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Changes in cardiac troponin I concentration and echocardiographic parameters after semen collection in stallions

By

Adrienne Viljoen

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Dedication

To my mom, for sharing her gift as a mother with her three children and who has dedicated all of her time and effort to the upbringing of us three. For always believing and encouraging me to fulfil my dream of becoming an equine veterinarian.

"For I know the plans I have for you, declares the Lord. Plans to prosper you and not to harm you. Plans to give you hope, and a future." Jeremiah 29:11

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List of Abbreviations

AoV	aortic valve
AST	aspartate aminotransferase
bpm	beats per minute
CK	creatine kinase
CK-MB	creatine kinase myocardial band
cTn	cardiac troponin
cTnI	cardiac troponin I
ECG	electrocardiography
FS	fractional shortening
HR	heart rate
IQR	interquartile range
IVSd	interventricular septum in diastole
IVSs	interventricular septum in systole
LDH	lactate dehydrogenase
LVFWd	left ventricular free wall in diastole
LVFWs	left ventricular free wall in systole
LVIDd	left ventricular internal diameter in diastole
LVIDs	left ventricular internal diameter in systole

MV	mitral valve
OVAH	Onderstepoort Veterinary Academic Hospital
PV	pulmonary valve
SD	spectral Doppler
TV	tricuspid valve

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Summary

CHANGES IN CARDIAC TROPONIN I CONCENTRATION AND ECHOCARDIOGRAPHIC PARAMETERS AFTER SEMEN COLLECTION IN STALLIONS

By

ADRIENNE VILJOEN

Supervisor: Prof. M.N. Saulez

Co-supervisor: Prof. A. Carstens

Department: Companion Animal Clinical Studies

Degree: MSc

This dissertation focuses on the effect of breeding on the myocardium of stallions using cardiac troponin I (cTnI) as the preferred blood biomarker for detection of myocardial cell injury. Haematological, echocardiographic and heart rate (HR) parameters are also reported.

Fourteen clinically healthy phantom-trained stallions were assessed. Cardiac troponin I concentration was determined pre-semen collection and at 4, 6, 12 and 24 hours following semen collection. Predictors that were measured included mean HR during each stage of semen collection, maximum HR, area under the curve for HR and fractional shortening (FS).

Pre-semen collection cTnI concentrations were within reported reference ranges for all stallions. Following semen collection, cTnI concentration was significantly increased at 4 and 6 hours post-semen collection. Results also suggest that the HR of stallions peak during mounting of the phantom. Five stallions failed to show an expected increase in FS immediately post-semen collection. No association was identified between the measured predictors and increased cTnI concentration.

This study concluded that cTnI concentration may increase in stallions following semen collection although the clinical significance of this observed increase in healthy stallions is unclear without histopathological evaluation of the myocardium. No conclusion regarding FS immediately post-semen collection could be drawn from results in this study. The research reported in this dissertation can serve as a reference for future studies investigating cTnI concentrations in stallions.

Key words: *cardiac troponin I, myocardium, stallion, breeding, biomarker, heart rate, cardiac telemetry*

Chapter 1: Literature review

1.1 CARDIAC TROPONIN

1.1.1 General introduction:

In the past, traditional methods such as electrocardiography (ECG) and echocardiography have been used in conjunction with cardiac markers for the detection of myocardial injury. Serum levels of the muscle enzymes aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and creatine kinase (CK) were regarded as diagnostic cardiac biomarkers until cardiac muscle-specific creatine kinase myocardial band (CK-MB) and LDH-1 and -2 isoenzymes were identified.[5] It was later recognised that these enzymes lack both specificity and sensitivity in the presence of skeletal muscle injury, in both humans[2] and animals.[33] Specific cardiac markers have improved and progressed from measuring enzymes such as glutamate oxaloacetate transaminase, CK and LDH isoenzymes, to structural proteins such as cardiac troponin (cTn).[1]

As declared by the American College of Cardiology and the European Society of Cardiology, cTn has become well established as the preferred biomarker for the detection of myocardial infarction in human medicine.[3,34] Since then, cTn has supplanted CK-MB as the biomarker of choice for the detection of myocardial injury in several other species because of its high sensitivity and tissue specificity.[6,28,34]

Currently, cardiac troponin I is the most sensitive and specific marker, even in the presence of skeletal muscle damage.[41] The molecular structure of cTnI differs from the skeletal form. With a molecular weight of 24,000 Dalton, cTnI is larger than the other isoforms and contains an additional 32 amino acid N-terminal peptide.[6,66] It is not markedly affected by exercise, skeletal muscle injury or renal failure, making it not only a more sensitive, but also more specific cardiac biomarker than CK-MB.[6,28,34,39]

1.1.2 Biology of cardiac troponin:

Cardiac troponin is a 3-unit protein complex consisting of troponin I, -T and -C subunits respectively and was first described in 1965 by Ebashi.[13,23] This complex, together with tropomyosin, is located on the myofibrillar thin (actin) filament of striated muscle, and is essential for the calcium-mediated regulation of skeletal and cardiac muscle contraction (Figure 1.1).[6,34] Cardiac troponin C, a calcium binding protein, regulates the activation of the actin filaments for muscle contraction.[34] Cardiac troponin I inhibits contraction in

the absence cTnC. The whole troponin complex attaches to the thin actin filaments and tropomyosin via the largest troponin, cTnT.[34] Cardiac isoforms -T and -I are uniquely expressed in the myocardium and have been widely recognized as being highly sensitive and specific markers for the non-invasive diagnosis of increased myocyte permeability and necrosis.[34] In contrast, cTnC is shared by both cardiac and slow-twitch skeletal muscle, and do not discriminate between cardiac and skeletal muscle injury.[6]

Cardiac troponins are mostly found as structural-bound proteins, and as a small free cytosolic pool of unbound troponin that is released acutely following myocardial injury. The cytosolic pool represents about 6-8% of cTnT and 3% of cTnI.[6,34] Circulating levels of cTn in healthy mammals are extremely low or undetectable by current assay methods.[5]

The actual half life ($T_{1/2}$) of circulating cTnI is relatively short (2 hours).[6] Small amounts are initially released from the cytoplasm, resulting in early diagnostic sensitivity, while cTn reaches and persists in circulation following myocardial cell necrosis due to the continual slow release and degradation of the structural pool.[6] The amount of cTn release following insult is affected by myocardial tissue content and severity of injury and reperfusion.[15,39,56] Increases in blood cTn correlates with the severity of histopathological findings, while previous myocardial injury, weight loss and heart failure may be associated with depletion of tissue cTn and ultimately decreased amount of release.[39] In humans with acute myocardial infarction, cTn concentrations are not detectable for three

hours post-injury, are detected 4-6 hours post-myocardial damage, peaking 12-18 hours later, and remain elevated for 5-9 days depending on the extent of myocardial damage.[2,61]

1.1.3 Assays for cardiac troponin I analysis:

Cardiac troponin assays are generally more sensitive and specific than CK-MB assays as the latter assay has cross-reactivity with skeletal muscle and a short $T_{1/2}$ in serum (24-36 hours).[6] Monoclonal antibodies have been developed to detect cTnI that shows no cross-reactivity with the skeletal form of cTnI. More than twenty immunometric commercial assays are currently available for the detection of cTnI and cTnT in the serum or plasma of human patients.[43]

