The effect of Rift Valley fever virus clone 13 vaccine on semen quality in rams

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Declaration

I, Geoffrey James Brown, hereby declare that the experiments presented in this dissertation, entitled "The effect of Rift Valley fever virus clone 13 vaccine on semen quality in rams" were conceived, planned and executed by myself, and apart from the normal guidance from my supervisors, I received no assistance.

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

This dissertation is presented in partial fulfilment of the requirements for the degree MMedVet (Gyn) in animal reproduction.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

AV	artificial vagina
BHK	baby hamster kidney
cRNA	complementary RNA
DAFF	Department Agriculture, Forestry and Fisheries
DNA	deoxyribonucleic acid
DVTD	Department of Veterinary Tropical Diseases
EE	electroejaculation
ELISA	enzyme-linked immunosorbent assay
EVA	equine viral arteritis
FVS	Faculty of Veterinary Science
MEM	minimum essential medium
MLV	modified live
mRNA	messenger RNA
OBP	Onderstepoort Biological Products
OIE	Office International des Épizooties
PBS	phosphate buffered saline
PRRSV	porcine reproductive and respiratory syndrome virus
PVC	polyvinyl chloride
RH	relative humidity
RNA	ribonucleic acid
RVF	Rift Valley fever
RVFV	Rift Valley fever virus
SD	standard deviation
SNT	serum neutralisation test
TCID	tissue culture infectious dose
TUNEL	terminal deoxynucleotidyl transferase-mediated deoxy-UTP nick end labelling
UP	University of Pretoria
VNT	Virus neutralisation test

Summary

Title: **The effect of Rift Valley fever virus clone 13 vaccine on semen quality in rams**

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Abstract

Rift Valley fever (RVF) is an arthropod-borne viral disease of significant importance in both livestock and humans. Epidemics occur periodically in domestic ruminants, typically after heavy rains, which encourage rapid multiplication of mosquito vectors. Clinical symptoms in livestock vary from inapparent infection to abortions and peracute deaths.

The disease has significant zoonotic potential. People in contact with infected livestock may develop disease that varies from mild flu-like symptoms to severe neurological and haemorrhagic disorders and death.

An important way of controlling the disease is through vaccination of susceptible livestock. Rift Valley fever virus (RVFV) clone 13 is a relatively new livestock vaccine against RVF that is derived from an avirulent natural mutant strain of RVFV. This vaccine has been shown in previous studies to confer protective immunity against infection with live virus.

The effect of this vaccine on semen quality in male animals has never been tested.

The purpose of the current trial was to determine whether RVFV clone 13 vaccine had any effect on semen quality in rams. The hypothesis tested was that animals vaccinated with RVFV clone 13 vaccine would not experience a reduction in semen quality (measured by evaluating the percentage progressively motile and percentage morphologically normal spermatozoa in successive ejaculates) relative to unvaccinated control animals.

A test/control model was used to evaluate the effect of this vaccine on semen quality.

A group of peripubertal ram lambs were tested for antibodies to RVFV using a serum neutralisation test (SNT). Animals without detectable antibodies (n=23) were then randomly allocated to either a test group (n=12) or a control group (n=11). Daily rectal temperature measurements were taken and weekly semen evaluations were conducted. Blood samples were drawn weekly to assess serum antibody titres.

Seven animals were subsequently eliminated from the statistical analysis because of potential confounding factors. Of these seven, five animals had extremely poor semen quality at the start of the trial, one animal was found to have a persistent febrile response commencing at the start of

the trial, and one animal had seroconverted to Rift Valley fever virus in the period between the initial screening and onset of the trial.

Logistic regression analysis was performed on data gathered from the remaining animals to determine whether an association existed between animal group, rectal temperature and semen quality parameters. It was found that no correlation existed between treatment group and values obtained for the semen quality parameters measured. There was no statistically significant post-vaccination temporal decline in the percentage of live morphologically normal spermatozoa, or the percentage of progressively motile spermatozoa, either when assessed amongst all animals or when assessed within individual groups. Based on the data from this trial, the hypothesis was not rejected.

Despite this finding, it should be stated that the elimination of animals from the analysis had some effect on the statistical power of the study. A repeat of the trial with a larger sample size and a more comprehensive pre-screening process to avoid the inclusion of animals with poor semen quality may be indicated.

Chapter 1 Introduction and literature review

1.1. Introduction

Rift Valley fever (RVF) is a mosquito-borne, viral infectious disease of ruminants that has significant economic and public health effects. It can induce disease with high mortality in juvenile and adult animals and can cause abortion in pregnant animals (Flick & Bouloy, 2005). In addition, it is an important zoonosis that may have debilitating or life-threatening effects in humans (Gerdes, 2004). People at particular risk of contracting this disease are those who work in the fields of agriculture and animal health (Swanepoel & Coetzer, 2004).

Measures aimed at controlling the disease need to be multi-faceted in their approach, with the goal of predicting the occurrence of the disease so that control strategies can be timeously implemented to minimise the effect of outbreaks.

Meteorological conditions can be monitored and predictions made regarding climatic conditions favourable to disease outbreak (Martin *et al.*, 2008). Once an outbreak has occurred, severity of an epidemic can be reduced by managing potential exposure of susceptible animals, implementing vector control strategies and vaccinating at-risk animals. Vaccination assists in preventing the spread of disease by reducing the population of infected animals that can infect arthropod vectors. Vaccination of dams confers colostral immunity to offspring, which results in a reduction in juvenile mortality (Gerdes, 2004).

In South Africa, three vaccines are commercially available: a modified-live (MLV) vaccine, a killed vaccine, and a vaccine derived from an avirulent natural mutant strain. The latter vaccine, Rift Valley fever virus clone 13 vaccine (Onderstepoort Biological Products, Onderstepoort, South Africa), has been marketed for several years. The clinical efficacy of this vaccine in inducing seroconversion in sheep has been proven in other studies (Dungu *et al.*, 2010). The purpose of the current study was to evaluate the effects (if any) of this vaccine on semen quality in rams, and to quantify such effects by measuring differences in semen motility, sperm morphology and rectal temperature over a period of approximately one ovine spermatogenic cycle of 42 days.

1.2. Hypothesis

The hypothesis for this research study was as follows:

Animals vaccinated with Rift Valley clone 13 vaccine do not experience a reduction in semen quality (measured by evaluating the percentage progressively motile and percentage morphologically normal spermatozoa in successive ejaculates) relative to unvaccinated control animals.

1.3. Literature review

1.3.1 Aetiological agent

Rift Valley fever virus (RVFV) (Genus *Phlebovirus*, family *Bunyaviridae*) is responsible for RVF, a disease of importance in southern Africa, where it is cyclically endemic (Swanepoel & Coetzer, 2004; Flick & Bouloy, 2005). The family *Bunyaviridae* consists of more than 300 enveloped RNA viruses, most of which are arthropod-transmitted, and includes the genera *Phlebovirus*, *Hantavirus* and *Nairovirus* (Elliot, 1990; Pepin *et al.*, 2010).

The *Phlebovirus* genus consists of 68 serotypes, several of which may cause clinical syndromes in animals and man (Elliot, 1990). Rift Valley fever virus is the type species of this genus, but other viruses within the genus include the Sicilian sandfly fever serocomplex, Naples virus, the Uukuniemi virus serocomplex, Candiru virus, Punta Toro virus and others (Liu *et al.*, 2003). Phleboviruses consist of a single stranded RNA genome with three segments: large (L), medium (M) and small (S). The L and M segments are negative-sense, and code for the L protein (a viral polymerase). In addition, the M segment codes for a precursor to the viral glycoproteins. The S segment has an ambisense polarity: it encodes for two proteins, the nucleoprotein N and a non-structural protein known as NSs (Ikegami & Makino, 2009; Pepin *et al.*, 2010).

During viral replication, each of the three segments is transcribed to messenger RNA (mRNA) which is then replicated by synthesis of complementary RNA (cRNA). Importantly, in RVFV, the cRNA encoding for the S segment operates as a template for synthesis of NSs mRNA (Pepin *et al*, 2010).

The NSs encoded for by the S segment of the RVFV RNA genome has been identified as a major factor of virulence. It is believed that NSs assists in avoiding the immune response of the

host by acting as an interferon antagonist (Pepin *et al.*, 2010). NSs may also be the agent responsible for chromosomal defects seen in ovine and murine cells which may be the underlying cause of foetal deformities, abortions and placental necrosis seen in infected animals (Mansuroglu *et al.*, 2010).

Vaccine strains vary in virulence. The NSs may be altered by biological attenuation or chemical inactivation, as in the case of the Smithburn (live) vaccine and the formalin-inactivated vaccine.

In addition, NSs may also be altered by natural mutation, as in the case of the clone 13 vaccine (Pepin *et al.*, 2010).

These respective alterations will be discussed in more detail in Section 1.3.3.1.

1.3.2 Epidemiology

1.3.2.1. Disease occurrence

Rift Valley fever in sheep was first described in Kenya in 1931 (Daubney *et al.*, 1931) and has subsequently been isolated throughout sub-Saharan Africa (Swanepoel & Coetzer, 2004; Bird *et al.*, 2009), as well as Madagascar (Morvan *et al.*, 1992), Egypt (Meegan *et al.*, 1979) and the Arabian peninsula (Fagbo, 2002).

In South Africa significant outbreaks have occurred in 1950-1951, 1974-1975 (Gerdes, 2004) and in 2008 (Bird *et al.*, 2009).

RVF epidemics have been shown to have an association with rainfall. It has been noted by researchers that epidemics occur when significantly above-average rainfall occurs at several sites within a calendar year (Davies *et al.*, 1985) (Gerdes, 2004). Recently, models have been developed to predict RVF outbreaks using satellite-observed indicators of higher rainfall (Anyamba *et al*., 2009).

The global phenomenon of climate change may result in conditions favourable to the spread of tropical diseases to regions previously unaffected by them. The spread of RVF is highly dependent on conditions favourable to the proliferation of mosquito vectors. Global warming may result in an increase in habitat and range of these vectors, and hence spread of the disease (Martin *et al.*, 2008).

1.3.2.2. Transmission

RVFV is transmitted between mammalian hosts during blood-feeding of aedine and culicine mosquito vector species. A study was performed which evaluated the vector efficiency of eight African mosquito species from the genera *Culex* and *Aedes*. It was found that all species examined were capable of transmitting infection, however some variation in transmission rate occurred (Turrell *et al.*, 2008). The relative importance of different mosquito species in transmission of the disease is the subject of ongoing research.

In the early 1980s, 134 876 mosquitoes were trapped over a three year period, from varying ecological regions in Kenya. Mosquitoes were grouped into 3 383 pools. Of these pools, 19 were found to be positive for RVFV (Linthicum *et al.*, 1985).

Trans-ovarial transmission has been described to occur between successive generations of mosquito and is widely accepted as fact in review literature (Flick & Bouloy, 2005; Swanepoel & Coetzer, 2004); however field and laboratory research has not always been able to prove unequivocally that this is so.

One group of researchers suggested that *Aedes* spp. serve as the primary reservoirs of RVFV in inter-epidemic periods. When sufficient rainfall occurs to allow the *en masse* hatching of eggs embedded within the mud, an enormous number of *Aedes* spp. will emerge. Some of these mosquitoes will be infected with RVFV and will feed on vertebrate hosts, transmitting the virus. These infected vertebrate hosts may infect various mosquito genera (*Aedes, Culex, Eretmapodites, Anopheles, Mansonia* and others), which will then function as secondary vectors, transmitting the infection to other susceptible vertebrates and ensuring rapid RVFV dissemination. The inter-epidemic egg reservoir theory was supported by the finding that several specimens of *Aedes lineatopennis* tested positive for RVFV after being raised from field-collected pupae (Linthicum *et al.*, 1985; Gerdes, 2004).

Other workers have had different findings. Prior to the findings of Linthicum and co-workers (1985), studies by South African researchers failed to isolate any virus from the progeny of mosquitoes from populations with a presumed high prevalence of infection (McIntosh *et al.*, 1980). A later study extracted *Aedes* spp. eggs from the soil of dried up pans and vleis in the south-eastern Free State Province. These eggs were then hatched, larvae were reared under laboratory conditions and viral assays were performed on the resulting mosquitoes. No virus

was recovered from mosquito tissues and no RVF was transmitted to hamsters used as a mosquito food source (Gargan *et al.*, 1988).

In addition to a variety of mosquito vector species, RVFV has the capacity to be transmitted by biting flies, including members of *Simulium* spp. (blackflies), *Culicoides* spp. (midges) and *Phlebotomus* spp. (sandflies), as well as *Amblyomma* and *Rhipicephalus* tick species (Pepin *et al.*, 2010). The role of non-mosquito species in natural transmission of RVFV is not well described in the literature.

In 1999 the arbovirus, West Nile virus, was introduced to the United States and spread across North America. Due to concern that the same could occur with RVF, researchers evaluated the vector competence of North American mosquito species, and found that several species were capable of transmitting RVFV under laboratory conditions (Turell *et al.*, 2008).

Despite finding positive neutralising antibodies in wild ruminants in interepidemic periods, it is still unknown what role ruminants play in harbouring and amplifying infection between disease outbreaks (Evans *et al.*, 2008). Infection via oronasal and respiratory exposure may occur when a viraemic animal comes into contact with a susceptible animal. Aerosol infection has been shown to be capable of inducing infection in laboratory animals (Easterday & Murphy, 1962). Experimental infection of puppies and kittens via respiratory aerosols resulted in infection, whilst infection via the gastrointestinal route failed to do so (Keefer *et al.*, 1972). Performing a necropsy or obstetric procedure on an infected animal is a significant risk factor associated with human infection (Bird *et al.*, 2009).

1.3.2.3. Hosts

Rift Valley fever is predominantly a disease of wild and domestic ruminants; however, primates and rodents may become infected. Young immunologically naïve animals show more severe clinical manifestations with a higher incidence of mortality than older animals (Flick & Bouloy, 2005).

A variety of studies have found positive antibody titres to RVFV in wildlife. An RVFV seroprevalence survey was performed amongst Kenyan wildlife using 1 008 serum samples collected from 1999-2005. Sera were tested using a virus neutralisation test (VNT). Buffalo sera collected were tested both using VNT and an indirect enzyme-linked immunosorbant assay

(ELISA). Seven Kenyan species had sera with detectable neutralising antibodies: African buffalo (*Syncerus caffer)*, black rhino (*Diceros bicornis)*, kudu (*Tragelaphus strepsiceros)*, impala *(Aepyceros melampus)*, African elephant *(Loxodonta africana)*, kongoni (*Alcelaphus buselaphus)* and waterbuck (*Kobus ellipsiprymnus)* (Evans *et al.*, 2008).

A 1988 study of sero-prevalence among Namaqua rock rats (*Aethomys namaquensis)* revealed that 15 % of animals sampled had positive antibodies to RVFV (Pretorius *et al.*, 1997). This may indicate that rodents could play a role in harbouring the disease during inter-epidemic periods.

Laboratory animals have long been used in the study of RVFV. In a 1962 study, mice, hamsters and rats were evaluated for relative susceptibility to RVFV by exposing them to an aerosolised strain of the virus. Hamsters and mice had comparable mortality rates after aerosol exposure, whilst rats appeared to be resistant to infection by this route (Easterday & Murphy, 1962).

Whilst RVF is primarily a disease of ruminants, RVFV may also infect humans, particularly those who work closely with livestock or who lead a pastoral existence. During a 1977 Egyptian epidemic in domestic animals, it was estimated that between 10 000-20 000 human cases occurred, with 70 to 80 deaths recorded (McIntosh *et al.*,1980).

In 2000 an outbreak of RVF occurred on the southern portion of the Arabian Peninsula, affecting parts of Yemen and Saudi Arabia, with 882 human cases reported (Balkhy & Memish, 2003).

In September-November 2000, 165 human cases were seen at a referral hospital in Gizan province, Saudi Arabia. Patients under study at this referral centre experienced a mortality rate of 33.9 %, possibly reflecting the severity of referred cases. Of the total of 517 patients diagnosed with RVF in the Saudi outbreak, 87 died (Al-Hamzi *et al.*, 2003).

In 2008 several staff and veterinary students at the Faculty of Veterinary Science, University of Pretoria experienced symptoms after coming into contact with animal tissues infected with RVFV (Bird *et al.*, 2009).

