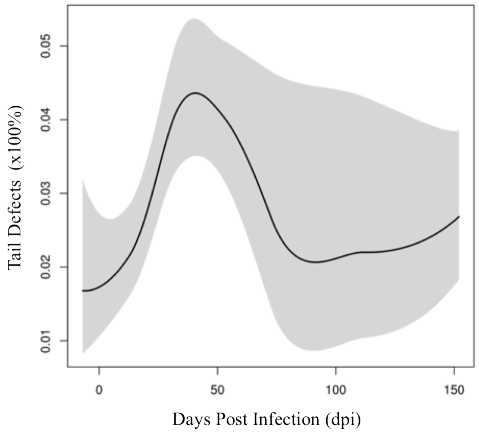
e. Mid-piece abnormalities



f. Acrosome abnormalities



g. Tail abnormalities



Even though there was an increase in acrosome and tail defects, those abnormalities were never detected at a frequency higher than 10%. On average, there was a 11-fold increase in the number of detached heads at 41 dpi (22.33%). The number of detached heads was increased from 15-72 dpi and it was higher than 10% from 20-58 dpi. At around 72 dpi the percentage of detached spermatozoa returned to baseline values (Table 3.5.3.). The head defects were observed to increase 6-fold at 51 dpi (23.17%) compared to the mean value before inoculation of the stallions with the virus. Abnormalities of sperm heads increased between 23-69 dpi ($p < 0.05$), however only between 23-58 dpi were values higher than 10% observed. A 3-fold increase in the presence of proximal droplets in spermatozoa was observed at 20 dpi (17.71%). The percentage of proximal droplets increased from 13-27 dpi ($p < 0.05$) being higher than 10% also between 13-27 dpi. An increase in the number of spermatozoa showing distal droplets was observed between 9-11 dpi (3-fold increase at 9dpi; 15.86%) ($p < 0.05$). At 9 and 11 dpi distal droplets were seen, on average, at a frequency higher than 10%. However, between 20-58 dpi a decrease in the percentage of distal droplets was seen ($p < 0.05$). Even though mid-piece abnormalities baseline values were observed at high frequency (25.10%), the percentage of mid-piece spermatozoa defects seen in the stallions' ejaculate was increased between 62-65 dpi, after inoculation of the stallions with the EAV KY84 ($p < 0.05$). During the period of the most dramatic decrease (23-58 dpi) in the percentage of MNS, individual sperm, frequently had 3 or more abnormalities, even though only the most proximal was counted (Figure 3.5.7.).

In summary, following inoculation of the stallions with the KY84 strain of EAV all stallions had a significant decrease in the number of morphological normal

spermatozoa accompanied by a consequent increase in the number of spermatozoa abnormalities. Stallion L141 did not show any change in the semen quality parameters evaluated, other than a decrease in the percentage of morphologically normal spermatozoa. On average, alteration in semen morphology occurred between 11 and 76 dpi.