The assays vary from each other by the type of antigen used for calibration, antibody specificities and method of detection which includes spectrophotometric, fluorescent, chemiluminescent and electrochemical methods.[43] Amino- and carboxyl terminal parts of the cTnI molecule are susceptible to proteolysis, and preferably, the antibodies used in cTnI assays should recognise only epitopes that are located in the stable part of the cTnI molecule.[43] The majority of assays currently available use antibodies directed towards the 30-110 amino acid sequence, which represents the most stable part of the cTnI molecule.[43] Assays are widely available and validated for various species.[40-42]

Cardiac troponin assays were originally developed for detection of myocardial infarction associated with substantial tissue necrosis. Current assay methods can detect small elevations in circulating cTnI reflecting <1g of myocardial tissue necrosis.[3] The evolvement of assay performance and increased sensitivity over the last decade has reduced rates of false positive cTn results related to analytical causes such as heparin interference and cross-reactivity with skeletal muscle.[34,42]

Due to the conservation of the structure and function of cTnI across several mammalian species (>95% homology between mammals), the equine myocardium has high reactivity with human immunoassays.[41,42,48,55] Antibodies used in cTnT and some cTnI tests can detect equine cTnT and cTnI respectively, allowing researchers to conclude that the cTn assays can detect equine troponins.[41,42] Recently, the amino acid sequence for equine cTnI has been characterized, further validating the use of commercial analyser systems to measure equine cTnI.[55]

Reports documenting cTnI values in equine literature have varied in the type of immunoassay and sample used including fresh and frozen serum samples with AxSYM Troponin I,[14,26] fresh serum with ADVIA Centaur cTnI assay,[61] frozen serum with Dimension Heterogenous Immunoassay Module,[49] plasma with i-STAT 1 analyser[35] and equine myocardial tissue with Stratus I assay[42]. Due to inter-assay variability, results may not be extrapolated to other studies unless the assays have been standardised. A selection of assays used to measure equine cTnI is summarised in Table 1.1.

Cardiac troponin is highly stable for up to 12 months when samples are stored and maintained at -70ºC.[7,40] Samples deteriorate significantly and progressively with time and temperature within 24 hours (approximately 3% loss of activity/day) when stored at refrigerator temperature (4ºC), while it deteriorates by approximately 10% activity per week when stored at -20ºC.[39] Significant loss of activity occurs with multiple freeze-thaw cycles.

1.1.4 Cardiac troponin in veterinary and human medicine:

Detecting and diagnosing myocardial injury in the horse is difficult.[38] Biochemical markers including isoenzymes of CK-MB and LDH have been used clinically to diagnose myocardial injury in horses. However, both these enzymes lack tissue specificity and sensitivity, which is of greater significance in use of animals as a result of mild to moderate release of skeletal muscle proteins in association with animal handling and stress restricting the value of biomarkers with low cardiac selectivity.[38] O'Brien *et al.*[42] established that equine myocardial tissue has a cTnI concentration equivalent to that found in human heart muscle and that the equine skeletal cTnI was only 0.05%-0.1% of the cardiac reactivity level, indicating that cTnI might be a useful test to diagnose myocardial damage in the horse.[42]

In humans, cTnI is mainly used for detection of myocardial infarction, but may also be used as a cardiac biomarker for angina, left ventricular hypertrophy, congestive

heart failure, myocarditis, atrial fibrillation, hypovolaemia, pulmonary embolism, blunt trauma, sepsis, renal failure, diabetes mellitus and cardiotoxicity related to anticancer drugs and sympathomimetics.[40]

Animal studies have shown that cTnI is affected by strenuous exercise,[31] piroplasmosis,[16] administration of dobutamine,[22] primary cardiac disease,[30] gastric dilatation volvulus[60] and hypertrophic cardiomyopathy.[30] Due to inter-assay variability, cTnI values cannot be compared between different assays, but within reference range reported cTnI concentrations have been recently described in several species including dogs, cats and horses.[48,63]

Investigation of biomarkers in the horse has been limited. Normal resting values for cTnI in both pastured and race-training healthy adult Thoroughbred horses have been reported.[8,48] Elevations of equine cTnI concentration is associated with several disease states occurring more commonly in horses. Reports exist of increased cTnI concentration in a horse with a ruptured aortic jet lesion and ventricular tachycardia,[14] and in a horse with myocardial necrosis and ventricular tachycardia.[61] Elevated cTnI has also been observed in horses with acute monensin toxicosis and cardiomyopathy,[47] in a 10-month-old filly with a double outlet right ventricle[24] and in a horse with piroplasmosis and cardiac arrhythmias.[16] Cardiac troponin I concentrations were measured in two

horses undergoing electrical cardioversion for atrial fibrillation and were found to be similar to healthy control horses.[26]

Cardiac troponin I has been evaluated in a number of human studies and has demonstrated increased concentrations in a small percentage of human athletes participating in endurance events.[37,57] However, no consistency have been found in these biochemical changes, and neither the mechanism nor the significance of these elevated troponins is clear. Elevated cTnI could result from sub-clinical underlying heart disease being unmasked by strenuous exercise leading to myocardial cell damage but not permanent myocyte necrosis.[34] Similarly to human medicine, equine endurance racing has also been associated with increased cTnI concentrations during and at the end of the competition with the mechanisms and significance thereof yet to be determined.[31]

Recent studies also reported on the effect of acute, short-term high intensity exercise on cTnI concentrations on a high-speed treadmill as well as during true race conditions and were in agreement with each other.[21,38] A tendency for increased plasma cTnI was seen in clinically healthy performance horses in training after short-term high intensity exercise. It is suspected that mild hypoxia during exercise causes an increased permeability of the myocardium with leakage of cTnI across the cell wall, supported by a study in rats that had shown short periods of hypoxia leading to increased cTnI concentrations without myocardial cell death.[49]

In human medicine, elevated cTnI and other cardiac biomarkers have been demonstrated during septic shock in both children and adults and have been reported to correlate with increased mortality.[4] Slack *et al.*[62] established normal reference ranges for cardiac biomarkers in non-septic neonatal foals, and compared these values to values obtained from septic neonatal foals. Their findings suggest that myocardial injury occurs during septicaemia in neonatal foals, but is not associated with survival among septic foals.[62]

Smith *et al.*[64] reported that although decreased cardiovascular function was associated with intravenous administration of fumonisin B_1, it was not accompanied by myocyte necrosis nor injury due to similar cTnI concentrations detected in fumonisin treated and control horses.

1.2 ECHOCARDIOGRAPHY

1.2.1 Evaluation of cardiovascular function:

Traditional methods used for evaluation of cardiovascular function in the horse include ECG and echocardiography.[11,12,25] Several other ancillary procedures are available for evaluation of cardiovascular function. These include both invasive (arterial blood pressure monitoring following cardiac catheterisation, lithium dilution for measurement of haemodynamic indices, angiography and transoesophageal Doppler echocardiography) and non-invasive procedures including radiography, computed tomography, magnetic resonance imaging and ambulatory and radiotelemetric ECG.