1.3.3 Control

Control measures to reduce losses due to RVF include vaccination, vector control by biological or chemical means, or moving livestock from low-lying marshy areas to higher areas during the wet season (Swanepoel & Coetzer, 2004).

1.3.3.1. Vaccination

In South Africa, three vaccines are currently available. Due to the virulence and zoonotic nature of the virus, manufacture of vaccines requires an isolated production facility. Commercially available RVFV vaccines are summarised in Table 1.

Table 1 Rift Valley Fever virus vaccines currently available in South Africa (Von Teichman *et al.*, 2010)

Vaccine	Act 36/1947 registration number	Description
RVFV live	G0119	Freeze-dried live attenuated (Smithburn strain)
RVFV inactivated	G1349	Formalinised, aluminium hydroxide gel adjuvanted
RVFV clone 13	G3876	Freeze-dried, live attenuated (clone 13 strain)

1.3.3.2. Modified-live RVFV vaccine

The modified-live (Smithburn) strain was originally produced by serial intra-cerebral inoculation in suckling mice (Smithburn, 1949). This vaccine provides long-lasting protection against infection but has been shown in several studies to cause abortion and teratogenesis when administered to gravid animals in the first trimester of pregnancy (Coetzer & Barnard, 1977). Since it is a live vaccine, reversion to virulence of the Smithburn strain is theoretically possible and authors have recommended that it is not used in countries where RVFV has not been introduced (Swanepoel & Coetzer, 2004; Ikegami & Makino, 2009).

1.3.3.3. Inactivated RVFV vaccine

Inactivated RVFV vaccine is produced from an isolate obtained from a cow during a disease outbreak in the Free State Province of South Africa in 1974, which was then adapted to baby

hamster kidney (BHK) cells. Production of the inactivated RVF virus vaccine is labour intensive and slow, testing is cumbersome and protracted, resulting in increased expense and a long lead time. In addition, clinical use requires a booster for adequate immunity (Von Teichman *et al.*, 2010).

1.3.3.4. RVFV clone 13 vaccine

RVFV clone 13 vaccine is produced from the avirulent strain 74HB59 of RVFV. The original virus was derived from a non-fatal human case of RVF in the Central African Republic, and was then passaged through mice and Vero cells. This strain lacks approximately 70 % of the S-segment RNA coding for the non-structural protein NSs, which was found to be the determinant of virulence (Ikegami & Makino, 2009; Von Teichman *et al.*, 2010). This large deletion renders the virus unable to revert to virulence *in vivo* (Pepin *et al.*, 2010).

RVFV clone 13 vaccine was shown to provide protection against experimental infection of pregnant ewes and prevented symptoms such as pyrexia, abortion, teratogenesis and death in a vaccinated group when compared to a control group (Dungu *et al.*, 2010). This vaccine is produced using standard freeze-dried live vaccine production processes, which are safe, cost-effective and relatively simple (Von Teichman *et al.*, 2010). It is essential that this vaccine is proven in field trials to be effective and safe for animals at all production stages, including potential sires.

1.3.4 The process of spermatogenesis

1.3.4.1. Physiological and histological characteristics of spermatogenesis

In animals that reproduce by sexual reproduction, spermatogenesis occurs within the seminiferous tubules of the testes.

The process of spermatogenesis has been defined as

‘A long process of cellular differentiation in which a spermatogonial stem cell proceeds through several mitotic divisions, a meiotic division and numerous cytological transformations leading to the generation of mature elongated spermatids’ (Barth & Oko, 1989) and

'The sum of all cellular transformations in developing germ cells that occur in the seminiferous epithelium' (Senger, 2003).

The precise histological and physiological changes that occur during the production of spermatozoa in the mammalian testes have been a topic of study for many decades, with study primarily concentrating on rodent models (Leblond & Clermont, 1952). However, the intricacies of bovine spermatogenesis has also been well described and summarised (Barth & Oko, 1989).

The correct interpretation of sperm morphology smears requires a working knowledge of the spermatogenic process. Evaluation of sperm morphology smears may assist in diagnosing testicular, epididymal or accessory gland pathology. Workers have classified the cells of the bovine spermatogenic series into 13 different types: spermatogonial cell types (A0, A1, A2, A3, intermediate, B1 and B2 spermatogonia), primary spermatocytes (pre-leptotene, leptotene, zygotene and pachytene primary spermatocytes), secondary spermatocytes, and spermatids. These cells were classed based on their histological appearance, their location within the spermatogenic epithelium, and their associations with other cells (De Rooij & Russell, 2000).

Spermatogonia are relatively unspecialised cells that give rise to primary spermatocytes after several cell divisions (De Rooij & Russell, 2000). The development of spermatozoa from spermatogonia occurs in a predictable, orderly fashion. As this process occurs, the developing spermatozoa gradually make their way from the periphery of the seminiferous tubule toward the lumen, where they will eventually be released by the process of spermiation (Barth & Oko, 1989; Senger, 2003).

The process of spermatogenesis comprises two principle processes which occur sequentially: spermatocytogenesis and spermiogenesis. During the process of spermatocytogenesis, the diploid germ cells (spermatogonia) differentiate and replicate by mitosis. Some are retained to form a reserve of cells for further duplication, others proceed with meiotic development to form primary, then secondary spermatocytes. Secondary spermatocytes are those cells that have completed meiosis I, and need to undergo meiosis II in order to proceed with development into haploid germ cells. The cell types and processes of spermatocytogenesis are represented in Table 2.

Table 2 Summary of cell types seen in spermatocytogenesis

Cell type	Haploid (n) or Diploid (2n)	Comments
Type A spermatogonia	2n	Elaborated by mitosis, has subtypes A0, A1, A2,
Type B spermatogonia	2n	Intermediate stage prior to formation of spermatocytes
Preleptotene spermatocytes	2n	
Leptotene spermatocytes	2n	First stage of meiotic prophase
Zygotene spermatocytes	2n	Second stage of meiotic prophase
Pachytene spermatocyte	2n	Third stage of meiotic prophase
Diplotene spermatocyte	2n	Fourth stage of meiotic prophase
Diakinetic spermatocyte	2n	Final stage of meiotic prophase
Secondary spermatocyte	2n	Enters meiosis II to produce haploid round spermatids
Round spermatid	n	Continues with spermiogenesis to produce spermatozoa, to be released from the seminiferous epithelium.

Spermiogenesis follows spermatocytogenesis and is the process whereupon the haploid round spermatid differentiates into a functional, motile spermatozoon (Senger, 2003). Spermiogenesis has been divided into four stages: The Golgi stage, cap stage, the acrosome stage and the maturation stage. These stages are summarised in Table 3.

Table 3 Summary of the stages of spermiogenesis (adapted from a description by Barth and Oko, 1989)

Stage of spermiogenesis	Description
Golgi phase	From formation of round spermatid, until formation of acrosomal granule
Cap phase	Acrosomic head cap is growing and enlarging
Acrosome phase	Nucleus and acrosome undergo the greatest amount of morphological transformation
Maturation phase	Formation of flagellum and neck piece, shaping of the neck piece

Certain cell types within the spermatogenic series are particularly sensitive to disruption and damage by chemical agents, heat and radiation, possibly resulting in subfertility or infertility. The premise upon which this research was based was that if RVFV clone 13 vaccine was to cause any abnormalities in sperm production that these abnormalities would be mediated by the effects of pyrexia on testicular tissue. The mechanisms of this heat-induced damage will be elaborated in Section 1.3.5.4.

1.3.5 The concept of heat stress and reproductive function

1.3.5.1. Physiology of thermoregulation in mammals

Changes in core body temperature in mammals can have adverse effects on normal physiological processes, including enzyme function, muscle activity and energy metabolism (Akin, 2011). Mammals and birds are classified as homeothermic - they maintain a relatively constant body temperature, regardless of significant variation in environmental temperature. By contrast, reptiles, fish and amphibians are poikilothermic - their body temperature varies with the temperature of the environment (Cunningham, 2002). Whilst poikilotherms rely solely on behavioural modification to regulate their body temperature during adverse environmental circumstances, homeotherms are able to employ physiological mechanisms to maintain body temperature relatively independently of ambient temperature (Akin, 2011). Body temperature of mammals and birds is maintained within a narrow range by controlling metabolic heat production as well as heat loss. Thermoregulation is regulated by nervous feedback from diverse tissues and organs. Central nervous integration of these signals occurs within the thermoregulatory centre of the hypothalamus. The hypothalamus of an animal determines a thermoregulatory set-point. When core body temperature rises above this set point, mechanisms of heat loss are initiated. When core temperature decreases below the set point, heat-producing mechanisms are activated (Cunningham, 2002).

1.3.5.2. The concept of 'heat stress'

Heat stress is a term used to refer to a physiological process whereby environmental temperature drives core body temperature above the thermoregulatory set-point (Hansen, 2009). Importantly, pyrexia occurs when the thermoregulatory set point is reset to a higher temperature, resulting in a disturbance in normal thermoregulatory function (Cunningham, 2002). The difference between these two concepts is important. Substances that may induce pyrexia are known as pyrogens, and have been divided into two categories - those that originate from within host cells, such as cytokines, and those that originate from other sources, such as bacteria, viruses or exogenous toxins (Mackowiak, 1998).

1.3.5.3. The physiological function of the scrotum

Mammals are the only animals in which the testes descend from a position within the abdominal cavity into the outpouching of skin and peritoneum that is the scrotum (McEntee, 1990). There

is considerable controversy regarding the evolutionary pressures that resulted in the development of the scrotum, with no apparent unifying explanation (Bedford, 2004). Evolutionary biologists have advanced a number of hypotheses for the precise reasons behind scrotal development. An in-depth evaluation of these hypotheses is beyond the scope of this dissertation; however a basic tabulated summary based on a recent review article by Kleisner *et al.*, (2010) can be found in Table 4.

Table 4 Theories for evolution of the scrotum (Kleisner *et al*., 2010)

Hypothesis	Brief explanation
Cooling hypothesis	A temperature that is lower than core body temperature is physiologically necessary for normal spermatogenesis
Temperature trigger hypothesis	The storage of sperm at a temperature significantly lower than that of female core body temperature may result in a biochemical trigger mechanism which is activated when sperm is ejaculated into the warmer female reproductive tract, resulting in activated sperm capable of fertilisation.
'Galloping' hypothesis	The evolution of rapid locomotion (galloping) by certain species caused an increase in intra-abdominal pressure due to strong muscle contractions. This resulted in variations in intra-testicular pressure incompatible with spermatogenesis and sperm storage. Hence, the positioning of the testes in an extra-abdominal position evolved simultaneously to counteract these effects.
Behavioural/signalling hypothesis	The scrotum serves a signalling function. In some mammalian groups it is brightly coloured and serves as a sexual signal

In most mammals the process of spermatogenesis occurs within the intrascrotal testes at a temperature that is measurably lower than core body temperature.

Notable exceptions include certain marine mammals, the elephant, rhinoceros, hyrax and certain small mammals (for example elephant shrews and golden moles) (Bedford, 2004). A description of the thermoregulatory function of the scrotum first occurred in the early 20th century (Moore & Quick, 1924).

In most species, if the testes are maintained at core body temperature, spermatogenesis does not occur (Roberts, 1986). Importantly, the fact that mammalian species which normally have abdominally retained testes are able to produce healthy sperm, indicates that a higher testicular temperature is not fundamentally incompatible with spermatogenesis. The temperature at which testicular tissue optimally produces sperm appears to be species-dependent (Bedford, 2004). Interestingly, in dolphins, blood flowing to the intra-abdominal testes is cooled by a vascular

countercurrent exchange mechanism, whereby spermatic arteries are in close proximity to veins draining the dorsal surface of the tail flukes (Pabst *et al.*, 1995).

The ram has a prominent, pendulous scrotum which facilitates heat loss in hot environments. It is provisioned with abundant quantities of temperature receptors which have an effect on systemic thermoregulatory responses. In a 1962 study, local heating of ovine scrota was shown to cause a marked increase in respiratory rate. When a comparable area of skin on the flank of the animal was heated, no such increase occurred. This elevated respiratory response could be abolished by severing sensory nerves from the scrotum (Waites, 1962).

A variety of physiological mechanisms exist to ensure that the temperature of the testes within the scrotum is lower than the core body temperature. The scrotal skin of the ram contains numerous sweat glands, which assist in evaporative cooling during hot periods (Waites & Voglmayr, 1963). The lining of the scrotum contains a layer of smooth muscle known as the tunica dartos which contracts in cold environments, causing puckering of the scrotal skin and elevation of the scrotal contents towards the body wall. In addition, the cremaster muscle, extending from a leaf of the internal abdominal oblique muscle to the tunica vaginalis, is able to retract the testes towards the body (Setchell, 2006).

It was suggested by early authors that the testicular artery and vein form a countercurrent exchange network which facilitates heat exchange between the warm arterial blood originating from the systemic circulation, and the cooler venous blood draining the testes (Harrison & Weiner, 1949). This viewpoint was disputed by Waites and Moule (1961), who suggested instead that the function of the venous plexus within the neck of the scrotum was to ensure even distribution of scrotal thermoregulation to superficial and deep regions of the testis.

Maloney and Mitchell (1996) performed an experiment in which telemetric thermocouples were placed within the peritoneal cavity and scrotum of five Dorper-cross rams. Rams were then serially exposed to hot and cold environments, exercised on a treadmill and injected with *Salmonella typhosa* lipopolysaccharide endotoxin. The effect of these separate interventions was then assessed by measuring and comparing systemic and intrascrotal temperatures, and evaluating variations in scrotal retraction. Circadian variations of both scrotal and core temperature were noted in unrestrained animals whilst in their home pens at an ambient temperature of 21-23 °C. Scrotal temperature was maintained at an average of 3.30 ± 0.03 °C below body temperature under these controlled conditions. Exposure to cold (6 °C, 70 %

relative humidity (RH)) for 5 h had no significant effect on core temperature but led to a significant fall in scrotal temperature. Scrotal retraction began immediately upon reduction of ambient temperature, and reached a maximum after 1.5 h.

Exposure to heat (40 °C, 25 % RH) for 5 h caused an initial rise in core body temperature over the first 1.5 h, followed by a gradual decline for the remainder of the exposure. Heat exposure caused a significant rise in scrotal temperature to 37 °C, with scrotal retraction remaining minimal.

Exposure to endotoxin resulted in a biphasic increase in body temperature up to a maximum of 2.5 °C above the resting temperature; however scrotal temperature did not increase significantly. Scrotal retraction remained unchanged for 3 h post-injection, but then decreased significantly.

Exercise for 40 min on a treadmill at 2 $m.s^{-1}$ and 10 % gradient had a significant effect on core body temperature (resulting in an average increase of 1.3 °C), but had no significant effect on scrotal temperature. Scrotal retraction was reduced significantly during exercise.

Since the scrotal temperature remained unchanged during the experimentally-induced fever, the researchers suggested that local scrotal thermoregulation was not affected by endotoxin injection. The authors posited that the explanation of a single, simple thermoregulation pathway for regulating scrotal temperature based on core body temperature was possibly an oversimplification of a system that likely consists of a complex hierarchy of inputs and modulating effector signals (Maloney & Mitchell, 1996).

1.3.5.4. Mechanisms of heat-induced impairment of spermatogenesis

In many mammalian species, prolonged pyrexia, insulation of the scrotum or prolonged exposure to high atmospheric temperature and humidity may result in impairment of spermatogenesis (Roberts, 1986; Setchell, 2006). Pigs with congenital unilateral cryptorchidism were shown to have retarded spermatogenesis in the retained testis, which could be alleviated by the application of a water-cooling device (Frankenhuis & Wensing, 1979).

In an early study, cells of the spermatogenic series most sensitive to heat were determined to be the pachytene, diakinetic and dividing spermatocytes, as well as round spermatids: researchers

suggested that exposure to heat affected the nuclear reorganisation occurring during meiosis in these cells (Chowdhury & Steinberger, 1970)..

In male rats, most germ cell death occurs during spermatogonial development (up to 75 %), with a lesser amount occurring during subsequent maturations of spermatocytes and spermatid development. A certain amount of germ cell death occurs under normal spermatogenic conditions. An increased rate of germ cell death may occur under specific conditions: namely deprivation of hormonal support (gonadotrophins or androgens), oestradiol treatment, exposure to chemotherapeutic agents, or local testicular heating (Chowdhury & Steinberger, 1970).