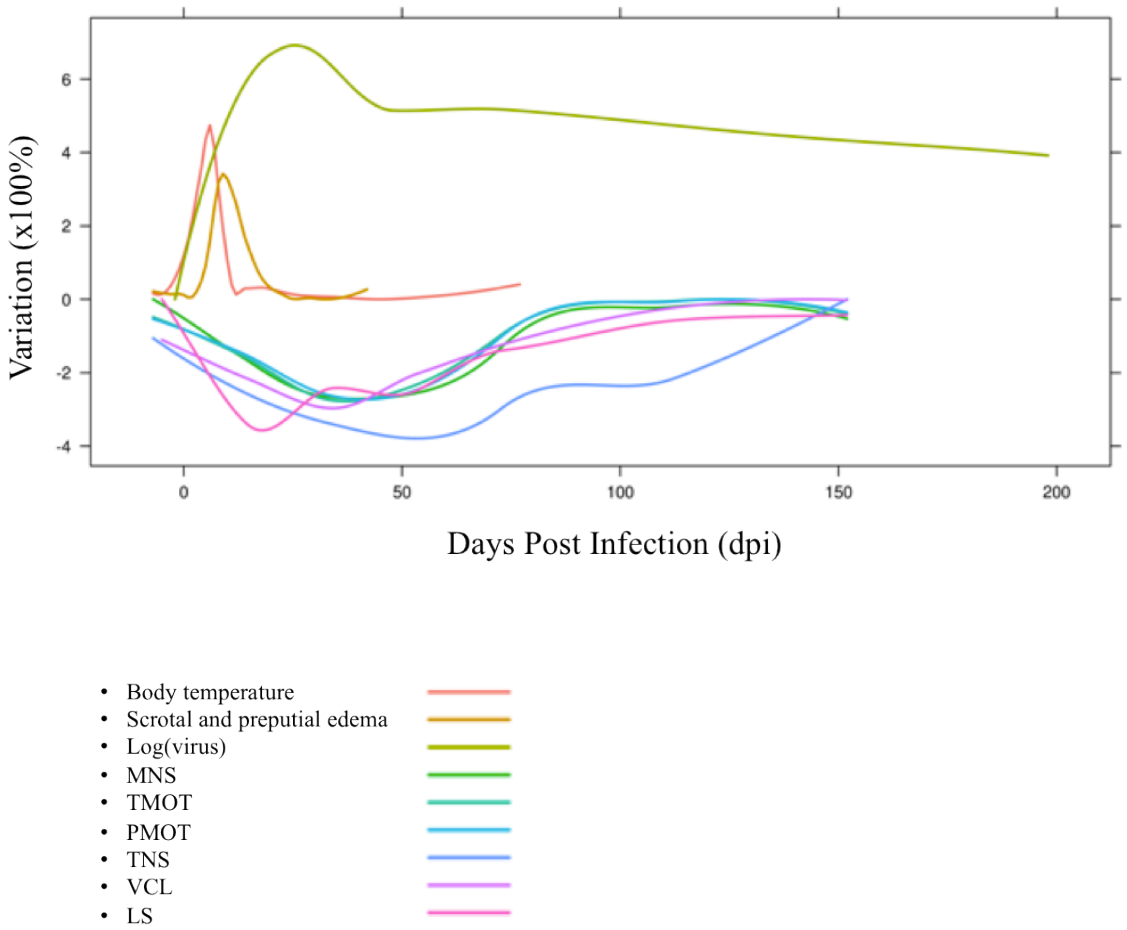
Effects of fever, edema and virus on semen quality

The statistical data analysis demonstrated strong evidence that high body temperature as well scrotal and preputial edema exert independent effects on all the semen quality parameters. However high titer virus in semen during acute or persistent infection of the stallions has little or no effect on semen quality (Figure 3.5.9.). Although increased virus concentrations were consistently associated with lower semen quality, the effect was usually not significant.

Scrotal edema and fever seem to have a larger effect on semen quality than virus titers in semen. The statistical models estimate that a 1 standard deviation (SD) change in temperature exposure, keeping edema and virus constant, would result in approximately 15% drop in normal morphology ($p < 0.0001$), 10% in TMOT ($p < 0.001$), and 12% in PMOT ($p < 0.001$) compared to baseline. Whereas, a estimated change of 1 SD in edema exposure keeping temperature exposure and virus concentrations constant, would result in a drop of 23% normal spermatozoa morphology ($p < 0.0001$), 17% TMOT ($p < 0.0001$), 20% PMOT ($p < 0.0001$), 7% TNS ($p < 0.01$), 4% in the number of LS ($p < 0.01$), and 8% in VCL ($p < 0.001$) compared to baseline. A one standard deviation change in virus concentration, though, keeping temperature and edema constant, would result in

only 7.5% drop in TNS ($p < 0.01$) and, 2% in the number of LS ($p < 0.03$) but would not have a significant effect in other semen parameters, such as, MNS, TMOT, PMOT and, VCL.