1.2.2 Echocardiographic evaluation of cardiac function in horses:

The evolvement of echocardiography lead to its first application in horses in 1977.[50] As an excellent, non-invasive diagnostic tool, it allows investigation of the morphology and function of cardiac structures and measurement of cardiac dimensions. It is used in horses presenting with cardiac murmurs, poor performance, and is also useful in diagnosing structural heart disease in horses presenting with arrhythmias.[19] It cannot, however, identify all focal abnormalities of myocardial dysfunction.[19] Information obtained from an echocardiographic examination can be used in evaluating a horse's current and future athletic ability, as well as the effect abnormalities may have on the normal life expectancy of the horse.[51]

1.2.3 B (brightness)-mode/two dimensional echocardiography:

Cardiac anatomy is readily identifiable with B-mode echocardiography which generates cardiac images with both depth and width.[53] These images are frequently updated and provide a real-time view of the heart in motion. This allows assessment of cardiac anatomy. Long-and short-axis images of the heart are standard and these images are the template from which all other echocardiographic examinations are performed.[19]

1.2.4 Evaluation of left ventricular function (M-mode echocardiography):

Left ventricular function can be assessed using M-mode echocardiography which provides a one-dimensional view of the heart representative of the cardiac structures moving over time (Figure 1.2).[19,53] A right parasternal short-axis view is used to obtain M-mode images of the left ventricle and this is used to measure left ventricular internal diameter, septal and left ventricular free wall thickness at end-diastole and peak systole respectively, and to calculate FS.[45,53]

Measurements are made from the leading edge of the near side to the leading edge of the far side of the structure being measured and should be performed routinely for detection of cardiac enlargement. The trailing-inner inner-leading edge method may also be used to measure the LVID.[59] Normal reference ranges of M-mode measurements of the myocardial wall, the interventricular septum, the diameter of the cardiac chambers and various valvular parameters have been determined in normal Thoroughbred and Standardbred horses and serve as reference values in cases of cardiac pathology in these breeds.[46,65,67]

1.2.5 Doppler Echocardiography:

Doppler echocardiography is a non-invasive means of evaluating blood flow within cardiac chambers and blood vessels and provides valuable information about the velocity, direction and timing of the blood flow and can estimate pressure gradients.[9,10,53] There are three forms of Doppler echocardiographic examinations that are commonly used.[53] Pulsed-wave spectral Doppler (SD) is combined with 2-D echocardiography to allow more precise localisation of abnormal flow in a specific area within the heart. It is useful for evaluating the nature of blood flow within the heart with optimal imaging planes as parallel to blood flow as possible.[53] It is not as accurate in recording high velocity flow as is continuous-wave Doppler. The latter should be combined with pulsed-wave Doppler because of loss of specific anatomical location when continuous-wave Doppler is used alone. Colour-flow Doppler is used to determine blood flow velocity, direction and turbulence, displayed with different colour-flow maps.[53]

1.2.6 Fractional shortening:

Fractional shortening is a parameter of ventricular performance and is used as an estimate of myocardial contractility. It can be calculated as the percentage change in end diastolic left ventricular internal diameter (LVIDd) and end systolic left ventricular internal diameter (LVIDs).[53]

$$FS\ (\%) = \frac{LVIDd - LVIDs}{LVIDd} \times 100$$

Fractional shortening has several significant shortcomings and is not only dependent on left ventricular contractility, but also preload, afterload and HR.[17,36,59] A reduction in FS may be found in cases of myocarditis or cardiomyopathy, as well as following administration of sedative drugs, while a significant reduction in afterload, or increased preload will give rise to an increased FS.[45]

1.2.7 Stress echocardiography:

Poor performance is commonly encountered in equine medicine, and cardiac disease is considered the third most common cause of poor performance in the equine athlete after diseases of the musculoskeletal and respiratory systems respectively.[52] These cardiovascular abnormalities often go undetected due to the subclinical presence of myocardial disease at rest and may only become apparent when there is a demand for maximal cardiac output leading to alterations in cardiac dimensions and

function, which can then be detected echocardiographically.[19] Echocardiographic evaluation at rest gives little information regarding cardiac function under stress load, therefore performing echocardiography immediately after a stress test may contribute to the evaluation of horses with poor performance or suspected subclinical myocardial dysfunction at rest.[54]

Exercise stress echocardiography involves an echocardiographic examination of the left ventricle at rest and immediately after a near-maximal to maximal treadmill test.[52] Physiologically, increases in myocardial contractility will only persist briefly following exercise, and stress echocardiography should be performed within 2-3 minutes following high intensity exercise while the heart rate is still in excess of 100 beats per minute (bpm).[20,27,52] A pharmacological stress test has been investigated as an alternative to exercise stress echocardiography.[58]

The normal response of the equine myocardium after high-intensity exercise during systole is an increase in the thickening of the interventricular septum and left ventricular free-wall.[52] The FS should be at least the same or increased relative to pre-stress echocardiography.[52] Horses with low normal or slightly decreased resting myocardial function may show a normal post-exercise response or marked post-exercise left ventricular dysfunction, seen as decreased FS, dyskinesis or akinesis.[20,27,52] As the HR slows, FS will return to normal and it is therefore essential that stress echocardiography be performed in the immediate post-exercise period.[52,54]

1.3 ELECTROCARDIOGRAPHY

Co-ordinated myocardial contraction and relaxation depends on normal electrical excitation via the specialized conduction pathway within the heart. Myocytes within the atria and ventricles possess properties of excitability, refractoriness and conductivity.[44] These electrophysiological features of myocytes result from specific properties of the cardiac cell membrane. The spontaneous generation of electrical activity is caused by ion fluxes across the cell membrane resulting in different concentrations of ions and proteins either side of the cell membrane generating a potential difference in electrical charge across the cell membrane.[44] The ECG records these changes in electrical potential difference across the heart during depolarization (systole) and repolarisation (diastole) respectively.[12]

1.3.1 Interpretation of the electrocardiogram:

In horses, ECGs are most useful to detect dysrhythmias and are critical in identifying abnormalities in heart rhythm. The clinical application of equine electrocardiography has been studied extensively over the years. Understanding the underlying physiological principles facilitates interpretation of the equine ECG.

The mono- or bi-phasic P wave on the ECG represents depolarisation and contraction of the atria, caused by depolarisation of the sino-atrial node in the right atrial wall. The

impulse generated from the SA node will travel to the atrioventricular node and the bundle of His. This phase corresponds with the P-R interval on the ECG. The QRS complex represents depolarisation of the ventricular syncytium, while the T-wave seen on the ECG represents repolarisation.

Sustained, or frequently occurring arrhythmias are easily detected in the resting horse, however, intermittent or exercise-induced arrhythmias are more difficult to detect.[54] Horses have large fluctuations in sympathetic and parasympathetic tone in the recovery period following exercise and significant arrhythmias are most frequently detected in the immediate post-exercise period. Detection of arrhythmias often requires additional diagnostic methods, and a variety of ambulatory ECG systems are available for use in horses, including 24-hour Holter monitor and radiotelemetric monitoring systems.[19] Ambulatory electrocardiography is a term used to describe any system that records the ECG over a certain time period, with analysis only performed once the monitor has been removed from the patient.