In all mammalian cells, two major pathways are involved in the process of programmed cell death: a so-called intrinsic pathway, and an extrinsic pathway (Hikim *et al.,* 2003). Such cell death has been shown to occur in rat testes affected by heat stress. Heating of the testes may increase metabolism of spermatogenic cells, leading to localised hypoxia. This may occur due to inadequate blood flow into the testes or reduced oxygen transport which in turn may lead to the generation of reactive oxygen species or direct damage to DNA (Paul, *et al.,* 2009). A specialised technique, namely terminal deoxynucleotidyl transferase-mediated deoxy-UTP nick end labelling (TUNEL) was used in work performed by Hikim and co-workers on rat testes, for the in situ detection of cells with breaks in their DNA strands indicative of apoptosis. By this method, it was found that spontaneous apoptosis of germ cells in control rats (i.e. those rats whose testes were not subjected to hyperthermic stress) occurred primarily in type A spermatogonia and in a few spermatocytes late in meiosis. By contrast, in rat testes subjected to hyperthermic stress, significant numbers of pachytene, diplotene and dividing spermatocytes as well as early spermatids were affected (Hikim, *et al*., 2003). This finding confirmed the histopathological findings of other workers made more than 30 years earlier (Chowdhury & Steinberger, 1970).

1.3.6 Prior work on the effect of heat on semen quality in mammals

The effect of duration of general body heating to 40.5 °C with 45 % relative humidity on the fertility of Merino rams has been evaluated. In one study two rams were exposed to two consecutive days of 8 h in a hot chamber, another two rams were exposed to four consecutive days of 8 h in a hot chamber. Two further animals served as controls, and were not heated. These animals were then mated to 20 synchronised ewes per group (10 ewes per ram), such that rams were 10-16 days post-heating at the time of mating. On evaluation of the spermiogram, a marked decline in sperm concentration was noted in animals subjected to heating. The percentage of live cells was lower in heated animals, with a markedly larger number of pyriform cells and tailless sperm. In the four-day heated rams, there was a significant increase in the number of acrosomal abnormalities noted 10-16 days after heating. The results of the semen evaluation was reflected in the subsequent conception rates of the ewes: 12 of 20 ewes mated to control rams lambed, 3 of 20 ewes mated to two-day heated rams lambed, and 0 of 20 ewes mated to four-day heated rams lambed (Rathore, 1968).

In another study, the same author evaluated the fertility of four rams heated in a warm chamber at 40.5 °C with 45 % relative humidity for 8 h for 1, 2, 3 and 4 days respectively. A fifth ram was not exposed to the hot chamber and was used as a control. Eight ewes were assigned to each ram, and were superovulated using a gonadotropin. Semen was collected by an artificial vagina (AV) and morphological characteristics of the sperm from each ram were evaluated. It was found that animals exposed to heat had an increasing incidence of abnormalities as duration of heat exposure increased. Collected semen was used to inseminate superovulated ewes. These ewes were then slaughtered 60-70 h after insemination, the reproductive tracts were excised and fertilised oocytes were counted and evaluated. Fertilisation rates declined markedly as duration of ram heating increased (Rathore, 1970).

1.3.7 Effects of infectious diseases on semen quality in mammals

Several bacterial, viral and protozoal infectious diseases have been shown to cause reduction in semen quality in ruminants. Normal rectal temperature in the sheep has been established by prior authors as 39.1 ± 0.5 °C (The Merck Veterinary Manual, Ninth Edition, 2005). Raising the temperature of the testes above this value for prolonged periods may result in abnormal sperm production as discussed in Section 1.3.5.4. The underlying mechanism may be as a result of systemic pyrexia, local inhibition of thermoregulation, or direct damage to germ cells.

1.3.7.1. Local effect on thermoregulation

The effect of infection may affect the thermoregulatory mechanism directly. In the case of bovine scrotal cutaneous *Dermatophilus congolensis* infection, the invasion and thickening of scrotal skin as a result of infection was found to have a sustained effect on the ability of the scrotum to regulate testicular temperature (Sekoni, 1993).

In one case study, a bull that developed fever and scrotal swelling as a result of *Eperythrozoon wenyonii* infection was shown to have a transient decline in semen quality. It was hypothesised that marked scrotal oedema resulted in a failure of testicular thermoregulation (Montes *et al.*, 1994).

1.3.7.2. Possible direct effects on testicular tissue

In ovine orchitis induced by *Arcanobacterium pyogenes*, semen quality was found to be affected by infection of the testicular tissue. It was unclear whether this was due to a direct effect on spermatogenesis by bacterial invasion, or to the local heat and swelling brought about by a septic epididymo-orchitis. In the case of *A. pyogenes* infection, some improvement in semen quality was seen in the chronic phase of infection (Gouletsou *et al.*, 2004).

Arteriviruses, including porcine respiratory and reproductive syndrome virus (PRRSV) and equine viral arteritis (EVA), have been shown to have a tropism for testicular tissue, which provides the virus with a venereal mechanism of spread. PRRSV has been shown to manifest as loss of libido, alterations in semen quality, decrease in sperm motility, increase in morphological abnormalities and a reduction in the proportion of spermatozoa with normal acrosomes (Prieto & Castro, 2005).

Bluetongue disease has been associated with infertility of male ruminants. This infertility has been described both anecdotally by practitioners, and formally by researchers. It has been hypothesised that infertility comes about either as a result of the effect of pyrexia on spermatogenesis, or as a result of microvascular lesions in the testis brought about by orbivirus infection. Natural bluetongue virus serotype 8 infection was associated with significant changes in semen quality (Kirschvink *et al.*, 2009).

1.3.7.3. Effect of pyrexia on sperm production

Whilst the effect of pyrexia induced by infectious disease is generally accepted to be a cause of poor semen quality, there are few actual studies that evaluate semen quality in male animals after the known occurrence of a disease.

In a Nigerian study, Yankasa rams experimentally infected with *Trypanosoma vivax* showed rapid elevation in abnormal sperm when compared to a control group. A significant temperature reaction was noted in infected rams compared to uninfected controls (Sekoni, 1993). By contrast, *T. gondii* infection was shown to have minimal effect on semen quality in rams after experimental infection, despite experimental animals showing increased serum antibodies and clinical signs of infection (anorexia, hypothermia). Notably in this study, no pyrexia was noted in trial animals (Lopes *et al.*, 2009).

A human case report was published in which a volunteer of proven fertility and semen quality was evaluated prior to and after an influenza-induced febrile episode. The subject developed a fever of 39-40 °C during the febrile episode. Total sperm count decreased significantly from day 15 to day 58 after the fever and returned to normal by day 79. Abnormal sperm chromatin (measured by sperm chromatin structure assay and flow cytometry) was shown to increase significantly, from 9 % prior to the fever, to 24 % and 36 % at days 15 and 37 post-fever (Sergerie *et al.*, 2007).

1.3.8 Vaccine reactions that affect semen quality in rams

Vaccination of male animals with modified-live vaccines of various infectious diseases have been cited by anecdotal accounts and proven by controlled trials to cause alterations in the spermiogram. Vaccination with a modified-live (MLV) strain of bluetongue was shown by researchers to cause significant transient reduction in semen quality (Bréard *et al.*, 2007). By contrast, the use of an inactivated bluetongue vaccine was reported to have no detrimental effect on semen quality (Leemans *et al.*, 2012). The authors attributed this result to the absence of a pyrexic response to vaccination.

Unfortunately, there are no studies on the effect of RVFV (natural infection or vaccine strains) on semen quality in ruminants with which to compare the present study. One review article refers to original research presented at a congress in which it was found that RVFV can be isolated from bull semen a week after vaccination with the live attenuated (Smithburn) RVF

vaccine. In addition, it was found that bull semen was 'severely affected' by the administration of RVFV Smithburn strain. The review article is vague and poorly worded and the original text could not be accessed at the time of writing, so it is difficult to verify the research methodology involved (Saad *et al.*, 1997) (Kamal, 2011).

Chapter 2 Materials and methods

2.1. Model

Merino ram lambs (n=23) were used for the study. Animals were aged by evaluating the eruption of permanent teeth. All animals had two permanent teeth or fewer. These rams were divided into two groups of approximately equal size:

Group 1: Animals vaccinated with Rift Valley fever virus (RVFV) clone 13 vaccine (n=12)
Group 2: Unvaccinated control animals (n=11)

Semen was collected from all animals (test and control groups) prior to vaccination of test animals. Semen was then collected at weekly intervals for 42 days after vaccination. The parameters of daily temperature, progressively motile spermatozoa and morphologically normal spermatozoa were compared between vaccinated and control groups using descriptive statistics and a repeated measures logistic regression model.

2.2. Experimental procedures

2.2.1 Preparation of animals

The facilities used were at Knoffelfontein Farm, Philipstown, Northern Cape, South Africa. This is a Department of Agriculture, Forestry and Fisheries (DAFF) approved private facility for assisted livestock reproduction (DAFF facility certificate: ZA15/14, expiry date 24 October 2014). Animals were housed in a large outdoor pen with sufficient shelter. Test and control animals were housed together. *Ad libitum* access to lucerne hay and fresh water was provided. Ethical approval for the study was obtained from the University of Pretoria Research Ethics Committee, protocol approval number V020-12.

Prior to enrolling animals in the study, ram lambs from a reserve flock were tested for antibodies specific to RVFV using a serum neutralisation test (SNT) at the Virology Laboratory of the Department of Veterinary Tropical Diseases (DVTD), Faculty of Veterinary Science (FVS), University of Pretoria (UP), Onderstepoort. Seropositive animals were excluded from participation in the study to ensure that only immunologically naïve animals were used.

2.2.2 Allocation to control and test groups

On Day 0 of the study, all animals were allowed to mix freely within a holding pen and then driven into a narrow handling race in single file. As each animal emerged from the front of the handling race, it was allocated by simple binary alternation to either a red or blue group. This method of allocation represents a form of simple random allocation (Roberts & Torgerson, 1998). Animals allocated to the blue group (n=12) were marked on the forehead with a blue livestock marking crayon, while animals allocated to the red group (n=11) were marked on the forehead with a red livestock marking crayon for easier identification. Prior to vaccinating one of the groups with RVFV clone 13 vaccine, it was decided without any forethought or consideration that the blue group would serve as the vaccinated group, and that the red group would serve as the control group.

2.2.3 Clinical examination

On Day 0 of the study, all animals were thoroughly clinically assessed. Rectal temperature, femoral pulse, respiration and body condition score were assessed and recorded. Body condition score was allocated on a scale of 1-5, where a score of 1 indicated a severely cachectic animal and a score of 5 indicated an obese animal.

A specific examination of external genitalia, including palpation of the scrotum and its contents was conducted. Scrotal circumference was measured and recorded. Thereafter body temperature was measured daily for the duration of the trial. Temperature was assessed using a digital thermometer (Thermoval-Rapid® - Hartmann, Northriding, Gauteng, South Africa). The thermometer was inserted into the rectum up to the window of the digital display, equating to a distance of approximately seven centimetres. Prior to first use, the thermometer was calibrated against a laboratory thermometer in a water bath at 37 °C.

2.2.4 Vaccination procedure

On Day 0 of the study, animals in the test group (n=12) were vaccinated by subcutaneous injection with the Rift Valley fever virus clone 13 vaccine, manufactured by Onderstepoort Biological Products (OBP). Vaccine administration was performed as directed by the manufacturer. An unopened sterile syringe and needle were used for each animal. Animals in the control group (n=11) were not vaccinated, and no inert placebo was used. Animals were injected beneath a wool-free fold of skin adjacent to an axilla.

2.2.5 Blood collection

Blood was collected by jugular venipuncture. An assistant restrained the animal in a standing position, with the head elevated. In animals with thick wool on the neck, the region of the jugular groove was clipped with manual scissors-type sheep shears. An alcohol-soaked swab was used to disinfect the region and to moisten the remaining wool in order to facilitate visualisation of the jugular vein. Venipuncture was achieved with a 20 gauge needle and approximately 3 ml of blood was aspirated into a plain serum tube (Vacutainer, Becton-Dickinson, Woodmead, Gauteng, South Africa). Blood samples thus collected were clearly marked with the animal's identification number and the date of collection, and were stored upright in a refrigerator at 4 °C for 48 h until separation of serum and cellular components of blood occurred. The serum was collected and the clot of blood cells was discarded. The serum was placed in an unused, clearly marked plain serum tube (Vacutainer, Becton-Dickinson, Woodmead, Gauteng, South Africa), which was then kept frozen at -18 °C until it was possible to deliver the samples to the laboratory.

2.2.6 Antibody testing

Antibody testing was performed by means of a SNT at the Virology Laboratory of the DVTD, FVS, UP. The SNT was done according to the OIE Terrestrial Manual (2013) and slightly modified to the Standard Operating Procedures (SOP) of the Virology Section of the DVTD (OIE, 2013).

Serum to be tested was diluted at a ratio of 1:5 with laboratory-prepared phosphate-buffered saline (PBS) and inactivated in a water bath for 30 min at 56 °C. From this dilution a series of six, two-fold dilutions in laboratory-prepared Eagles Minimum Essential Medium (MEM) with 5 % gamma-irradiated foetal calf serum (Highveld Biological, Lyndhurst, South Africa) was made in 96-well microtitre plates (Biolite Multidishes®, AEC Amersham, Halfway House, South Africa). A volume of 100 µl was used per well. The stock virus used as antigen was a live virus vaccine strain (Rift Valley fever virus Smithburn vaccine, OBP , Onderstepoort, South Africa). This antigen was diluted at a ratio of 1:500 in MEM containing 5 % foetal calf serum, in order to obtain $100TCID_{50}$. A series of four, ten-fold dilutions were made from the $100TCID_{50}$ antigen, to be used as the virus control. A volume of 100 µl of the $100TCID_{50}$ antigen was added to all the wells containing the diluted test sera. A virus control was then set up over three rows and six columns of the microtitre plate. A volume of 100 µl MEM, containing 5 % foetal calf serum was added to all the control wells. A volume of 100 µl of the

100TCID$_{50}$ virus antigen was then added to the first two columns, and a further volume of 100 µl of the four dilutions was added to the remaining four columns. The plates were then incubated for 1 h at 37 ± 2 °C in a humid atmosphere of 5 % CO_2. A cell suspension of Vero cells (ATCC, Virginia, USA) containing 480 000 cells/ml was added to all the wells. A cell control was set up in duplicate rows: one containing 200 µl MEM and 5 % foetal calf serum, and another containing 80 µl of the cell suspension. The plates were then incubated at 37 ± 2 °C in a humid atmosphere of 5 % CO_2, until the back titration indicated that the stock virus showed 50 % cytopathic effect (CPE) at the 10^{-2} dilution - this took three days. The end point of the test was taken as the dilution at which 50 % of the cells in that particular well showed CPE.

2.2.7 Semen collection

Semen was collected either by means of an artificial vagina (AV), or by electrostimulation.

2.2.7.1. Use of an artificial vagina for semen collection

Where an AV was used, a ewe in oestrus was restrained in a neck clamp within an enclosed pen. One ram at a time was introduced into the pen and allowed to interact with the restrained ewe. A portion of rams showed normal sexual behaviour and attempted to mount the ewe. In these instances the penis was diverted into the AV and semen was collected in a glass vial attached to the AV. The AV used consisted of a PVC plumbing pipe with a latex inner liner and a glass collection vial. Prior to use, the glass collection vial was warmed to approximately 37 °C by placing it in a water bath for at least 5 min. The cavity between the latex liner and the PVC pipe was filled with approximately 150-200 ml of recently boiled tap water with a temperature between 80 and 90 °C. The hot water was retained within the cavity by a modified bicycle inner tube valve. The AV cavity was then inflated further by the operator's breath, until a tight fit occurred which would admit an index finger. This was empirically estimated to exert approximately the same pressure as a ewe's vaginal cavity. No lubrication of any type was used on the AV liner.