Figure 3.5.9. Depiction of body temperature, scrotal and preputial edema and semen virus titers along with semen quality parameters.



3.6. DISCUSSION

The results of the present study demonstrated that semen parameters are negatively affected following experimental infection of stallions with the KY84 strain of EAV. These results agree with the findings of Neu *et al*. (1991) that deterioration in semen quality following experimental EAV inoculation of stallions appears to be temporary and that the duration of reduced semen quality corresponded approximately with the duration of spermatogenesis in the horse (Brito, 2007).

Following inoculation with EAV KY84, all stallions shed virus in the semen starting from 3 dpi until at least 107 dpi. Thus, presence of virus in semen was constant throughout the experimental observation period. High body temperature and/or scrotal edema caused by infection of the stallions with EAV KY84, were not a constant though, and were observed sometime between 1-15 dpi during the acute phase of the disease. Following 5-14 days of injury exposure (fever and/or scrotal edema), deleterious effects on spermatogenesis were noticed. Statistical data analysis demonstrated strong evidence that the edema and fever exerted independent deleterious effects on the quality of the semen of all stallions evaluated, but virus seemed to exert little to no direct effect, as virus concentrations remained high long after semen quality returned to baseline. However, it is not possible to entirely rule out the direct effect of the virus infection on semen quality given that, in a previous study, the KY84 strain of EAV was isolated from the testis of stallions killed up to 20 dpi (Neu and Timoney, 1988). Nonetheless, it is unlikely that virus, rather than the combined effect of fever and scrotal and preputial edema, was responsible for the detrimental effect seen in semen quality, since microscopic lesions observed in the reproductive tract of stallions during the acute phase

of KY84 EAV infection were a result of viral replication and lysis of endothelial cells and changes in germ cells were never reported (Neu, 1988). In contrast, histopathological changes in germ cells of testis exposed to heat were detect in different species (Setchell, 1998).

It is well known that testicular temperature, 3 to 5^0C cooler than the body, is required in most mammals in order for normal spermatogenesis to occur (Setchell, 1998; Brito, 2007; Hansen, 2009). When testicular temperature is elevated due to conditions such as fever, testicular trauma, inflammation, and edema, the thermoregulatory mechanism necessary to cool the testis and allow for normal spermatogenesis is disrupted (Johnson et al., 1997). In these conditions, metabolism and oxygen demand increase at a greater rate than blood flow from the testicular artery to the testes, and testicular hypoxia occurs with consequent detrimental effects on sperm production and quality (Setchell, 1998; Brito, 2007). Previous studies assessing the effects of increased testicular temperature in different species such as, mice (Pérez-Crespo et al., 2008), rams (Rasooli et al., 2010), boars (Wettemann et al., 1979), bulls (Karabinus et al., 1997) and stallions (Freidman et al., 1991; Love and Kenney, 1999) have shown that transient, as well as, chronic exposure of the testis to elevated temperatures can disrupt spermatogenesis.

In this study, with the exception of one stallion (L141) that showed a drop only in the number of MNS, semen of the other 6 stallions experimentally infected with EAV showed a significant decrease in all parameters evaluated for semen quality (TMOT and PMOT; TNS; LS; MNS; VCL) between 11-76 dpi. Stallion L141 had fever but not edema during the acute phase of the disease. Perhaps, the fever in this animal was not sufficient to elevate the intrascrotal temperature to a degree needed to interfere with

motility, TNS, LS and VCL, but it was sufficient to decrease the percentage of MNS. Neu *et al*. (1991) observed a similar outcome in a group of stallions experimentally infected with EAV, which experienced a decrease in the percentage of MNS but not in motility, and suggested that "mechanisms important in the formation or maintenance of normal sperm structure may be the most sensitive to increased temperature".