1.3.2 Telemetric electrocardiography:

Telemetric ECG is regarded as the technique of choice to accurately diagnose arrhythmias present in the exercising horse.[19] It consists of electrodes with leads attached to a small radio transmitter that sends the electrocardiographic signal to a distant receiver. The system utilises a modified base-apex system with one electrode placed near the

withers on the left side and the second electrode placed near the apex of the horse's heart, also on the left side. The electrodes are applied underneath a surcingle or saddle around the horse's thorax. It provides instant, continuous streaming that can be viewed, while information can simultaneously be stored to be analysed at a later stage with additional software programs.[54] It has the advantage of identifying arrhythmias immediately, while simultaneously providing combined functionality as an ambulatory system due to the system's data storage capability.

1.4 FIGURES AND TABLES

Figure 1.1 Components of the sarcomere illustrating the position of cardiac troponin I, -C and -T respectively. (Modified from: http://www.thailabonline.com/lab-tropnin.htm)

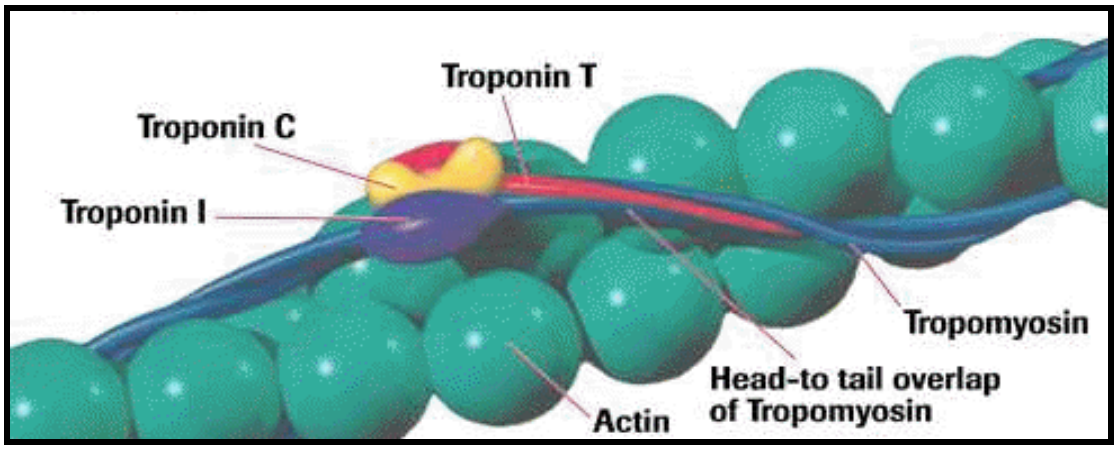
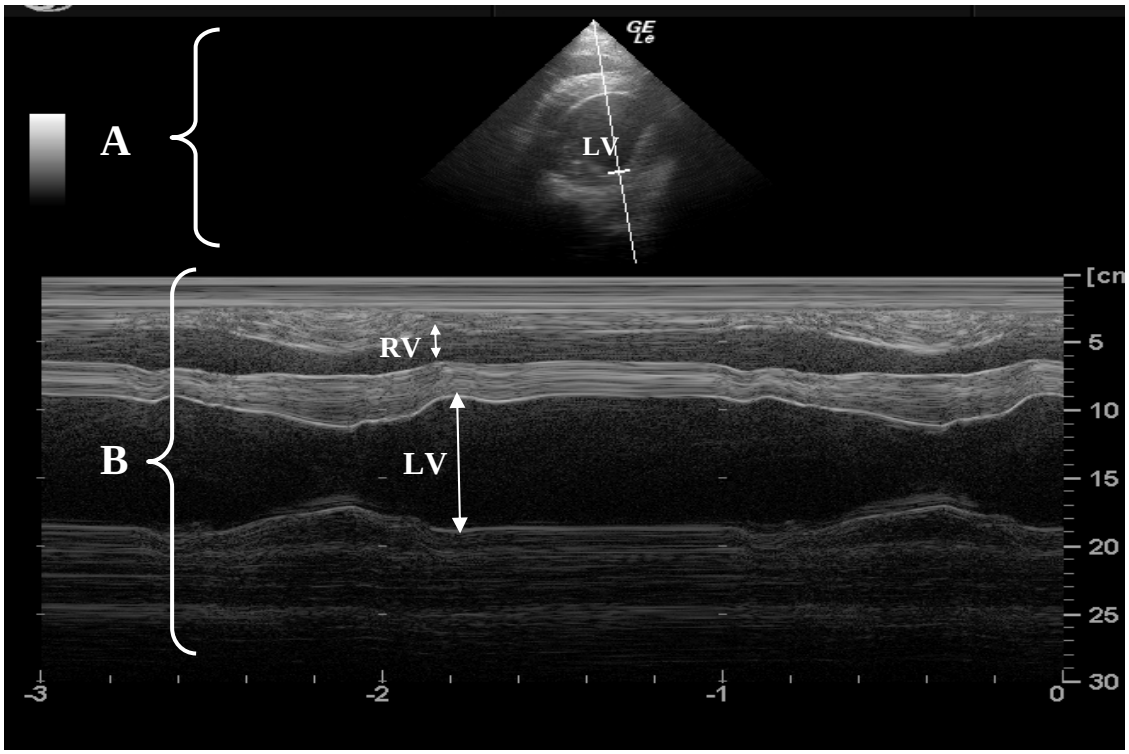


Figure 1.2 Right parasternal short-axis B-mode echocardiogram and corresponding M-mode echocardiogram of the left ventricle obtained from a 15-year-old Lipizzaner stallion.



A, B-mode echocardiogram; B, M-mode echocardiogram; LV, left ventricle; RV, right ventricle.

Table 1.1 Selection of assays used to measure cardiac troponin I in horses.

Study and Reference Number	Year	Assay used	Analytical sensitivity	Lowest [cTnI] reported in study	Highest [cTnI] reported in study
Kraus, M.S. *et al.*[35]	2010	AB[a] SHC[d]	0.01ng/mL 0.02ng/mL	0.00ng/mL	3.68ng/mL
Divers, T.J. *et al.*[18]	2009	AB[a]	0.01ng/mL	<0.08ng/mL	>60ng/mL
Jesty, S.A. *et al.*[22]	2009	AB[a] SHC[d]	0.01ng/mL 0.02ng/mL	0.00ng/mL	3.73ng/mL
Fennell, L.C. *et al.*[24]	2009	NR	NR	NA	0.3µg/L
Nostell, K. *et al.*[38]	2008	ACA[b]	<0.022µg/L	<0.022µg/L	0.035µg/L
Durando, M.M. *et al.*[19]	2007	CS[c]	0.03ng/mL	<0.08ng/mL	0.18ng/mL
Hallowell, G.D. *et al.*[29]	2007	NR	NR	0.56ng/mL	0.19ng/mL
Diana, A. *et al.*[16]	2006	NR	NR	NA	0.27ng/mL
Begg, L.M. *et al.*[8]	2006	ACB[e]	0.15µg/L	<0.15µg/L	NA
Holbrook, T.C. *et al.*[31]	2006	CS[c]	<0.03ng/mL	0.023ng/mL	0.32ng/mL
Slack, J.A. *et al.*[62]	2005	AB[a]	0.01ng/mL	0.01ng/mL	0.51ng/mL
Peek, S.F. *et al.*[47]	2004	DDB[f]	0.04ng/mL	NR	0.6ng/mL
Philips, W. *et al.*[48]	2003	DDB[f]	0.04ng/mL	<0.15ng/mL	0.35ng/mL
Schwarzwald, C.C. *et al.*[61]	2003	ACB[e]	NR	NA	404ng/mL
Smith, G.W. *et al.*[64]	2002	CS[c]	0.03ng/mL	<0.003ng/mL	NR
Frye, M.A. *et al.*[26]	2002	AA[g]	0.3ng/mL	0.097ng/mL	<0.3ng/mL
Cornelisse, C.J. *et al.*[14]	2000	AA[g]	0.3ng/mL	<0.3ng/mL	5.9ng/mL
O'Brien, P.J. *et al.*[42]	1997	CS[c]	NR	NA	NA