2.2.7.2. Use of electrostimulation for semen collection

In those animals that failed to mount the restrained teaser ewe and ejaculate into an AV, electrostimulation was used to collect semen. Rams were restrained in a 'sitting' position and the penis was exteriorised by digital manipulation. The exteriorised penis was then encircled by

a gauze swab immediately behind the glans penis, and held firmly. The ram was then moved into lateral recumbency, and the penis was held over a warmed glass collection vial in preparation for ejaculation. Collection vials were warmed by placing them in a water bath at 37 °C for at least 5 min, until their temperature was approximately equal to that of the surrounding water. A methylcellulose-lubricated electroejaculation probe (Ruakara Ram Probe®, Shoof International, Cambridge, New Zealand) was then introduced into the rectum to the approximate level of the prostate gland. Electrical stimulation was then intermittently applied until ejaculation occurred. After collection, the semen was maintained at 37 °C for several minutes until semen motility evaluation could be conducted. The semen collection was done by an assistant, and the semen evaluation by the author. After some practice it became possible to evaluate approximately one sample every 10 min. Hence the longest time that a collected semen sample sat in a water bath at 37 °C before evaluation was 10 to 15 min.

2.2.8 Semen evaluation

Semen evaluation consisted of subjective evaluation of mass motility and individual progressive motility, as well as objective evaluation of an eosin-nigrosin stained raw semen sample. All laboratory glassware used in semen handling and evaluation was warmed to 37 °C on a thermostat-controlled laboratory heating pad. Findings were recorded on a paper semen evaluation form according to the format set out by Nöthling & Irons, (2008) and then entered into a computer spreadsheet (Excel® 2010, Microsoft Corporation, Redmond, Washington).

2.2.8.1. Mass motility evaluation

A pre-warmed 22 x 22 mm coverslip was stuck to a warmed glass microscope slide by a drop of the operator's saliva. A warmed Pasteur pipette was then used to place a single, small globose drop of undiluted fresh semen on the coverslip. This sample was then placed inverted on a heated microscope stage, so that the drop hung through a specially designed hole in the stage. The sample was then examined at 40x bright-field magnification, and a subjective score out of five was allocated to describe the observed mass motility. For details on criteria for allocation of mass motility score, refer to Table 5.

Table 5 Method of assigning mass motility score

Score	Characteristics
0	No motility whatsoever
1	Individual sperm motility visible, no wave motion, approximately 10 % of sperm are motile
2	No wave motion, but more than 10 % of sperm are motile
3	Slow wave motion visible
4	Well defined, strong wave motion. Wave motion reaches the edge of the droplet
5	Very well defined wave motion, with a whip-lash effect that extends to the edge of the droplet

2.2.8.2. Individual motility evaluation

Once mass motility had been evaluated, as defined in Section 2.2.8.1, the sample droplet of semen was extended using a commercial semen extender. (Triladyl®, Minitüb, Tiefenbach, Germany).

A single droplet of this extended sample was placed on a coverslip, which was then placed inverted on a warmed microscope slide, allowing the extended semen to spread evenly between coverslip and slide. Ten fields were evaluated at 200x magnification using phase-contrast microscopy, proceeding from the edge of the coverslip to the centre, according to a method previously described in the literature (Nöthling & Dos Santos, 2012). The average of ten consecutive fields was calculated, and expressed as the percentage individual progressive, aberrant and immotile sperm respectively.

2.2.8.3. Sperm morphology evaluation

Sperm morphology was evaluated using bright-field light microscopy of an eosin-nigrosin stained smear of raw semen. The eosin-nigrosin stain used was prepared in-house by veterinary technologists at the Section Reproduction, FVS, UP according to laboratory standards laid out in ISO 17025:2005 (International Organisation for Standardization, 2005). After preparation, the stain solution was checked for osmolality and pH before being declared fit for use. Acceptable ranges were an osmolarity of 290-320 mOsm and a pH value of between 6.8 and 7.2. Semen was mixed with the stain at a ratio determined subjectively by the concentration of the original semen sample. A highly concentrated semen sample was mixed with a greater volume of eosin-nigrosin stain, with the intention of creating a smear consisting of a monolayer of spermatozoa with good differentiation in uptake of stain between living and dead spermatozoa. Once made,

smears were allowed to air-dry and were checked for quality using 100x bright field microscopy. If a morphology smear consisted of a monolayer of randomly orientated sperm cells with good differentiation between stained and unstained spermatozoa, it was deemed to be of acceptable diagnostic quality.

After drying for 24 h, sperm morphology smears were fixed under a 22 x 40 mm glass coverslip using a mounting medium for later evaluation (Entellan® Merck Millipore International, Billerica, Massachusetts). Evaluation of sperm morphology was carried out at 1000x magnification using an oil-immersion lens.

Sperm morphology was evaluated according to the method of Nöthling and Irons (2008). Two hundred spermatozoa were evaluated per ejaculate collected. Using the aforementioned method, defects were classified as affecting the nucleus, the acrosome or tail; or both the nucleus and the acrosome or tail. A computer spreadsheet was used to assist in the calculation of percentage morphologically normal sperm, percentage live morphologically normal sperm, percentage sperm with nuclear defects, and percentage sperm with tail/acrosomal defects.

2.2.9 Statistical analysis

2.2.9.1. Criteria for exclusion of animals from evaluation

For the purposes of data analysis, animals with Day 0 progressive motility of less than 50 % were excluded from the statistical analysis. It was decided that animals with such poor progressive motility would be unlikely to pass a breeding soundness examination under 'real-world' conditions, and were unlikely to exhibit a significant improvement in semen quality with repeated collections.

In addition, animals that showed antibodies to RVFV prior to the onset of the trial were excluded from statistical analysis.

Day 0 rectal temperature was another criterion used to exclude animals suspected of having a persistent febrile reaction prior to the onset of the trial. Animals that displayed a fever response for five or more consecutive daily measurements were excluded. The definition of 'fever' was that followed by the Merck manual (The Merck Veterinary Manual, Ninth Edition, 2005).

2.2.9.2. Statistical procedures

Data was analysed using Statistical Analysis Software (SAS) (SAS Institute, Cary, North Carolina). Descriptive statistics were generated from the data using SAS plugin for MS Excel. The effect of vaccination on percentage progressively motile spermatozoa was assessed using repeated-measures logistic regression analysis (SAS GENMOD function).

The primary outcome assessed was whether or not vaccination affected breeding soundness. This could not be directly assessed, since the intervening variable of temperature existed, and therefore it was assessed whether animals with higher body temperatures also had poorer semen progressive motility.

A secondary outcome assessed was to determine whether vaccination had any effect on temperature. From this, a conclusion could then be drawn on whether vaccinated animals had poor semen quality relative to unvaccinated animals.

Chapter 3 Results

3.1. Clinical findings on first day of trial (Day 0)

3.1.1 General clinical examination

Animals used in the study were young, peripubertal ram lambs. The majority of animals had no permanent teeth. The modal body condition score for the entire group of rams was a score of 2.5 out of five. A summary of dental ages and body condition scores is shown in Table 6.

Table 6 Dental ages and body condition scores of all animals at the start of the trial

Ram	Number of permanent teeth	Body condition score
B016	0	2.5
B049	2	not recorded
B054	not recorded	2.5
B057	not recorded	2.5
B070	0	2.5
B100	0	2.5
B205	0	2.5
B208	not recorded	2.5
B214	0	2.5
B260	0	2.5
B341	0	not recorded
B043	0	not recorded
B104	2	2.5
B122	0	not recorded
B134	2	2.5
B253	0	not recorded
B259	0	2.5
C002	0	2.5
C003	0	2.5
C006	0	2.5
C009	0	2.5
C013	0	2.5
C014	0	2.5

3.1.2 Specific genital examination

The scrotal circumferences of all animals is summarised in Table 7. The average scrotal circumference of all animals was 29.1 cm. One animal (Ram ID B341) had a small abscess on the scrotal skin, likely subsequent to a bite from a tick, but otherwise no animals showed any evidence of external genital pathology.

Table 7 Scrotal circumference of all animals at the start of the trial

Ram	Scrotal circumference (cm) at start of trial
B016	32
B049	30
B054	31
B057	33
B070	28
B100	28
B205	27
B208	30
B214	27
B260	32
B341	28
B043	30
B104	32
B122	32
B134	34
B253	26
B259	32
C002	26
C003	28
C006	25
C009	26
C013	26
C014	26

3.2. Clinical findings

No local swelling, pain or redness was noted at vaccination sites in any vaccinated animals at any time during the study period. No animal became sufficiently ill at any time during the trial to warrant treatment or exclusion from the trial.

3.2.1 Temperature variation

The complete data containing all temperature measurements throughout the trial, including those animals excluded from the statistical analysis, is presented in Appendix H. As discussed in Section 2.2.9.1, Animal C009 was excluded from the statistical analysis, since it exhibited a Day 0 rectal temperature of 39.90 °C which persisted for five days from the start of the trial.

The lowest rectal temperature recorded for any animal (vaccinated or control) at any point during the 42 day measurement period was a reading of 37.4 °C in ram B057 on day 24 of the study. The highest rectal temperature recorded of any animal (vaccinated or control) at any point during the 42 day measurement period was a temperature of 41.4 °C in ram B259 (a vaccinated animal) on day 2 of the study. The mean rectal temperature of all animals (vaccinated and control groups) throughout the study period was 39.34 ± 0.41 °C (Mean ± SD).

The mean rectal temperature of all animals in the control group throughout the study period was 39.27 ± 0.36 °C (Mean ± SD). The mean rectal temperature of all animals in the vaccinated group throughout the study period was 39.41 ± 0.43 °C (Mean ± SD). The highest daily average rectal temperature recorded in all animals (vaccinated and control) throughout the study period was a temperature of 39.73 ± 0.35 °C (Mean ± SD), recorded on day 29 of the study.

The lowest daily average rectal temperature recorded in all animals (vaccinated and control) throughout the study period was a temperature of 38.79 ± 0.23 °C (Mean ± SD), recorded on day 41 of the study. Further temperature findings will be discussed in Section 3.2.4.

3.2.2 Antibody testing

One animal from the control group (Ram ID B205) tested positive for RVFV antibodies without being exposed to vaccine antigen. Otherwise, none of the control animals displayed an antibody response that was detected by SNT. Of the vaccinated animals, all except those with ID C003 and C009 exhibited an antibody response. Most vaccinated animals displayed a measurable

antibody titre on the third test (Day 21 after vaccination).A summary of SNT results from all animals is shown in Table 8.

Table 8 Summary of serum neutralisation test results

Control Animals							
Ram ID	**Day 0**	**Day 6**	**Day 14**	**Day 21**	**Day 28**	**Day 34**	**Day 43**
B016	Neg	Neg	Neg	Neg	Neg	Neg	-**
B049	Neg	Neg	-	Neg	Neg	Neg	Neg
B054	Neg	Neg	-	Neg	Neg	Neg	Neg
B057	Neg	Neg	Neg	Neg	Neg	Neg	Neg
B070	Neg	Neg	Neg	Neg	Neg	Neg	Neg
B100	Neg	Neg	Neg	Neg	Neg	Neg	Neg
B205	1:5	1:10	-	1:14	1:10	1:14	Neg
B208	Neg	Neg	Neg	Neg	Neg	Neg	Neg
B214	Neg	Neg	Neg	Neg	Neg	Neg	Neg
B260	Neg	Neg	-	Neg	Neg	Neg	Neg
B341	Neg	Neg	Neg	Neg	Neg	Neg	-
Vaccinated Animals							
Ram ID	**Day 0**	**Day 6**	**Day 14**	**Day 21**	**Day 28**	**Day 34**	**Day 43**
B043	Neg	Neg	1:40	1:28	1:14	1:28	1:20
B104	Neg	Neg	1:20	1:14	1:20	1:20	1:20
B122	Neg	Neg	-	1:28	1:80	1:112	1:80
B134	Neg	Neg	1:10	1:7	1:7	1:14	Neg
B253	Neg	Neg	1:20	1:20	1:28	1:14	1:10
B259	Neg	Neg	1:40	1:40	1:56	1:28	1:40
C002	Neg	Neg	-	1:112	1:80	1:56	1:28
C003	Neg	Neg	Neg	Neg	Neg	Neg	Neg
C006	Neg	Neg	1:28	1:20	1:10	1:10	Neg
C009	Neg	Neg	Neg	Neg	Neg	Neg	Neg
C013	Neg	Neg	1:14	1:20	1:28	1:20	1:40
C014	Neg	Neg	Neg	1:14	1:14	1:7	1:7

**No value indicates that sample was of inadequate quality for testing

3.2.3 Semen evaluation

Despite attempts to randomise allocation of animals to vaccinated and control groups, a disproportionately large number of very young animals (animal identification numbers starting with C) were allocated to the vaccinated group. Animals that had a Day 0 progressive motility score of less than 50 % were excluded from the statistical analysis. It was decided that animals with such a poor score were unlikely to pass a repeated breeding soundness evaluation. Younger animals were overrepresented among excluded animals. Therefore animals B016, C002, C003, C006, C013 and C014 were excluded due to poor semen quality on Day 0 of trial.

For further details of exclusions due to other criteria, refer to Sections 2.2.9.1, 3.2.1 and 3.2.2.

3.2.3.1. Semen collection

Ideally, all animals would have attempted mounting of a teaser ewe in oestrus and a semen sample would have been collected using an AV. Unfortunately, many animals failed to do so, and semen had to be collected by electroejaculation (EE). Electroejaculation was the predominant means of semen collection throughout the study. Of the 161 semen collections performed during the study, 115 were by EE and 46 were by AV. Only a single animal (B134) could have semen collected by AV on every attempt.

Rams B208 and B104 were collectible by AV on all attempts except on day 43. Younger animals (identification numbers beginning with C) tended to require EE collection on every attempt, with the exception of ram C006, which ejaculated into the AV on Day 28 and Day 34, but subsequently required EE on day 43. A summary of collection methods is presented in Appendix I.

3.2.3.2. Semen mass motility evaluation

The results of the semen mass motility evaluation are shown in Table 9. Raw data is presented in Appendix C, including that from animals excluded from the statistical analysis. Non-integer scores were allocated where it was difficult to clearly allocate an animal into a specific ordinal category. Mass motility was used primarily as a clinical, indicative score of sperm concentration and motility and was not used in any statistical calculations.

Table 9 Semen mass motility evaluation

Control Animals							
Ram ID	**Day 0**	**Day 6**	**Day 14**	**Day 21**	**Day 28**	**Day 34**	**Day 43**
B049	5	3.5	4	3.5	4	3.5	2
B054	5	4.5	4	2	4.5	2.5	3
B057	4	4.5	4	4	4.5	5	3.5
B070	4	4	2	4	4	4	3
B100	5	5	4	1.5	4.5	2	4.5
B208	5	4.5	4	4	4	4	3.5
B214	4	5	4.5	5	3.5	3	4
B260	5	4	5	4.5	4	4.5	4.5
B341	3	1	1	4	0.5	2.5	2

Vaccinated Animals							
Ram ID	**Day 0**	**Day 6**	**Day 14**	**Day 21**	**Day 28**	**Day 34**	**Day 43**
B043	5	5	4.5	5	4.5	4	4
B104	5	4	4.5	4	4.5	5	4.5
B122	4	4	4	3	4.5	3	4.5
B134	5	5	4.5	4	5	4.5	4
B253	4	5	4	2	4	4	4.5
B259	4	4	5	5	4.5	5	4
C009	4	3	3	4	4	3.5	0

3.2.3.3. Individual sperm motility evaluation

A summary of individual progressive motility of animals included in the statistical analysis can be seen in Table 10. A complete table of raw data can be found in Appendix D, including data from animals excluded from the statistical analysis.