Statistical analysis predicted that semen quality of stallions inoculated with EAV KY84 return to baseline values at around 76 dpi. This contrasts with the results obtained by Neu *et al.* (Neu and Timoney, 1988) in which semen quality improved to preinoculation values around 16 weeks post infection with EAV KY84 (112 dpi). Perhaps, in that study, the effects of body temperature and scrotal edema more dramatically affected spermatogonia, and thus, recovery of preinoculation semen characteristics took longer to occur. Spermatogenesis in stallions is 57 days (Brito, 2007) and is followed by the epididymal transit of spermatozoa, a process that lasts 8-11 days (Swierstra et al., 1975). Therefore, spermatozoa evaluated anytime between 11 and 76 dpi were at different stages of development at the time of injury exposure (1-15 dpi). So, it is possible to conclude that various stages of developing spermatozoa (spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids and mature sperm cells) were sensitive to the effects of high body temperature and scrotal edema observed during the acute phase of EAV infection. However, most of the morphological defects were observed at around 41 dpi (nadir). Taking into consideration the length of spermatogenesis, the lifespans of primary (19 days) and secondary spermatocytes (0.7 days), spermatids with round nuclei (8.7 days) and elongated spermatids (10.1 days), as well as knowing that epidydimal transit is approximately 9 days (Swierstra et al., 1974),

the appearance of morphological abnormal cells mostly at 41 dpi, suggests that the cells more dramatically affected at the time of testicular injuries were primary and secondary spermatocytes. These results are similar to the results found by Freidman *et al*. (1991) in which primary and secondary spermatocytes were the germ cells mostly affected following 24 and 48h insulation of stallions' testis.

In the assessment of morphological abnormalities, spermatozoa were assigned only to one morphological category and the most proximal defect was prioritized over the distal defects. The defects seen in spermatozoa were varied and all were increased following experimental inoculation of the stallions with EAV KY84, with the exception of the percentage of distal droplets that was decreased. The spermatozoa with distal droplets decreased significantly between 23-58 dpi and this decrease did not mean necessarily that their incidence had dropped, but rather, another abnormality had increased. The period in which distal droplet abnormalities were significantly decreased coincided with the period of most dramatic decrease (23-58 dpi) in the percentage of MNS, when an increase in the multiple defects in the spermatozoa was seen. Spermatozoa with detached heads had the most dramatic increase, with a 11-fold increase occurring at 41 dpi, compared to baseline, followed by head abnormalities with a 4-fold increase at 51 dpi. Detached heads and head defects are among those abnormalities that appear to have a deleterious effect on fertility, and, even though, proximal droplets were also observed on a high frequency after experimental infection of the stallions with EAV KY84, the droplets seem to have a minor effect on fertility (Love et al., 2000).

It is important to mention that the values for onset of semen quality parameters as well as for semen quality recovery are an oversimplification of interaction between the

virus and host factors that occur during *in vivo* infection. Drop in semen quality was gradual over time and it occurred at different times for different stallions as well as a return to baseline values. As can be conclude from the graphics, there is an individual variation in semen quality parameters among stallions following experimental inoculation with the KY84 strain of EAV. Some stallions, for example, never experienced edema; however, a mean exposure to edema was calculated (Fig 3.5.2.) and considered to occur from 4-15 dpi.

In conclusion, fever and scrotal edema are the most likely cause of deterioration of semen quality following experimental infection of stallions with the KY84 stain of EVA. However, the direct effects of the virus on semen quality, even though unlikely, cannot be excluded. The spermatozoa deterioration observed following experimental inoculation of stallions with EAV KY84 were of enough magnitude to cause temporary infertility of the stallions. In addition to the risk of acutely EAV infected stallions becoming long-term carriers and shedding virus for years in semen, the deterioration in semen quality of acutely EAV infected stallions can cause great economic loss associate with the temporary removal of these animals from the breeding activities for as long as 3 to 4 months, until semen quality return to normal.