[a]AB = ACCESS® Immunoassay, Beckman Instruments,Fullerton, CA; [b]ACA = Architect C18200, Abbott Laboratories, Diagnostic Division, Abbott Park, IL; [c]CS = Stratus CS, Dade Behring, Bear, DE; [d]SHC = i-STAT 1, Heska Corporation, Loveland, CO; [e]ACB = AVIDA Centaur cTnI assay, Bayer Corporation, Pittsburgh, PA; [f]DDB = Dimension® Heterogeneous Immunoassay Module, Dade Behring, Newark, DE; [g]AA = AXSYM Troponin I, Abbott Laboratories, Abbott Park, IL; NR = not recorded; NA = not available and [cTnI] = cTnI concentration.

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Chapter 2: Changes in cardiac troponin I concentration and echocardiographic parameters after semen collection in stallions

2.1 ABSTRACT

Increased concentration of cTnI is associated with piroplasmosis, ventricular tachycardia and short-term high intensity exercise in horses. Anecdotal reports exist of acute death in stallions during and immediately after breeding and this may be due to myocardial injury. We hypothesized that cTnI and FS would increase following semen collection.

Fourteen clinically healthy, phantom-trained stallions were studied. Echocardiography was performed prior to semen collection and heart rate recorded prior to, during and after semen collection using cardiac telemetry. Fractional shortening was determined within five minutes following semen collection. cTnI concentrations were measured using a chemiluminescent immunoassay pre-semen collection and at 4, 6, 12 and 24 hours post-semen collection. Linear regression analysis was used to determine the associations between the predictors (HR, age, weight and FS) and change in cTnI concentration.

cTnI concentrations were available for eleven stallions. Compared with pre-semen collection cTnI concentration (0.001 [interquartile range 0.001-0.03] ng/ml), cTnI concentration was significantly increased at 4 (0.01 [0.001-0.09] ng/ml, $P = 0.016$) and 6 hours (0.01 [0.001-0.11] ng/ml, $P = 0.010$), but not at 12 (0.01 [0.001-0.06] ng/ml, $P = 0.396$) and 24 hours (0.001 [0.001-0.06] ng/ml, $P = 0.623$) post-semen collection. No significant association was found between the measured predictors and change in cTnI concentration.

Data suggest that cTnI concentration may increase after semen collection. This may not be of clinical significance, but should be considered in stallions with suspected cardiac disease.

2.2 INTRODUCTION

Cardiac troponin I is a sensitive and tissue-specific diagnostic biomarker and is regarded as the biomarker of choice for detection of myocardial injury in human medicine.[1,10] Its structure is highly conserved among mammalian species and researchers have concluded that commercial immunoassays designed to detect human cTnI can be used in veterinary medicine.[16,17,21,22]

Investigation of cardiac biomarkers in horses has been limited. In 1997, O'Brien *et al.*[17] identified cTnI as a potential useful test for detecting myocardial cell injury in horses.

Normal reference ranges for equine cTnI have recently been established and the value of cTnI as a cardiac biomarker in horses is supported by studies demonstrating increased cTnI concentrations in horses with a wide range of clinical conditions.[2,4-7,19,26,27]

Anecdotal reports exist on acute death in stallions during and immediately following breeding, which may be attributable to myocardial injury. There is a paucity of literature regarding cardiac and physiological indices associated with breeding in stallions, with only report on continuous HR and sympathetic nerve activity during and after copulation in stallions.[28] However, cardiac biomarkers were not investigated.

2.3 OBJECTIVES

To mimic the effect of breeding on physiological indices in stallions, a study was designed that closely resembles natural breeding conditions of stallions. The objective of this study was to determine whether myocardial injury occurs in stallions following semen collection through the use of echocardiography, telemetric electrocardiography and determination of serum cTnI concentrations.

2.4 HYPOTHESES

We hypothesized that:

1. cTnI increases following semen collection.
2. FS increases in the immediate post-semen collection period.

2.5 MATERIALS AND METHODS

2.5.1 Stallions and selection criteria:

To determine the effect of semen collection on the myocardium of breeding stallions, 14 clinically healthy phantom-trained stallions with no history of cardiac or systemic disease were selected for study. Enrolment was based on owner consent. Stallions were determined to be clinically healthy based on physical examination at rest (including cardiac and pulmonary auscultation), haematology and echocardiography. Stallions were admitted to the Onderstepoort Veterinary Academic Hospital (OVAH) between April and September 2009 and hospitalised until data collection of each individual stallion was completed. Stallions were individually housed and fed free choice teff hay and lucerne, while fresh water was always available. Concentrates were given according to owner's instructions. This study was approved by the University of Pretoria Animal Use and Care Committee (protocol number V011-09).

2.5.2 Experimental protocol:

Complete physical and cardiac examination at rest was followed by blood collection via left jugular venipuncture for haematology.

2.5.2.1 Echocardiography:

A complete echocardiographic examination was performed by a board certified radiologist at all times using a Logic E compact ultrasound system (Logiq E Console, GE Healthcare UK Ltd., St. Giles, UK) with a 3S-Rs Sector Phased Array Comfort Scan Probe. All B-mode, M-mode and Doppler measurements were recorded and results compared to normal published values for horses.[3,18,30] Echocardiographic examinations were performed without sedation. With the exception of one stallion, all echocardiographic examinations were performed within the stable to minimise stress and restrict movement.

B-mode

The heart was examined using B-mode in the left and right parasternal long and short axis views to evaluate the anatomy, particularly the echogenicity of the myocardium, the valve leaflets (mitral valve [MV], tricuspid valve [TV], aortic valve [AoV], pulmonary valve [PV]), and the movement of the heart or presence of any pathology. B-mode measurements were made of the left atrium width (LA), pulmonary artery (PA) width and maximal aortic width at the sinus of Valsalva.

M-mode

M-mode measurements were made from the right parasternal short axis including LVIDd, left ventricular free wall in diastole (LVFWd), interventricular septum in diastole (IVSd), LVIDs, left ventricular free wall in systole (LVFWs), interventricular septum in systole (IVSs) and FS.

Doppler

The E and A peak velocities in cm/s at the MV and TV were measured as well as the peak forward flow velocities at the AoV and PV. Colour flow Doppler was used to determine the presence of turbulence or valvular regurgitation, and if present, the width and length of the latter was measured. Using SD, the peak velocity and nature (laminar or turbulent) of the regurgitant jet was measured.

Fractional shortening was determined within five minutes of semen collection (Figure 2.1) and again at two hours post-semen collection at rest in the stable. Both measurements were taken from a right parasternal axis. The right forelimb was extended as far forward as the stallion would tolerate to allow quick and easy access for optimal left ventricular M-Mode echocardiography.