Table 10 Percentage progressively motile spermatozoa

Control Animals								
Ram ID	**Day 0**	**Day 6**	**Day 14**	**Day 21**	**Day 28**	**Day 34**	**Day 43**	**AVERAGE**
B049	75.50	72.50	68.00	30.00	78.50	83.00	81.50	69.86
B054	64.00	76.50	64.50	64.00	84.50	84.00	83.00	74.36
B057	58.50	83.50	63.50	81.00	90.00	70.00	84.00	75.79
B070	67.50	61.50	74.50	76.50	86.50	76.00	69.50	73.14
B100	51.50	84.50	81.00	9.60	84.00	63.00	80.50	64.87
B208	60.00	78.00	31.50	22.00	20.00	72.00	65.00	49.79
B214	69.50	78.00	81.00	81.00	78.00	80.50	68.00	76.57
B260	56.50	34.50	35.00	34.50	47.00	60.00	76.00	49.07
B341	54.00	17.50	10.50	66.00	40.00	59.50	54.00	43.07

Vaccinated Animals								
Ram ID	**Day 0**	**Day 6**	**Day 14**	**Day 21**	**Day 28**	**Day 34**	**Day 43**	**AVERAGE**
B043	82.00	76.00	81.00	82.00	74.00	80.00	68.00	77.57
B104	81.50	72.50	74.00	48.50	79.00	83.00	80.00	74.07
B122	58.00	89.00	62.00	72.50	86.00	85.00	88.00	77.21
B134	63.50	79.00	69.50	28.50	85.00	70.00	29.50	60.71
B253	59.00	70.00	70.00	39.50	75.00	81.50	84.00	68.43
B259	74.00	64.00	80.00	77.50	75.00	78.50	76.50	75.07
C009	50.50	21.00	59.00	27.00	37.50	30.00	3.50	32.64

3.2.3.4. Sperm morphology evaluation

A summary of sperm morphology evaluation of animals included in the statistical analysis can be seen in Table 11 (percentage morphologically normal sperm), Table 12 (percentage nuclear defects) and Table 13 (percentage tail/acrosomal defects).

Raw data, including that from animals excluded from the statistical analysis, can be found in Appendix E, Appendix F and Appendix G.

Table 11 Percentage morphologically normal sperm throughout trial

Control Animals								
Ram ID	**Day 0**	**Day 6**	**Day 14**	**Day 21**	**Day 28**	**Day 34**	**Day 43**	**AVERAGE**
B049	93.00	92.00	92.50	93.50	97.50	98.50	96.50	94.79
B054	96.50	98.00	98.50	91.00	98.50	94.50	99.00	96.57
B057	97.50	98.50	92.50	89.00	95.00	96.00	98.50	95.29
B070	98.00	97.00	93.50	94.00	97.50	97.00	98.50	96.50
B100	97.50	99.00	99.50	99.00	96.50	97.00	100.00	98.36
B208	96.50	98.00	94.00	95.00	75.00	95.50	94.50	92.64
B214	85.00	93.50	98.50	91.00	80.50	87.50	81.00	88.14
B260	93.50	97.50	99.50	99.00	94.50	96.50	99.00	97.07
B341	41.50	44.50	38.00	87.50	62.50	81.00	68.50	60.50

Vaccinated Animals								
Ram ID	**Day 0**	**Day 6**	**Day 14**	**Day 21**	**Day 28**	**Day 34**	**Day 43**	**AVERAGE**
B043	98.00	98.00	97.00	99.50	94.50	98.00	95.00	97.14
B104	98.00	98.50	98.50	97.00	92.50	96.00	99.00	97.07
B122	99.00	97.50	90.00	89.50	95.50	98.50	96.50	95.21
B134	96.00	97.50	94.50	97.00	97.00	96.50	97.50	96.57
B253	78.50	90.00	82.00	76.50	89.50	96.50	98.50	87.36
B259	89.00	89.00	98.00	92.00	96.50	96.00	98.00	94.07
C009	89.50	87.00	62.50	66.50	68.50	62.00	61.00	71.00

Table 12 Percentage sperm with nuclear defects

Control Animals

Ram ID	Day 0	Day 6	Day 14	Day 21	Day 28	Day 34	Day 43	AVERAGE
B049	0.00	0.00	0.50	2.00	0.50	0.50	0.00	0.50
B054	0.00	0.00	0.00	0.50	0.00	0.50	0.50	0.21
B057	0.00	0.00	0.50	0.00	1.00	0.00	0.00	0.21
B070	0.50	0.50	0.00	1.50	0.00	0.50	0.00	0.43
B100	0.00	0.00	0.00	0.00	0.00	0.50	0.00	0.07
B208	0.00	0.00	0.00	0.00	0.00	0.50	0.00	0.07
B214	0.00	0.00	0.00	0.00	0.00	2.00	2.00	0.57
B260	0.00	0.00	0.00	0.50	0.00	0.00	0.00	0.07
B341	0.50	0.00	0.00	0.00	4.00	0.50	1.00	0.86

Vaccinated Animals

Ram ID	Day 0	Day 6	Day 14	Day 21	Day 28	Day 34	Day 43	AVERAGE
B043	0.50	0.00	0.00	0.00	0.00	0.00	1.50	0.29
B104	0.00	0.00	0.00	0.00	0.50	0.00	0.00	0.07
B122	0.50	0.00	0.00	1.00	1.00	0.00	0.00	0.36
B134	0.00	0.00	1.00	0.00	0.00	0.50	0.50	0.29
B253	0.00	2.00	0.50	0.00	1.00	0.00	0.00	0.50
B259	1.00	0.00	0.50	0.00	0.00	1.50	0.00	0.43
C009	0.00	0.00	0.00	0.00	1.00	0.00	1.50	0.36

Table 13 Percentage sperm with tail or acrosomal defects

Control Animals								
Ram ID	**Day 0**	**Day 6**	**Day 14**	**Day 21**	**Day 28**	**Day 34**	**Day 43**	**AVERAGE**
B049	7.00	8.00	7.00	4.50	2.00	1.00	3.50	4.71
B054	3.50	2.00	1.50	8.50	1.50	5.00	0.50	3.21
B057	2.50	1.50	7.00	11.00	4.00	4.00	1.50	4.50
B070	1.50	2.50	6.50	4.50	2.50	2.50	1.50	3.07
B100	2.50	1.00	0.50	1.00	3.50	2.50	0.00	1.57
B208	3.50	2.00	6.00	5.00	25.00	4.00	5.50	7.29
B214	15.00	6.50	1.50	9.00	19.50	10.50	17.00	11.29
B260	6.50	2.50	0.50	0.50	5.50	3.50	1.00	2.86
B341	58.00	55.50	62.00	12.50	33.50	18.50	30.50	38.64

Vaccinated Animals								
Ram ID	**Day 0**	**Day 6**	**Day 14**	**Day 21**	**Day 28**	**Day 34**	**Day 43**	**AVERAGE**
B043	1.50	2.00	3.00	0.50	5.50	2.00	3.50	2.57
B104	2.00	1.50	1.50	3.00	7.00	4.00	1.00	2.86
B122	0.50	2.50	10.00	9.50	3.50	1.50	3.50	4.43
B134	4.00	2.50	4.50	3.00	3.00	3.00	2.00	3.14
B253	21.50	8.00	17.50	23.50	9.50	3.50	1.50	12.14
B259	10.00	11.00	1.50	8.00	3.50	2.50	2.00	5.50
C009	10.50	13.00	37.50	33.50	30.50	38.00	37.50	28.64

3.2.4 Statistical analysis

3.2.4.1. Review of criteria for exclusion of animals

As discussed in Section 2.2.9.1, certain animals were excluded from statistical analysis. In summary, these animals were as follows:

Ram B205: excluded due to seroconversion prior to onset of trial

Rams B016, C002, C003, C006, C013: excluded due to poor semen quality on Day 0 of the trial

Ram C009: excluded due to an apparent persistent febrile response noted from Day 0 of the trial

3.2.4.2. Descriptive statistics: progressive motility through the trial period

Once animals had been excluded based on the criteria as discussed in Sections 2.2.9.1 and 3.2.4.1, the data collected from the remaining animals was used for statistical analysis. The information that follows pertains only to animals remaining in the trial.

Table 14 contains a summary of descriptive statistics for Day 0 and cumulative findings throughout the trial. These statistics can be appreciated in graphical form in Figure 1 and Figure 2. The values represented in Table 14, Figure 1 and Figure 2, include all animals (both case and control groups) assessed cumulatively.

Table 14 Percentage progressive motility of vaccinated and control groups assessed together

	Day 0 value	**Cumulative values across entire study**
Mean	63.94	68.60
Standard Deviation	11.26	19.98
Minimum value	37.5	9.6
Maximum value	82	90

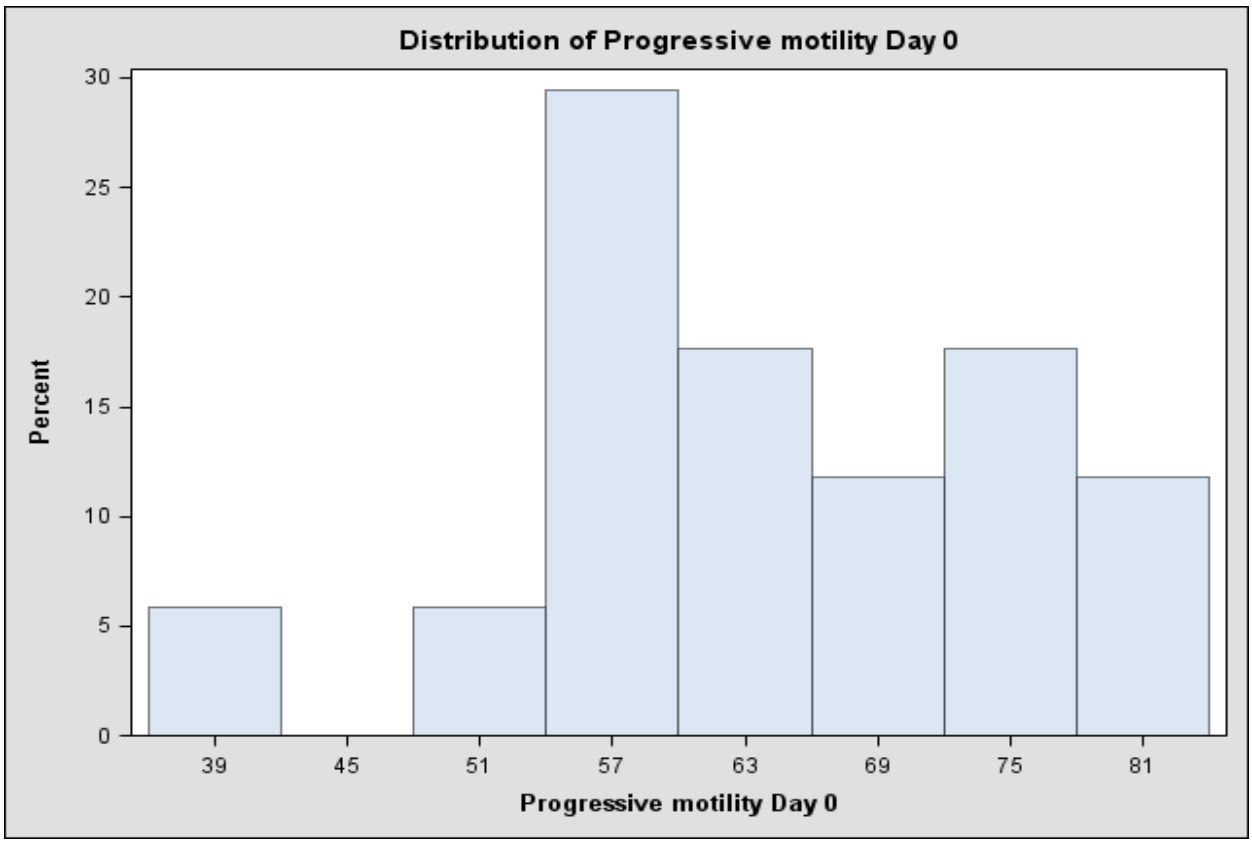

Figure 1 Distribution of progressive motility on Day 0 of the trial period

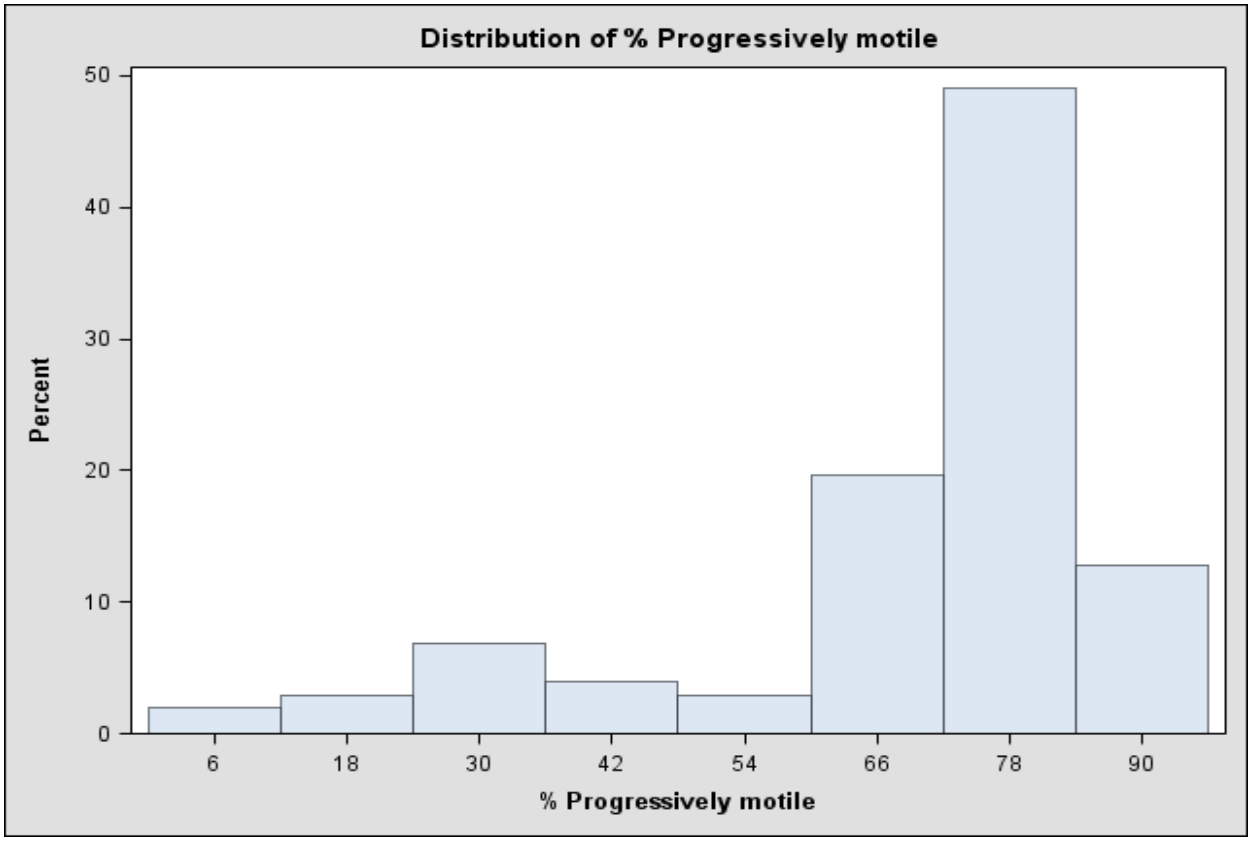

Figure 2 Distribution of progressive motility throughout the trial period

As can be seen from the summary statistics and figures above, the distribution of percentage progressively motile spermatozoa shifted to the right as the trial progressed. Therefore, when all animals (case and control animals) were measured together, there was a relative improvement, with more animals obtaining higher percentages of progressively motile spermatozoa.

3.2.4.3. Descriptive statistics: percentage live normal through trial period

A summary of statistics describing percentage live normal spermatozoa through the study period is presented in Table 15.

Similarly, as for percentage progressively motile spermatozoa, there was no appreciable decline in percentage live normal spermatozoa among all animals in the trial, from Day 0 onwards.

Table 15 Percentage live morphologically normal spermatozoa: vaccinated and control groups assessed together

	Day 0 value	**Cumulative value across entire study**
Mean	70.05	68.76
Standard Deviation	14.76	14.38
Minimum value	29.5	16.5
Maximum value	99	93.5

These findings are illustrated in graphical form in Figure 3 and Figure 4.