CHAPTER FOUR

Summary of thesis

EAV is the causative agent of equine viral arteritis (EVA) and is distributed in many equine populations throughout the world (Doll et al., 1957b; McCollum and Bryans, 1973; Timoney and McCollum, 1993a). While most EAV infections are asymptomatic or subclinical, some infected horses exhibit clinical manifestations characteristic of EVA such as influenza-like illness in adult horses, abortion in pregnant mares, pneumonia and/or enteritis in young foals, and persistent infection in stallions (Doll, Bryans, and Knappenberger, 1957; Timoney, McCollum, Roberts, et al., 1986; McCollum et al., 1999).

Over the years, the importation of carrier stallions and virus infective semen into the U.S.A. has been responsible for the introduction of new strains of EAV and the occurrence of new outbreaks of EVA. Experience over the past 20 plus years would indicate that EAV infection is of increasing economic significance to the $102 billion/annum horse industry in the USA. Economic losses attributable to EAV infection mainly include abortion, illness and death in young foals, the carrier state in stallions, and restricted export markets for carrier stallions as well as virus infective semen or embryos (Owens, 2005).

The establishment of persistent infection in the reproductive tract of the infected stallions is a unique feature of EAV infection. Following EAV infection, a variable proportion (30-60%) of stallions can become persistently infected and continuously shed the virus in their semen for varying time periods, from weeks to years (Timoney,

McCollum, Roberts, et al., 1986; Timoney, McCollum, and Roberts, 1986; Timoney et al., 1987; Neu and Timoney, 1988). In 2006, a multistate occurrence of EVA was confirmed in Quarter Horses for the first time, resulting primarily from the shipment of EAV infective semen and the interstate movement of donor or embryo recipient mares. During this 2006-2007 multistate EVA event, the disease also spread and affected other horse breeds such as, Thoroughbreds, Warmbloods, Arabians, American Saddlebreds, Paint Andalusian and led to the establishment of the carrier state in a significant number of stallions (Timoney et al., 2008; Zhang et al., 2010).

Although multiple factors may contribute to the development of the carrier state, host genetic variation is assumed to explain the individual differences in the occurrence of the carrier state and virus clearance, because such variations exist even after exposure of stallions to the same virus strain and dose. The profound differences in the breed-specific seroprevalence of EAV infections also might reflect inherent genetic differences that confer resistance or susceptibility to infection (McCollum and Bryans, 1973). Understanding of the nature of the interaction between the virus and the host is critical for understanding the mechanism of virus persistence.

Recently, it has been demonstrated, with the use of flow cytometry, that the $CD3^+$ T lymphocyte subpopulation of individual horses varied in their susceptibility to *in vitro* VBS EAV infection (Go et al., 2010). In a parallel retrospective study our lab showed that there is a correlation between the stallion carrier state of natural infected stallions and the *in vitro* $CD3^+$ T cell susceptible phenotype (Go et al., 2012). The data presented in the second chapter of this thesis strongly suggests that stallions that have the $CD3^+$ T cell

phenotype seem to be at a higher risk of becoming persistently infected with EAV following inoculation with the KY84 strain of EAV.

Furthermore, we investigated the effect of virus on semen quality of acutely and persistently infected stallions. In the third chapter of this thesis it was demonstrated that semen parameters are negatively affected following experimental infection of stallions with the KY84 strain of EAV. High body temperature as well scrotal edema observed during acute phase of infection exerted deleterious effects on all the semen quality parameters. However high titers of virus in semen during acute or persistent infection of the stallions seemed to have little or no effect on semen quality. The spermatozoa deterioration observed following experimental inoculation of stallions with EAV KY84 were of enough magnitude to cause temporary infertility of the stallions. Thus, in addition to the risk of acutely EAV infected stallions to become long-term carriers and shed virus for years or entire life in semen, the deterioration in semen quality of acutely EAV infected stallions can cause great economical loss associate with the temporary removal of these animals from the breeding activities for as long as 3 to 4 months, until semen quality return to normal.

The data presented in this dissertation suggest new directions for future EAV research to enhance our understanding of the carrier state, as well as susceptibility to the disease.

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