2.5.2.2 Telemetric electrocardiographic recordings:

On the morning following admission to the hospital, a telemetric ECG device (Kruuse Televet 100, Rosch and Associates Information Engineering, Frankfurt, Germany) was attached to each stallion and the HR monitored via cardiac telemetry. The antenna side of the device was positioned away from the stallion to ensure maximum range. The green electrode was placed on the sternum of the stallion, the red electrode on the left side of the thorax about 15-20cm below the top of the thorax. The black electrode was placed halfway between the green and red electrode (Figure 2.2), while the yellow electrode was placed on the right side of the stallion's thorax in a position similar to the red electrode. All electrodes were tightly fixed with 100 mm adhesive tape and a girth (Figure 2.3). Care was taken to position the electrodes in a way to avoid interference with semen collection and echocardiographic measurements immediately post-semen collection. The device was connected to a notebook and data were continuously streamed and recorded for the period from 15 minutes prior to semen collection to 20 minutes after semen collection. Different stages of semen collection were identified and included:

Stage 1; at rest in the stable prior to semen collection,

Stage 2; in a holding area, prior to entering the collecting area, but still out of sight of the phantom (Figure 2.4),

Stage 3; entering the collecting area with visual stimulation by a mare in oestrus,

Stage 4; successful mounting until dismount of the phantom,

Stage 5; ejaculation, and

Stage 6; at rest in the stable post-semen collection.

2.5.2.3 Semen collection and evaluation:

After visual stimulation of the stallion to a mare in oestrus, the stallion was allowed to mount the phantom (Figure 2.5). All ejaculates were collected using a Missouri-model artificial vagina (Mintub, Tiefenbach, Germany). The fresh ejaculated semen was filtered using sterile gauze immediately after collection while the gel-free semen was used for evaluation and processing. The evaluation of the raw semen included: gel-free semen volume, determination of sperm concentration (x 10^6 sperm/ml) using SpermaCue® (Minitüs Tiefenbach, Germany), sperm morphology by Eosin Nigrosin staining, and individual sperm motility after dilution in modified Kenney's extender by phase contrast microscopy at 37ºC.

2.5.2.4 Cardiac troponin I analysis:

Serum samples for cTnI analysis were collected by left jugular venipuncture prior to semen collection, and at 4, 6, 12, and 24 hours post-semen collection. All samples were centrifuged for 8 minutes at 2,080*g* within 60 minutes after collection at room temperature (24ºC). The serum obtained was separated and stored at -80ºC for batched analysis within 6 months after collection. Cardiac troponin I measurements were performed with a chemiluminescent immunoassay (Access® AccuTnI™, Beckman Coulter, Inc., Fullerton, CA, USA) intended for the quantitative determination of cTnI levels in human serum and plasma. The assay has a lower limit of detection of 0.01ng/ml and an analytical sensitivity of 0-0.04ng/ml and has been validated for measurement of equine cTnI.[8]

2.5.2.5 Statistical analysis:

Statistical analyses were performed using a commercially available software program (Stata 10.1, StataCorp, College Station, TX, USA). Group data are presented as medians and interquartile range (IQR). For statistical purposes, cTnI concentration values below the limit of detection were assigned a value of 0.001ng/ml. cTnI concentrations at 4, 6, 12 and 24 hours post-semen collection were compared with pre-semen collection values using the Wilcoxon signed-ranks test. Following log-transformation of cTnI concentrations, linear regression analysis was used to determine associations between the predictors: age, weight, HR (mean HR during each stage of semen collection, maximum HR and area under the curve), FS and the outcomes. Outcomes included cTnI concentrations at 6 hours post-semen collection and change in cTnI concentrations from pre- to 4 and 6 hours post-semen collection. Mean HR for each stage of semen collection was obtained by dividing the total number of recorded beats by the duration of the stage in minutes. Maximum HR attained over the entire trial was determined based on a 10-beat moving average of the instantaneous heart rates derived from the R-R intervals. An area under the HR curve was calculated for the period from stage 2 to stage 5 of semen collection. Due to the small sample size, the use of multiple regression to control for confounding was not possible. Significance was set at $P = 0.05$.

2.6 RESULTS

Eleven stallions with a median age of 8 (IQR 6-17) years and a median body weight of 503 (IQR 467-550) kg completed the study. Three stallions did not ejaculate and were therefore excluded. The most commonly recruited breed was Lipizzaners (n = 5), followed by Warmbloods (n = 4), and an Arabian and Nooitgedacht stallion (n = 1, respectively). No abnormalities were detected upon complete physical examination. Summary statistics for the different cTnI concentrations and variables measured are shown in Table 2.1. Haematology results revealed no significant abnormalities and are summarised in Table 2.2. Electrocardiograms at rest and during the semen collection process were normal and no dysrhythmias or arrhythmias could be detected.

Echocardiographic measurements obtained at rest are shown in Table 2.3. Four out of 11 stallions had mild tricuspid valve regurgitation, 4/11 had mild aortic regurgitation while 2/11 had mild mitral valve regurgitation. One stallion had both mitral and aortic valve regurgitation, one stallion had both mitral and tricuspid valve regurgitation while one of the stallions had both a tricuspid and aortic valve regurgitation. Secondary cardiac changes as a result of valvular regurgitation were not detected clinically or echocardiographically. Regurgitation was therefore regarded as physiological and non-significant.

Resting concentrations of cTnI were within reported reference intervals for each stallion (range 0-0.15ng/ml).[19] Six out of eleven stallions had a resting cTnI concentration that was below the lower limit of detection. Compared with pre-semen collection cTnI concentration, cTnI concentration was significantly increased at 4 (P = 0.016) and 6 hours (P = 0.010), but not at 12 (P = 0.396) and 24 hours (P = 0.623) post-semen collection (Figure 2.7 and Table 2.1 respectively). The first hypothesis of this study was therefore proved to be true.

Mean heart rates during each stage of semen collection are shown in Figure 2.8 and 2.9 respectively. Nine stallions reached their peak HR during mounting. Five out of eleven stallions failed to show an expected increase in FS immediately post-semen collection. HR at the time of post-semen FS measurement is shown in Table 2.5. No significant associations were found between any of the measured predictors and change in cTnI concentration from pre- to 4 and 6 hours post-semen collection, or between the measured predictors and cTnI concentration at 6 hours post-semen collection.

2.7 DISCUSSION

The aim of the study was to report cTnI concentration and FS in stallions after semen collection. Stallions of all breeds and ages were included for this purpose. Although one breed would have lead to the ability to report cTnI within a specific population of stallions, we were limited to the availability of stallions within the vicinity of the OVAH and therefore no breed predilection was considered. Following normal physical examination and haematological parameters and echocardiographic parameters, stallions were considered clinically healthy and suitable for inclusion in the study.

2.7.1 Echocardiographic measurements of stallions:

Echocardiographic examination at rest was performed within the stable to limit stress and excitement associated with the new environment and surroundings. Despite an adequate period between admission and echocardigraphic examination, stallions were still restless and excited while echocardiographic measurements were taken. The interpretation of these measurements may be debatable as a result of excitation during measurements as well as the size of the stallions. Even though the left forelimb was extended cranially to create adequate room for the ultrasonographer to manipulate the probe for optimum measurements, aortic measurements deep to the olecranon were difficult to obtain in the Lipizzaner stallions. Velocities obtained for various valves varied significantly within stallions. The LogicE® ultrasound machine was used for echocardiographic

measurements due to a superior image quality and ease of use but continuous wave Doppler was not a feature of this machine. All flow velocities including regurgitation velocities were measured with pulse-waved Doppler, which may have underestimated velocity flow measurements.