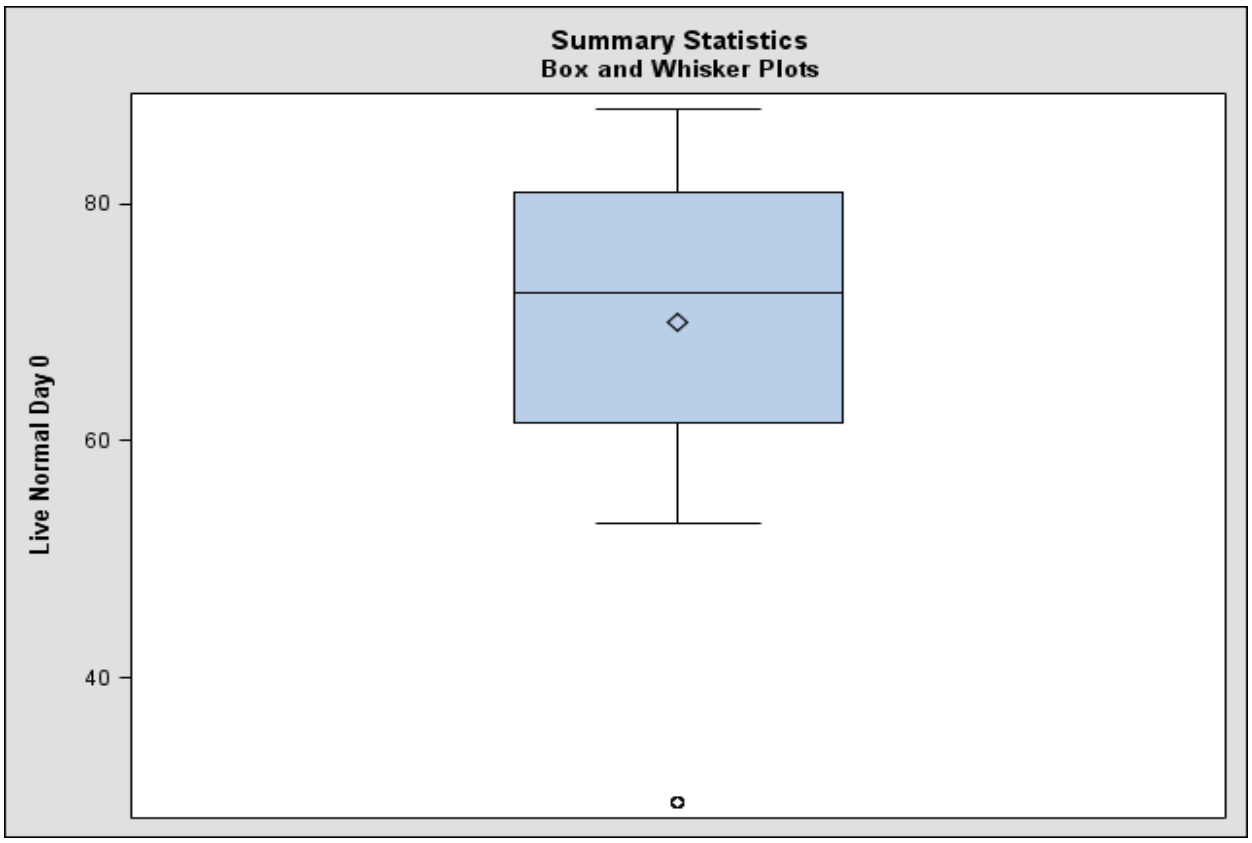

Figure 3 Distribution of percentage live normal sperm on Day 0 of the trial period

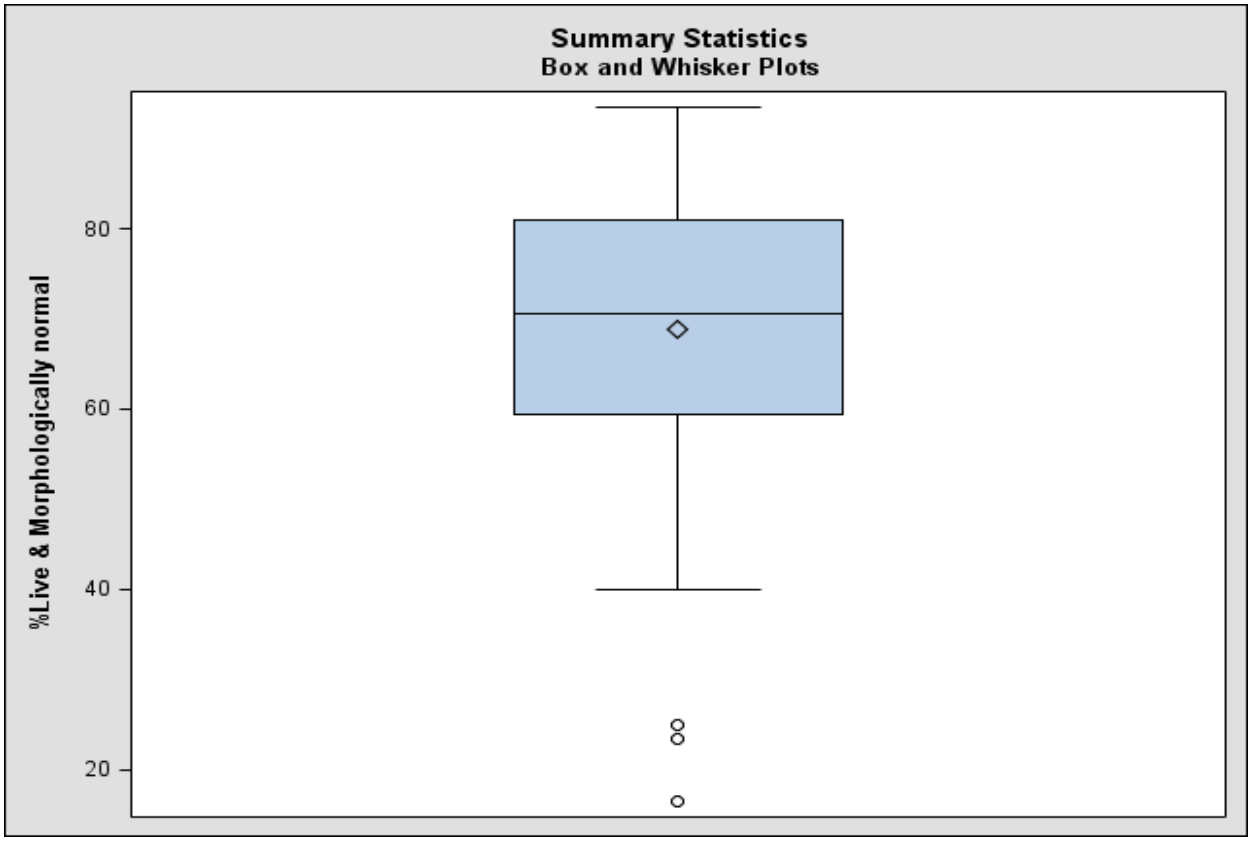

Figure 4 Distribution of percentage live normal sperm throughout the trial period

3.2.4.4. Descriptive statistics: temperature fluctuations

A full table of temperatures throughout the study is presented in Appendix H. As discussed previously, certain animals were excluded from the statistical analysis. Figure 5 and Figure 6 graphically illustrate differences in temperature fluctuations throughout the study before and after exclusion of animals.

As can be seen by comparing Figure 5 and Figure 6, exclusion of certain animals had minimal apparent effect on temperature correlation between case and control animals. In addition, these figures illustrate a daily variation in temperatures that appear consistent between vaccinated and control groups.

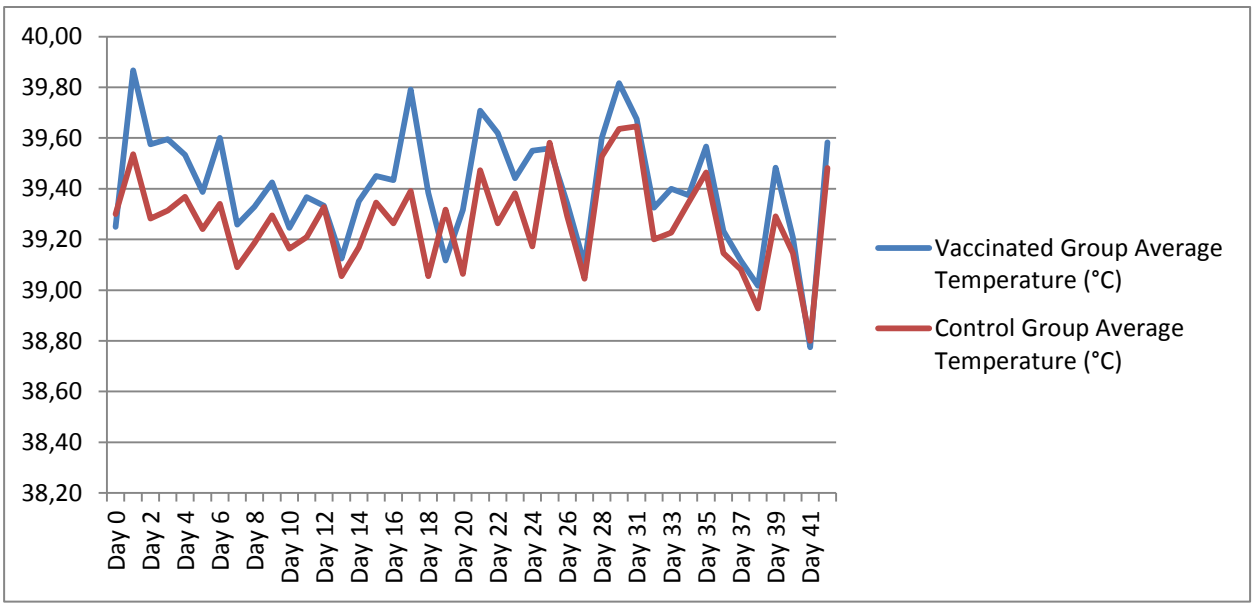

Figure 5 Fluctuations in daily temperature: all vaccinated and control animals

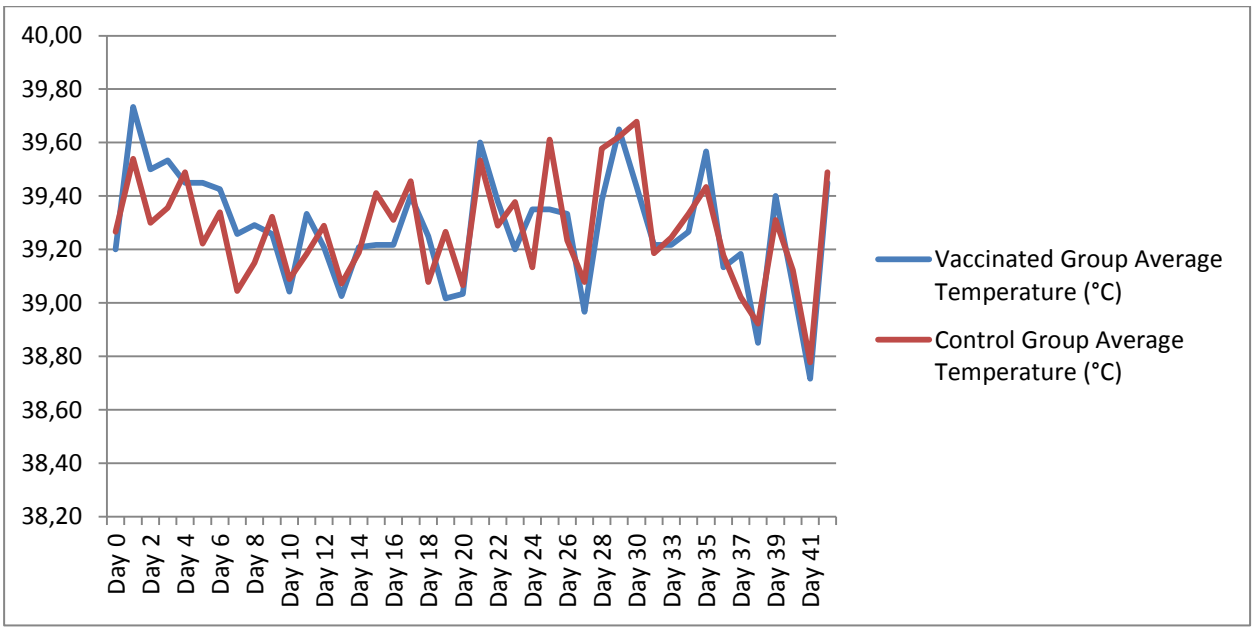

Figure 6 Fluctuations in daily temperature: after exclusion of animals for statistical analysis

3.2.4.5. Logistic regression analysis

Logistic regression analysis was performed to analyse statistical correlations between variables of interest. A summary of single-variable regression outcomes is displayed in Table 16.

When animals (both vaccinated and control) were grouped together and evaluated as a single group, Day 0 progressive sperm motility of all animals was found to correlate strongly with post-Day 0 progressive motility throughout the trial (P=0.0062). When progressive motility throughout the trial was evaluated according to group (vaccinated vs control), no significant difference was found between groups (P=0.0499).

When compared within groups, values for Day 0 progressively motile were shown to correspond significantly with subsequent values through the trial (P=0.0321).

The occurrence of fever (as discussed in Section 1.3.7) on any given day throughout the trial was not found to have significant association with animal group (vaccinated or control) (P=0.6665).

Table 16 Summary of correlations between measured variables: single variable models

Variable 1	Variable 2	P-value
Day 0 progressive motility	Post-day 0 progressive motility	0.0062
Post-day 0 progressive motility	Treatment group	0.0499
Post-day 0 live normal sperm	Treatment group	0.8901
Within-group day 0 progressively motile	Within-group post-day 0 progressively motile	0.0321
Daily temperature value	Treatment group	0.8606

When the influence of both the treatment group and Day 0 progressive motility were evaluated in the same multivariable regression model for their effects on progressive motility throughout the trial, it was found that treatment group was not significantly associated with a reduction in progressive motility (P=0.3325), but that Day 0 progressive motility correlated significantly with subsequent progressive motility (P=0.0321). A summary of these results is presented in Table 17.

Table 17 Effect of treatment group and Day 0 progressive motility on progressive motility through trial: 2-variable regression model

	Treatment group	Day 0 progressive motility
Progressive motility through trial	P=0.3325	P=0.0321

Body temperature (measured once a week, on the day of semen collection), and the values for Day 0 progressively motile sperm were evaluated in a two-variable regression model to assess their combined effect on the percentage of progressively motile sperm through the study. In this model it was found that the weekly measured temperature was not significantly correlated with progressive motility throughout the trial (P=0.3711), but that the values for Day 0 progressively motile sperm correlated highly with subsequent progressive motility evaluated during the trial (P=0.0002). A summary of these results is presented in Table 18.

Table 18 Effect of weekly body temperature and Day 0 progressive motility on progressive motility through trial: 2-variable regression model

	Weekly body temperature	Day 0 progressive motility
Progressive motility through trial	P=0.3711	P=0.0002

Similarly, body temperature (measured once a week, on the day of semen collection) and the values for Day 0 progressively motile sperm were evaluated in a two-variable regression model for their combined effect on percentage live morphologically sperm throughout the trial. It was found that the weekly temperature value was not significantly correlated with the value for live, morphologically normal sperm through the study (P=0.8785) but that Day 0 progressive motility correlated significantly with the value for live, morphologically normal sperm through the study (P=0.0190). A summary of these results is presented in Table 19.

Table 19 Effect of weekly body temperature and Day 0 progressive motility on percentage live morphologically normal sperm through trial: 2-variable regression model

	Treatment group	Day 0 progressive motility
Progressive motility through trial	P=0.3325	P=0.0321

Chapter 4 Discussion

Very few research trials have been performed involving Rift Valley fever virus clone 13 vaccine; so it is difficult to draw conclusions and make comparisons with known data. Most information has been published by researchers employed by OBP while conducting trials before commercial release of the product (Dungu *et al.,* 2010; Von Teichman *et al.,* 2011). The current trial did not evaluate the protective effect of RVFV clone 13 vaccine by exposing animals to a challenge trial. In this way it differs somewhat from the above research reports.

Obtaining sufficient numbers of animals for the trial proved difficult, particularly since the animals needed to be unvaccinated rams that had not been previously exposed to RVFV in the field. It is possible that some animals included in the study were too young to reliably pass a routine breeding soundness examination. This proved to be a complicating factor during data analysis, since a significant number of animals needed to be excluded from statistical calculations due to poor semen quality at the start of the trial.

Six animals were excluded from the statistical analysis due to poor semen quality after the first semen evaluation. A cut-off of 50 % progressively motile sperm was decided upon, since in a clinical setting it was deemed unlikely that an animal obtaining less than 50 % progressively motile spermatozoa on first evaluation would pass a breeding soundness examination, even if repeated collections were performed.

In the context of this study this theory proved to be partially correct. Animal B016 was excluded due to an initial Day 0 progressive motility of 37.5 % (Appendix D). After the semen evaluation on Day 0, progressive motility improved significantly, with a peak value of 83.5 % on Day 28. This would have been sufficient to pass the motility portion of a breeding soundness examination. A similar situation occurred with animal C002: a marked improvement in individual progressive motility was noted with semen collections after Day 0, although with this particular animal, the peak value was still insufficient to pass a breeding soundness examination.

Various factors may have caused poor semen quality in a large proportion of trial animals. Male animals store sperm in the epididymides prior to ejaculation. Prolonged sperm storage (for example lack of ejaculation due to absent or reduced sexual activity) may result in 'aged' spermatozoa that exhibit a lower than normal progressive motility and a higher proportion of epididymal defects and loose heads. This phenomenon was reviewed by Barth & Oko (1989), and an evolutionary biology review characterised such defects as 'post-meiotic senescence' (Pizzari *et al.*, 2007). Hence, repeated semen collections may have resulted in an improvement in semen quality as aged spermatozoa were ejaculated and replaced with newly produced spermatozoa.

Importantly, younger animals with small scrotal circumferences and no permanent teeth were over-represented among those excluded for poor semen quality (Appendix A and Appendix B). Ram lambs whose identification numbers began with 'C' were born several months after those whose identification numbers began with 'B'. This might suggest that these animals were not adequately mature prior to first semen collection and evaluation.

In addition to the physiological reasons for poor semen quality discussed above, events during collection and handling of the semen sample may affect its quality. Animals that have their semen collected by electroejaculation are more likely to contaminate the sample with urine or produce an oligospermic ejaculate that consists mainly of accessory gland fluid (Hulet *et al.*, 1964). However, such an oligospermic ejaculate need not necessarily also have a lower absolute proportion of progressively motile or live morphologically normal spermatozoa.

A single animal (B205) was excluded from the statistical analysis due to seroconversion to RVFV. This was interesting, since animals were tested for RVFV antibodies prior to enrolling them in the trial. This suggests that the animal was exposed to RVFV between the initial pre-enrolment screening test, and the first pre-vaccination test. Assuming perfect sensitivity and specificity of the SNT , the fact that no other animals exhibited antibody titres at this stage of the trial might indicate a low-intensity occurrence of RVFV in the region that the trial was conducted, as discussed in Section 1.3.2.2.

Animals were included by randomly allocating them to one group or another as they emerged from a handling race (discussed in Section 2.2.2). This proved to have some drawbacks, in that a disproportionately large number of younger animals (with correspondingly poor quality semen) were randomly allocated to the test group. This resulted in a smaller group of vaccinated

animals compared to control animals, which in turn had a negative effect on the statistical power of the study.

An improvement on the implemented randomisation model may have been to allocate animals to two groups based on the results of their initial semen quality analysis, to ensure an even distribution between groups. The assignment of a given group to either the vaccination or control group could then have been decided by a coin toss.

Some animals were collected by electroejaculation, and some by the use of an AV (discussed in Section 3.2.3.1). It is well known that ejaculates collected by AV provide a more consistent sample than electroejaculation, which is more reflective of true semen quality (Hulet *et al.*, 1964). Had a larger pool of animals been available, an alternative may have been to exclude animals that failed to ejaculate into an AV and then to randomise animals by quality as discussed in the previous paragraph.

When temperatures were averaged among groups and all animals were included (including those excluded from subsequent statistical analysis), it was found that throughout the trial vaccinated animals had a slightly higher average temperature (39.41 ± 0.43 °C) than control animals (39.27 ± 0.36 °C). The significance and underlying causes of this finding are open to debate, but the author speculates that it may well be as a result of natural variation, which would normalise with increased sample size.

Importantly, when logistic regression analysis was performed after exclusion of animals (Section 2.2.9.1), it was found that rectal temperature had no correlation with either progressive motility, or percentage live, morphologically normal sperm. This suggests that a febrile response to vaccination (if any) was minimal and was insufficient to induce abnormalities in the spermiogram.

Interestingly, in prior work by Dungu *et al.* (2010), no clone 13 vaccinated animals exhibited temperature reactions above 40 °C at any stage of the trial. There were several instances in the present study in which rectal temperatures in both control and vaccinated animals were elevated above 40 °C (Appendix H). This may be due to significant differences in environmental conditions under which animals were kept. In the trial by Dungu *et al.* (2010), animals were kept within an indoor, temperature-controlled confinement facility due to the use of virulent

infective virus. In the present trial, animals were kept outdoors during the early Karoo summer, and had to contend with significant variation in environmental temperature.

In prior work in sheep (Dungu *et al.*, 2010) and calves (Von Teichman *et al.*, 2011), animals vaccinated with the Smithburn (live) vaccine failed to develop a post-vaccination temperature response. It is known that the live vaccine may induce teratogenesis in pregnant animals and its use should be avoided in these animals if at all possible (Coetzer & Barnard, 1977).

The live vaccine is known to induce a strong protective immunity. The question remains: if the live vaccine does not induce a temperature response, will it have any effect on semen quality in rams? No previous studies exist to determine this.

The Day 0 value for percentage progressive motility was found to be highly predictive of subsequent values for progressive motility throughout the trial (P=0.0062) when both groups (vaccinated and control) were assessed together.

Similarly, the Day 0 value for percentage progressively motile sperm was found to be predictive of subsequent values for this parameter (P=0.0321) when assessed within-group (Table 16).

These findings suggested that an animal with high quality semen on day 0 was likely to continue to produce high quality semen throughout the trial, and an animal with poor quality semen on Day 0 was likely to continue to produce poor quality semen.

Interestingly, there was a right-shift in distribution of percentage progressive motility when comparing Day 0 values, and post-Day 0 values among both control and vaccinated groups (Table 14, Figure 1 and Figure 2).

As discussed previously, this apparent improvement may have occurred as a result of repeated ejaculations, thereby eliminating aged epididymal sperm which would have shown poor progressive motility.

Two animals from the vaccinated group failed to display measurable antibody titres to RVFV at any stage during the trial. These animals (Rams C003 and C009, refer to Table 8) were subsequently excluded from the trial due to poor semen quality. However, the fact that these animals failed to seroconvert after vaccination is worthy of special mention.

In previously reported work, two out of 17 vaccinated animals exhibited a weak antibody response to a 10^6 PFU dose of clone 13 vaccine, which was nonetheless able to protect them against challenge with live virus (Dungu *et al.*, 2010). In an earlier study by Barnard (1979), it was noted that after two inoculations with Smithburn's live virus vaccine, two out of five cattle failed to develop an antibody response detectable by the SNT . These animals were nonetheless immune when challenged with live virus. One reason for this might be that the SNT is not sensitive enough in detecting neutralising antibodies. Alternatively, antibody-mediated humoral immunity may not be the most important immune response that protects animals against RVFV infection.

Hypothesis

The findings of the trial did not support rejection of the hypothesis that animals vaccinated with clone 13 vaccine do not experience a reduction in semen quality relative to unvaccinated control animals. There was no statistically significant data to suggest that animals exposed to clone 13 vaccine produced semen that was poorer in quality than those animals not exposed to the vaccine.

Conclusion

As discussed previously, despite problems in animal selection and allocation to different trial groups, it is evident from the data collected in the present trial that semen quality was not adversely affected in vaccinated animals relative to unvaccinated controls.

In this study, RVFV clone 13 vaccine was found to be capable of inducing seroconversion in vaccinated animals. These animals did not experience significant deterioration in semen quality post-vaccination. Therefore, according to the findings of this research, RVFV clone 13 is a vaccine that can be used in breeding rams with no effect on breeding soundness.

It is important to note the conclusions drawn from this trial must be interpreted in the context of the small sample size used. Budgetary constraints and limited availability of animals and facilities needed to be balanced against the greater statistical power of a larger trial. The fact that two animals out of twelve failed to measurably seroconvert within 42 days after vaccination is concerning, and warrants further investigation. A challenge trial may assist in confirming whether or not the vaccine is protective. A repeat of the trial with a larger sample size may be

indicated to confirm, with greater statistical certainty, that the vaccine does indeed have no ill effect on semen quality parameters while remaining effective in protecting animals against clinical disease.

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Appendices

Appendix A **Dental ages of all animals at the start of the trial**

Ram	Number of permanent teeth
B016	0
B049	2
B054	not recorded
B057	not recorded
B070	0
B100	0
B205	0
B208	not recorded
B214	0
B260	0
B341	0
B043	0
B104	2
B122	0
B134	2
B253	0
B259	0
C002	0
C003	0
C006	0
C009	0
C013	0
C014	0

Appendix B **Scrotal circumference of all animals at Day 0 of trial**

Ram number	Scrotal circumference (cm)
B054	31
B057	33
B070	28
B100	28
B205	27
B208	30
B214	27
B260	32
B341	28
B043	30
B104	32
B122	32
B134	34
B253	26
B259	32
C002	26
C003	28
C006	25
C009	26
C013	26
C014	26
Average	**29.1**

Appendix C **Semen mass motility evaluation**

Control Animals							
Ram ID	**Day 0**	**Day 6**	**Day 14**	**Day 21**	**Day 28**	**Day 34**	**Day 43**
B016	4	5	4	4	4	4.5	4
B049	5	3.5	4	3.5	4	3.5	2
B054	5	4.5	4	2	4.5	2.5	3
B057	4	4.5	4	4	4.5	5	3.5
B070	4	4	2	4	4	4	3
B100	5	5	4	1.5	4.5	2	4.5
B205	4	4.5	5	4	4	3.5	3.5
B208	5	4.5	4	4	4	4	3.5
B214	4	5	4.5	5	3.5	3	4
B260	5	4	5	4.5	4	4.5	4.5
B341	3	1	1	4	0.5	2.5	2

Vaccinated Animals							
Ram ID	**Day 0**	**Day 6**	**Day 14**	**Day 21**	**Day 28**	**Day 34**	**Day 43**
B043	5	5	4.5	5	4.5	4	4
B104	5	4	4.5	4	4.5	5	4.5
B122	4	4	4	3	4.5	3	4.5
B134	5	5	4.5	4	5	4.5	4
B253	4	5	4	2	4	4	4.5
B259	4	4	5	5	4.5	5	4
C002	3	4	1	3	2.5	2.5	2
C003	0.5	2	0.5	1	3.5	3	3.5
C006	0.5	0.5	0	3	0.5	1	2
C009	4	3	3	4	4	3.5	0
C013	0	0.5	0.5	0.5	0.5	0.5	0
C014	0	0	0	0	0	0	0

Appendix D **Percentage progressively motile spermatozoa**

Control Animals

Ram ID	Day 0	Day 6	Day 14	Day 21	Day 28	Day 34	Day 43	AVERAGE
B016	37.50	46.00	66.50	77.00	83.50	82.50	74.50	66.79
B049	75.50	72.50	68.00	30.00	78.50	83.00	81.50	69.86
B054	64.00	76.50	64.50	64.00	84.50	84.00	83.00	74.36
B057	58.50	83.50	63.50	81.00	90.00	70.00	84.00	75.79
B070	67.50	61.50	74.50	76.50	86.50	76.00	69.50	73.14
B100	51.50	84.50	81.00	9.60	84.00	63.00	80.50	64.87
B205	74.50	73.50	77.00	83.00	79.50	79.50	83.00	78.57
B208	60.00	78.00	31.50	22.00	20.00	72.00	65.00	49.79
B214	69.50	78.00	81.00	81.00	78.00	80.50	68.00	76.57
B260	56.50	34.50	35.00	34.50	47.00	60.00	76.00	49.07
B341	54.00	17.50	10.50	66.00	40.00	59.50	54.00	43.07
AVERAGE	**60.82**	**64.18**	**59.36**	**56.78**	**70.14**	**73.64**	**74.45**	

Vaccinated Animals

Ram ID	Day 0	Day 6	Day 14	Day 21	Day 28	Day 34	Day 43	AVERAGE
B043	82.00	76.00	81.00	82.00	74.00	80.00	68.00	77.57
B104	81.50	72.50	74.00	48.50	79.00	83.00	80.00	74.07
B122	58.00	89.00	62.00	72.50	86.00	85.00	88.00	77.21
B134	63.50	79.00	69.50	28.50	85.00	70.00	29.50	60.71
B253	59.00	70.00	70.00	39.50	75.00	81.50	84.00	68.43
B259	74.00	64.00	80.00	77.50	75.00	78.50	76.50	75.07
C002	36.50	57.50	33.50	18.00	56.50	46.00	49.00	42.43
C003	5.80	14.50	19.00	9.50	38.50	59.00	45.50	27.40
C006	12.20	17.00	2.00	40.50	24.00	62.50	53.00	30.17
C009	50.50	21.00	59.00	27.00	37.50	30.00	3.50	32.64
C013	4.40	7.80	22.00	10.00	34.50	23.50	25.00	18.17
C014	5.00	16.00	0.00	0.00	4.60	22.00	42.00	12.80
AVERAGE	**44.37**	**48.69**	**47.67**	**37.79**	**55.80**	**60.08**	**53.67**	

Appendix E **Percentage morphologically normal spermatozoa**

Control Animals

Ram ID	Day 0	Day 6	Day 14	Day 21	Day 28	Day 34	Day 43	AVERAGE
B016	92.50	97.00	84.50	81.00	94.00	94.00	89.00	90.29
B049	93.00	92.00	92.50	93.50	97.50	98.50	96.50	94.79
B054	96.50	98.00	98.50	91.00	98.50	94.50	99.00	96.57
B057	97.50	98.50	92.50	89.00	95.00	96.00	98.50	95.29
B070	98.00	97.00	93.50	94.00	97.50	97.00	98.50	96.50
B100	97.50	99.00	99.50	99.00	96.50	97.00	100.00	98.36
B205	85.00	87.50	72.00	69.00	75.00	83.50	91.50	80.50
B208	96.50	98.00	94.00	95.00	75.00	95.50	94.50	92.64
B214	85.00	93.50	98.50	91.00	80.50	87.50	81.00	88.14
B260	93.50	97.50	99.50	99.00	94.50	96.50	99.00	97.07
B341	41.50	44.50	38.00	87.50	62.50	81.00	68.50	60.50
AVERAGE	**88.77**	**91.14**	**87.55**	**89.91**	**87.86**	**92.82**	**92.36**	

Vaccinated Animals

Ram ID	Day 0	Day 6	Day 14	Day 21	Day 28	Day 34	Day 43	AVERAGE
B043	98.00	98.00	97.00	99.50	94.50	98.00	95.00	97.14
B104	98.00	98.50	98.50	97.00	92.50	96.00	99.00	97.07
B122	99.00	97.50	90.00	89.50	95.50	98.50	96.50	95.21
B134	96.00	97.50	94.50	97.00	97.00	96.50	97.50	96.57
B253	78.50	90.00	82.00	76.50	89.50	96.50	98.50	87.36
B259	89.00	89.00	98.00	92.00	96.50	96.00	98.00	94.07
C002	59.00	81.50	77.00	68.00	51.50	79.00	73.00	69.86
C003	58.00	75.00	55.00	60.00	53.00	64.00	75.00	62.86
C006	51.00	48.00	20.50	60.00	64.50	68.50	78.00	55.79
C009	89.50	87.00	62.50	66.50	68.50	62.00	61.00	71.00
C013	48.00	87.00	73.50	60.50	61.00	71.50	65.50	66.71
C014	63.50	69.00	54.00	53.50	49.00	66.50	62.50	59.71
AVERAGE	**77.29**	**84.83**	**75.21**	**76.67**	**76.08**	**82.75**	**83.29**	

Appendix F **Percentage nuclear defects**

Control Animals

Ram ID	Day 0	Day 6	Day 14	Day 21	Day 28	Day 34	Day 43	AVERAGE
B016	0.00	0.50	0.00	0.50	0.50	0.50	0.50	0.36
B049	0.00	0.00	0.50	2.00	0.50	0.50	0.00	0.50
B054	0.00	0.00	0.00	0.50	0.00	0.50	0.50	0.21
B057	0.00	0.00	0.50	0.00	1.00	0.00	0.00	0.21
B070	0.50	0.50	0.00	1.50	0.00	0.50	0.00	0.43
B100	0.00	0.00	0.00	0.00	0.00	0.50	0.00	0.07
B205	0.00	0.50	0.00	1.50	2.00	3.50	0.00	1.07
B208	0.00	0.00	0.00	0.00	0.00	0.50	0.00	0.07
B214	0.00	0.00	0.00	0.00	0.00	2.00	2.00	0.57
B260	0.00	0.00	0.00	0.50	0.00	0.00	0.00	0.07
B341	0.50	0.00	0.00	0.00	4.00	0.50	1.00	0.86
AVERAGE	**0.09**	**0.14**	**0.09**	**0.59**	**0.73**	**0.82**	**0.36**	