2.7.2 Heart rate associated with semen collection:

By continually monitoring the HR of stallions during the semen collection process, we were able to calculate the maximum and average HR for each stallion during different stages of semen collection. The peak HR reached during mounting is similar to results reported by Terada *et al*.[28] Although specific time periods were identified for HR calculation, behavioural changes displayed by stallions differed and there were significant individual differences in the time from exposure to the mare in oestrus and ejaculation. Therefore an area under the curve for HR of each stallion was calculated to measure the effect of amplitude and time on cTnI concentrations post-semen collection.

2.7.3 Fractional shortening immediately post-semen collection:

Movement due to excitement of the stallions immediately post-ejaculation precluded optimal determination of FS immediately post-semen collection. Often, FS was only determined five minutes post-semen collection when the HR had usually already markedly decreased to below 100 bpm as is required for post-stress echocardiography,[20] with only four stallions displaying a HR ≥ 90 bpm (Table 2.5) at the time of post-semen collection FS measurement. The exact time elapsed between ejaculation and measurement of FS was not recorded and significant variations in FS post-semen collection was seen between stallions. In treadmill-exercised horses, Schefer *et al*.[25] reported that FS did not differ significantly post-exercise compared with baseline values and commented on several disadvantages of relying on FS as a single index of LV systolic function in horses. Due to the variable post-semen collection FS in this study and findings reported by Schefer *et al*.[25], FS alone does not accurately represent LV systolic function in horses, and the second hypothesis in this study proved to be more challenging. However, a tendency towards an increased FS post-semen collection compared to baseline was seen as three of the four stallions with higher heart rates also had an increased FS. M-mode echocardiography was performed in direct sunlight and screen glare reduced image quality affecting LVIDd and LVIDs measurements. This may have also contributed to the decreased FS observed in five stallions following semen collection.

2.7.4 The significance of increased cardiac troponin I concentration:

Results suggest that semen collection induces mild increases in circulating cTnI in stallions following semen collection. The precise etiopathogenesis is yet to be determined, but may due to myocardial strain associated with mounting and ejaculation subsequent to decreased myocardial cell integrity and transient leakage of cTnI into circulation. The observed increase in circulating cTnI may be a normal occurrence in stallions following semen collection, however this remains to be determined.

Copulation in stallions is reported to be of short-term, high-intensity activity.[28] Increased cTnI concentrations could be attributed to the short-term high intensity activity experienced by the stallion during mounting and ejaculation. The increase in cTnI concentrations as reported in this study is consistent with previous studies in horses describing increased cTnI concentrations after short-term high intensity exercise.[7,15] Sexual excitement and ejaculation is controlled by sympathetic nerve signals and norepinephrine is predominantly secreted by the nerve endings.[11] Although catecholamine toxicity is predominantly described in human medicine,[9,24] the same may be true for horses. It has been shown that the activity of the sympathetic nervous system peaks at mounting with 4.7-fold higher than baseline norepinephrine concentrations returning to normal within 10 minutes following ejaculation.[28] This could lead to mild myocardial cell necrosis or strain, resulting in leakage of cTnI that is transient. More severe myocardial injury and necrosis will lead to greater increases in cTnI concentrations as a result of structurally bound cTnI release.

Increased circulating cTnI could be due to release from skeletal muscle. As CK and AST were not determined in this study, uncertainty exists to whether the observed increase in cTnI was due to a post-ejaculation myopathy secondary to the muscular effort required to support the stallion during mounting and ejaculation. Cardiac troponin I contains a unique N-terminal amino acid sequence that is almost exclusively expressed in the myocardium, even in the presence of skeletal muscle injury.[12]

Additionally, skeletal muscle only contributes to 0.05-0.1% if cross reactivity in cTnI immunoassays.[17] No correlation between the increased cTnI concentrations and evidence of skeletal muscle damage, and therefore no evidence of skeletal cross-reactivity, could be found in humans competing in endurance events.[14,23] Cross-reactivity with skeletal isoforms therefore seems to be highly unlikely.

2.7.5 Limitations associated with this study:

This study had several limitations. The power of statistical analysis was reduced due to the small number of stallions studied. With more numbers, the statistical significance of increased cTnI concentration from baseline values could have either increased or normalised.

The study was performed outside the normal breeding season for horses with lower ambient temperatures and shorter photoperiods compared to the normal breeding season. The gonadotropin-releasing hormone (GnRH) pulse-regulator is negatively influenced by a shorter photoperiod in the stallion.[29] GnRH stimulates the release of luteinizing

hormone which, in return, is responsible for the synthesis of testosterone. Both GnRH (in the short-term) and testosterone (long-term), is responsible for libido in the stallion. Higher ambient temperatures and humidity could lead to increased HR and HR recovery times after semen collection with increased cardiac workload and possible myocardial strain.[13] Results obtained may have been different if this study was performed during the normal breeding season.

The quality of M-mode echocardiographic images and accompanying FS measurements in the immediate post-semen collection period may have been superior if this study was conducted indoors, eliminating screen glare as a possible contributor to decreased post-semen collection FS.

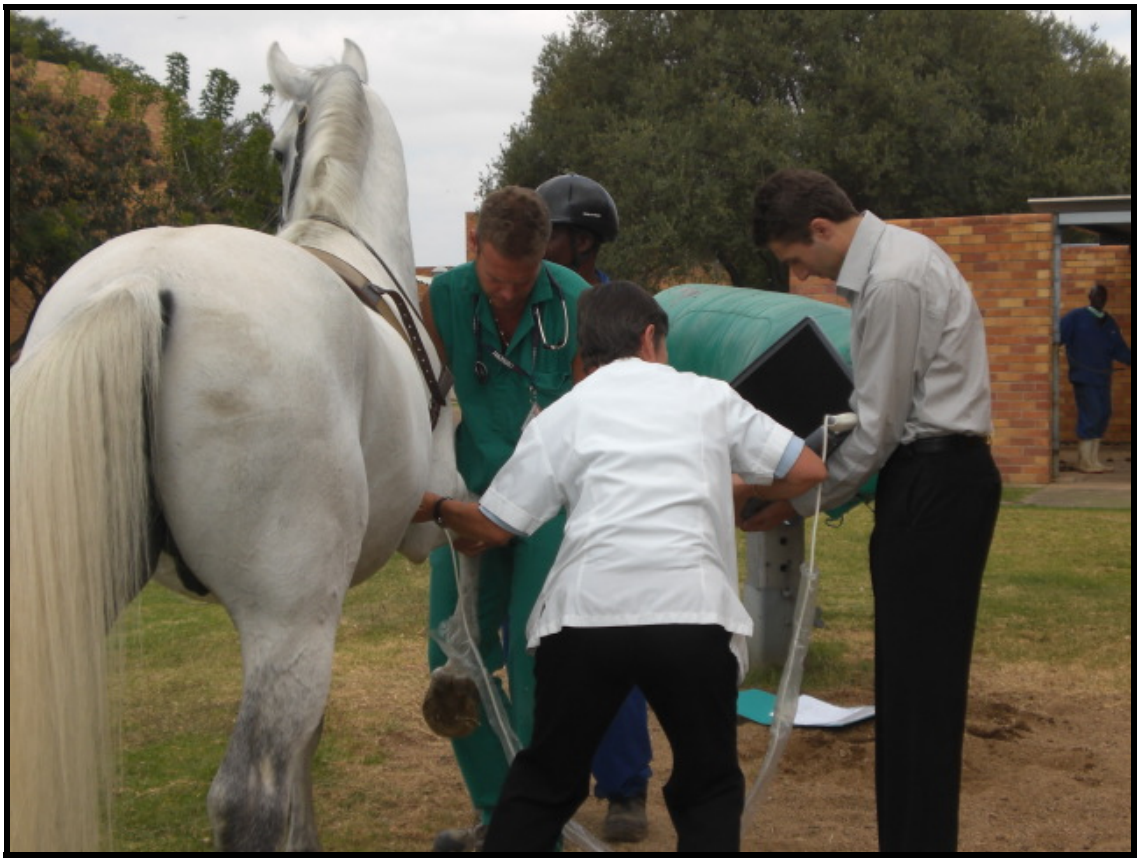
Additionally, species specific variations may exist in the kinetics of cTnI release into circulation and in the kinetics of clearance from the circulation. Appropriate sample collection time in horses is yet to be determined. In this study, the intervals of sample collection for cTnI analysis were chosen based on human literature[1] and studies reporting increased cTnI concentrations following short-term high intensity exercise in healthy horses.[7,15]