Vaccinated Animals

Ram ID	Day 0	Day 6	Day 14	Day 21	Day 28	Day 34	Day 43	AVERAGE
B043	0.50	0.00	0.00	0.00	0.00	0.00	1.50	0.29
B104	0.00	0.00	0.00	0.00	0.50	0.00	0.00	0.07
B122	0.50	0.00	0.00	1.00	1.00	0.00	0.00	0.36
B134	0.00	0.00	1.00	0.00	0.00	0.50	0.50	0.29
B253	0.00	2.00	0.50	0.00	1.00	0.00	0.00	0.50
B259	1.00	0.00	0.50	0.00	0.00	1.50	0.00	0.43
C002	0.00	0.00	0.50	3.00	4.00	2.50	5.50	2.21
C003	1.50	0.50	7.50	4.50	2.50	1.00	0.50	2.57
C006	7.50	3.50	4.00	11.50	11.00	10.00	1.50	7.00
C009	0.00	0.00	0.00	0.00	1.00	0.00	1.50	0.36
C013	8.00	0.00	0.50	3.00	1.00	5.00	13.00	4.36
C014	15.00	0.00	0.00	0.00	0.00	0.00	0.00	2.14
AVERAGE	**2.83**	**0.50**	**1.21**	**1.92**	**1.83**	**1.71**	**2.00**	

Appendix G **Percentage tail or acrosomal defects**

Control Animals

Ram ID	Day 0	Day 6	Day 14	Day 21	Day 28	Day 34	Day 43	AVERAGE
B016	7.50	2.50	15.50	18.50	5.50	5.50	10.50	9.36
B049	7.00	8.00	7.00	4.50	2.00	1.00	3.50	4.71
B054	3.50	2.00	1.50	8.50	1.50	5.00	0.50	3.21
B057	2.50	1.50	7.00	11.00	4.00	4.00	1.50	4.50
B070	1.50	2.50	6.50	4.50	2.50	2.50	1.50	3.07
B100	2.50	1.00	0.50	1.00	3.50	2.50	0.00	1.57
B205	15.00	12.00	28.00	29.50	23.00	13.00	8.50	18.43
B208	3.50	2.00	6.00	5.00	25.00	4.00	5.50	7.29
B214	15.00	6.50	1.50	9.00	19.50	10.50	17.00	11.29
B260	6.50	2.50	0.50	0.50	5.50	3.50	1.00	2.86
B341	58.00	55.50	62.00	12.50	33.50	18.50	30.50	38.64
AVERAGE	**11.14**	**8.73**	**12.36**	**9.50**	**11.41**	**6.36**	**7.27**	

Vaccinated Animals

Ram ID	Day 0	Day 6	Day 14	Day 21	Day 28	Day 34	Day 43	AVERAGE
B043	1.50	2.00	3.00	0.50	5.50	2.00	3.50	2.57
B104	2.00	1.50	1.50	3.00	7.00	4.00	1.00	2.86
B122	0.50	2.50	10.00	9.50	3.50	1.50	3.50	4.43
B134	4.00	2.50	4.50	3.00	3.00	3.00	2.00	3.14
B253	21.50	8.00	17.50	23.50	9.50	3.50	1.50	12.14
B259	10.00	11.00	1.50	8.00	3.50	2.50	2.00	5.50
C002	41.00	18.50	22.50	29.00	44.50	18.50	21.50	27.93
C003	40.50	24.50	37.50	35.50	44.50	35.00	24.50	34.57
C006	41.50	48.50	75.50	28.50	24.50	21.50	20.50	37.21
C009	10.50	13.00	37.50	33.50	30.50	38.00	37.50	28.64
C013	44.00	13.00	26.00	36.50	38.00	23.50	21.50	28.93
C014	45.00	0.00	0.00	0.00	0.00	0.00	0.00	6.43
AVERAGE	**21.83**	**12.08**	**19.75**	**17.54**	**17.83**	**12.75**	**11.58**	

Appendix H **Temperature variation throughout the trial (temperatures in °C)**

Control Animals											
Day	**B016**	**B049**	**B054**	**B057**	**B070**	**B100**	**B205**	**B208**	**B214**	**B260**	**B341**
0	39.5	38.8	39.4	39.5	39.0	39.7	39.4	39.2	39.4	39.1	39.3
1	39.5	39.8	39.1	39.9	39.4	39.8	39.6	39.7	39.6	39.6	39.2
2	39.5	39.2	39.1	39.0	39.5	39.7	39.0	39.7	39.4	39.3	39.1
3	39.2	39.1	39.4	39.7	39.3	39.3	39.1	39.6	39.3	39.2	39.6
4	39.3	39.2	39.6	39.8	39.5	39.7	38.4	40.0	39.2	39.2	39.5
5	39.2	39.0	39.0	39.1	39.5	39.4	39.5	39.8	39.0	39.3	39.2
6	39.3	39.1	39.1	39.0	40.0	39.4	39.4	39.4	39.4	39.6	39.4
7	39.3	38.5	38.9	38.9	39.7	38.7	39.4	39.4	39.4	39.2	38.9
8	39.6	39.0	38.6	39.1	39.3	39.3	39.1	39.5	39.5	39.0	39.2
9	38.9	39.1	39.1	39.1	39.4	39.7	39.5	39.7	39.3	39.4	39.3
10	39.3	38.6	38.8	38.8	39.1	39.9	39.7	39.6	39.2	38.8	39.3
11	39.2	38.8	38.9	39.1	39.6	38.8	39.5	39.8	39.3	39.1	39.4
12	39.3	39.0	39.1	39.2	39.2	39.4	39.7	39.8	39.2	39.6	39.2
13	39.0	39.0	38.7	38.9	39.3	39.3	39.0	39.8	38.8	39.2	38.9
14	39.1	39.0	39.0	39.1	39.4	39.4	39.1	39.6	39.0	39.2	39.2
15	39.3	38.9	39.3	39.2	39.3	39.0	38.8	39.6	40.5	39.5	39.4
16	39.1	39.1	39.1	39.2	39.3	39.6	39.0	39.7	39.4	38.9	39.5
17	39.1	39.1	39.5	39.4	39.3	39.5	39.1	39.5	39.7	39.5	39.6
18	39.1	39.0	38.6	39.0	38.6	39.3	38.8	39.4	39.2	39.0	39.6
19	39.3	39.2	39.2	38.6	39.7	39.6	39.8	39.6	39.1	39.1	39.3
20	39.7	38.9	39.0	38.7	39.4	38.9	38.4	39.8	38.9	38.6	39.4
21	38.8	39.1	39.4	39.6	40.0	39.4	39.6	39.7	39.6	39.3	39.7
22	39.2	39.2	38.9	39.3	39.4	38.9	39.1	39.8	39.0	39.4	39.7
23	39.3	38.9	39.5	39.1	39.6	39.2	39.5	39.7	39.4	39.5	39.5
24	39.1	38.8	39.3	37.4	39.3	39.3	39.6	39.6	39.6	39.5	39.4
25	39.8	39.1	39.6	39.5	39.4	40.1	39.1	39.8	39.4	39.4	40.2
26	39.5	39.0	39.3	38.8	39.5	39.5	39.6	39.4	39.6	38.6	39.4
27	38.8	38.8	39.0	39.0	39.3	38.7	39.0	39.1	39.0	39.2	39.6
28	39.0	39.4	39.5	39.7	39.7	39.8	39.6	39.4	39.6	39.6	39.5
29	39.2	39.6	39.1	39.9	39.5	39.7	40.2	39.8	39.9	39.5	39.6
30	**										
31	39.5	39.2	39.4	39.8	39.9	39.8	39.5	39.5	40.0	39.5	40.0
32	39.1	38.8	39.3	38.4	39.1		39.4	39.7	39.6	39.4	
33	39.0	39.3	39.3	38.9	39.5	39.7	39.3	39.6	39.3	38.3	39.3
34	39.3	39.1	39.6	39.0	39.3	39.3	39.5	39.4	39.6	39.4	39.3
35	39.3	39.2	39.3	39.6	39.5	39.6	39.9	39.5	39.3	39.3	39.6
36	39.2	39.2	39.4	37.8	39.5	39.9	38.8	39.5	39.4	38.7	39.2
37	39.2	39.0	39.4	39.2	39.1	39.5	39.5	38.4	38.9	38.8	38.9
38	38.7	38.9	38.7	38.7	38.3	39.4	39.2	39.2	39.0	39.2	38.9
39	39.0	39.0	39.1	39.3	39.6	39.3	39.4	39.3	39.4	39.7	39.1
40	39.3	38.4	39.1	39.1	39.4	38.9	39.2	39.5	39.2	39.3	39.2
41	39.0	38.4	39.1	38.8	38.9	38.8	38.8	39.1	38.6	38.9	38.4
42	39.4	39.1	39.3	39.3	39.6	39.8	39.5	39.9	39.7	39.3	39.4

Vaccinated group												
Day	**B043**	**B104**	**B122**	**B134**	**B253**	**B259**	**C002**	**C003**	**C006**	**C009**	**C013**	**C014**
0	38.6	39.2	39.2	39.4	39.4	39.4	39.1	39.7	39.0	39.9	39.1	39.0
1	39.8	39.1	39.8	39.6	39.5	40.7	39.8	40.0	40.0	40.1	40.1	40.1
2	39.1	39.1	39.4	39.0	39.1	41.4	39.3	39.6	39.8	39.9	40.0	39.4
3	39.2	39.2	39.1	39.3	39.3	41.3	39.0	39.5	39.4	40.4	39.6	40.2
4	39.5	39.3	39.6	39.3	39.2	39.9	39.0	39.3	39.3	40.2	40.1	39.9
5	39.3	39.3	39.4	39.3	39.3	40.3	39.3	39.5	39.1	39.7	39.7	38.8
6	39.0	39.2	39.4	39.1	39.4	40.5	39.5	39.6	39.6	39.6	39.6	40.9
7	39.2	39.1	39.2	39.6	39.3	39.4	38.8	39.4	39.0	39.3	39.4	39.8
8	39.0	38.9	39.5	39.5	39.4	39.5	39.2	39.4	39.4	39.6	39.7	39.1
9	39.3	39.4	39.5	39.6	38.8	39.1	39.7	39.6	39.4	39.7	39.7	39.7
10	38.4	38.9	39.5	39.3	39.5	38.8	39.4	39.7	39.2	40.1	39.4	39.0
11	39.4	39.4	39.5	39.5	39.1	39.2	39.5	39.5	39.3	39.8	39.4	39.1
12	39.2	39.2	39.6	39.3	39.0	39.1	39.6	39.6	39.3	39.7	39.6	39.1
13	39.0	38.9	39.1	39.3	38.9	39.1	38.9	39.3	39.3	39.6	39.3	39.0
14	39.1	39.5	39.4	39.3	38.9	39.2	39.4	39.5	39.5	39.9	39.6	39.3
15	39.1	39.3	39.4	39.2	39.0	39.3	38.8	39.5	38.9	40.9	40.7	39.3
16	38.9	39.0	39.4	39.1	39.2	39.7	39.6	39.3	39.5	40.1	39.9	39.5
17	39.7	39.3	39.1	39.4	39.6	39.3	40.0	40.0	39.5	41.0	40.9	39.7
18	39.2	39.4	39.5	39.2	38.9	39.3	39.6	39.5	39.0	39.9	40.1	39.0
19	39.2	38.5	39.3	39.0	39.2	38.9	39.7	39.5	38.8	38.5	39.7	39.1
20	39.1	39.5	39.1	38.6	39.1	38.8	39.2	39.4	39.4	40.7	40.1	38.8
21	39.7	39.5	39.6	39.5	39.9	39.4	39.7	39.7	39.9	40.2	39.9	39.5
22	39.0		39.5	39.3	39.6	39.5	39.6	39.9		40.7	39.7	39.4
23	38.7	39.5	39.3	38.9	39.5	39.3	39.7	39.9	39.4	39.8	39.7	39.6
24	39.4	39.5	39.4	39.3	39.3	39.2	39.8	39.8	39.3	39.9	40.0	39.7
25	39.9	39.3	39.2	39.2	39.3	39.2	39.9	39.9	39.3	39.9	40.1	39.5
26	39.9	38.6	39.8	39.4	39.2	39.1	39.0	39.4	39.2	39.3	39.9	39.3
27	39.0	39.0	38.8	39.1	39.0	38.9	39.1	39.3	39.1	39.2	39.2	39.5
28	39.4	39.5	39.4	39.1	39.4	39.5	39.9	40.0	39.2	39.9	40.1	39.8
29	39.4	39.3	40.2	39.7	39.8	39.5	39.9	39.9	39.4	39.9	40.2	40.6
30	**											
31	39.7	39.5	39.5	39.5	39.7	38.7	40.0	40.6	39.7	39.5	40.1	39.6
32	39.2	39.5	38.6	39.2	39.4	39.4	39.1	39.6	38.9	39.5	39.3	40.2
33	38.6	39.1	39.6	39.3	39.5	39.2	39.5	39.5	39.8	39.4	39.6	39.7
34	39.1	39.5	39.5	39.2	39.1	39.2	39.6	39.6	39.2	39.4	39.5	39.6
35	39.6	39.5	40.0	39.6	39.4	39.3	39.1	39.8	39.4	39.9	39.6	39.6
36	39.2	39.3	39.6	39.5	37.5	39.7	39.4	39.2	39.5	39.1	40.0	38.8
37	39.2	38.5	39.6	38.9	39.3	39.6	39.3	39.6	39.2	38.0	39.4	38.8
38	39.0	38.7	39.3	38.6	38.7	38.8	39.3	39.2	39.2	39.1	39.2	39.1
39	39.5	39.3	39.5	39.2	39.4	39.5	39.7	39.3	39.5	39.4	39.7	39.8
40	39.4	38.9	39.2	39.0	38.8	39.1	39.2	39.5	39.2	39.5	39.3	39.4
41	39.1	38.8	38.6	38.9	38.5	38.4	38.7	38.6	38.8	38.9	39.2	38.8
42	39.5	39.3	39.6	39.4	39.3	39.6	39.8	39.6	39.4	39.8	39.9	39.8

** No temperature measurement performed on day 30: severe weather

Appendix I **Methods of semen collection throughout the trial**

Control Animals							
Ram ID	**Day 0**	**Day 6**	**Day 14**	**Day 21**	**Day 28**	**Day 34**	**Day 43**
B016	EE	EE	EE	EE	EE	EE	EE
B049	AV	EE	EE	EE	EE	EE	EE
B054	AV	AV	EE	EE	EE	EE	EE
B057	AV	AV	EE	EE	EE	AV	AV
B070	EE	EE	EE	EE	EE	EE	EE
B100	EE	EE	EE	EE	EE	EE	EE
B205	EE	EE	EE	EE	EE	EE	EE
B208	AV	AV	AV	AV	AV	AV	EE
B214	EE	EE	EE	EE	EE	EE	EE
B260	AV	AV	AV	AV	AV	AV	AV
B341	EE	EE	EE	EE	EE	EE	EE

Vaccinated Animals							
Ram ID	**Day 0**	**Day 6**	**Day 14**	**Day 21**	**Day 28**	**Day 34**	**Day 43**
B043	AV	AV	EE	EE	AV	AV	AV
B104	AV	AV	AV	AV	AV	AV	EE
B122	EE	EE	EE	EE	EE	EE	EE
B134	AV	AV	AV	AV	AV	AV	AV
B253	EE	EE	EE	EE	EE	EE	EE
B259	AV	EE	AV	AV	AV	AV	AV
C002	EE	EE	EE	EE	EE	EE	EE
C003	EE	EE	EE	EE	EE	EE	EE
C006	EE	EE	EE	EE	AV	AV	EE
C009	EE	EE	EE	EE	EE	EE	EE
C013	EE	EE	EE	EE	EE	EE	EE
C014	EE	EE	EE	EE	EE	EE	EE