2.8 CONCLUSION

In conclusion, mounting and ejaculation results in increased cTnI concentrations in stallions. In the absence of myocardial histopathology, the significance of increased cTnI concentration observed in clinically healthy stallions following semen collection is difficult to determine. The increased cTnI concentration may not be of clinical relevance in healthy breeding stallions, but may be of importance in stallions with suspected cardiac disease. Analysis of cTnI concentration in stallions during the breeding season may have potential to identify stallions with possible myocardial disease. However, further evaluation of cTnI concentration in stallions following semen collection is needed. The second hypothesis of this study could not be proved with absolute certainty, but a tendency towards an increased FS post-semen collection was observed in stallions with higher heart rates, while great variability in FS occurred among stallions displaying lower heart rates in the immediate post-ejaculation period. The observed variability in post-semen collection FS in clinically healthy stallions highlights that FS should not be relied upon as a single index of LV systolic function in horses.

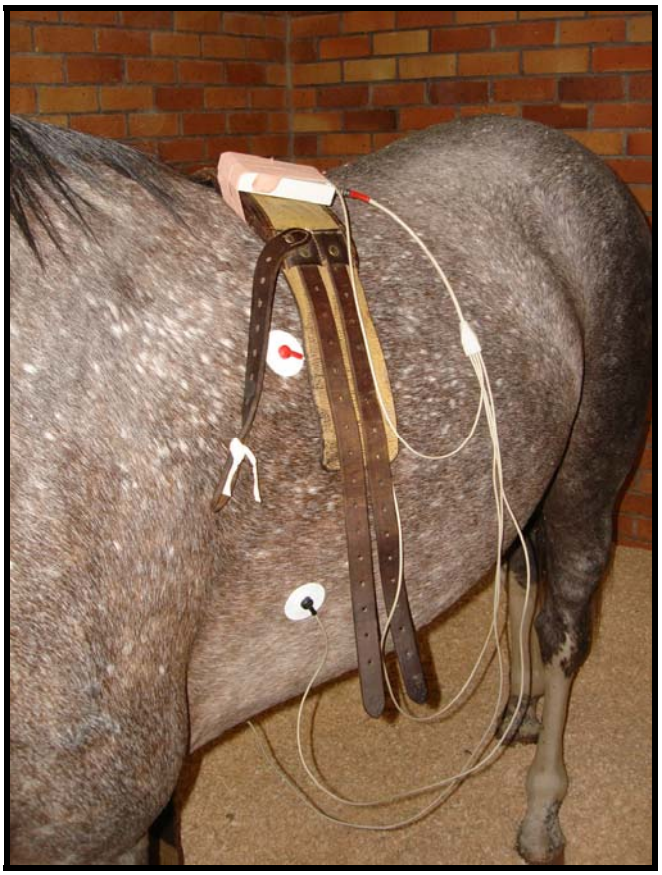
2.9 FIGURES AND TABLES

Figure 2.1 Right parasternal echocardiographic examination performed to determine the fractional shortening in the immediate post-semen collection period.



The right front leg is extended forward to facilitate echocardiographic examination.

Figure 2.2 Accurate placement of telemetry device and electrodes.



The antenna side of the device is positioned away from the animal to ensure maximum range. The red electrode is placed on the left side of the thorax about 15-20 cm below the top of the thorax while the green electrode was placed on the sternum. The black electrode was placed halfway between the green and red electrode.

Figure 2.3 Telemetry device and electrodes tightly secured around the stallion's chest and positioned in a way to avoid interference with the semen collection process.

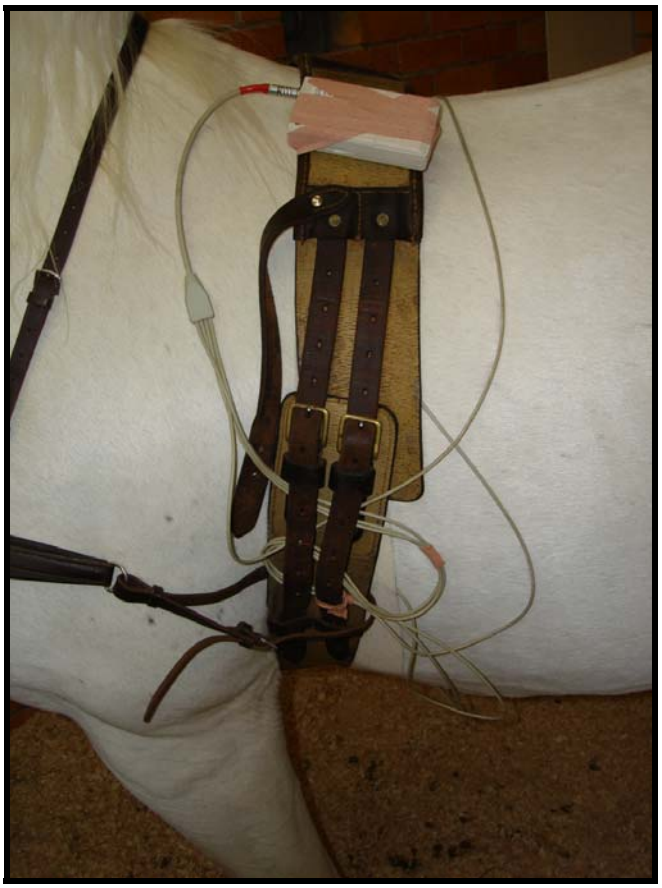


Figure 2.4 Telemetric electrocardiographic tracing obtained from a Lipizzaner stallion.

The instantaneous heart rate is displayed in the upper left hand corner.

Figure 2.5 Stage 3 of semen collection.

The stallion is in a holding area, prior to entering the collecting area, but still out of sight of the phantom.

Figure 2.6 Stage 5 of semen collection (ejaculation) after visual stimulation by a mare in oestrus.

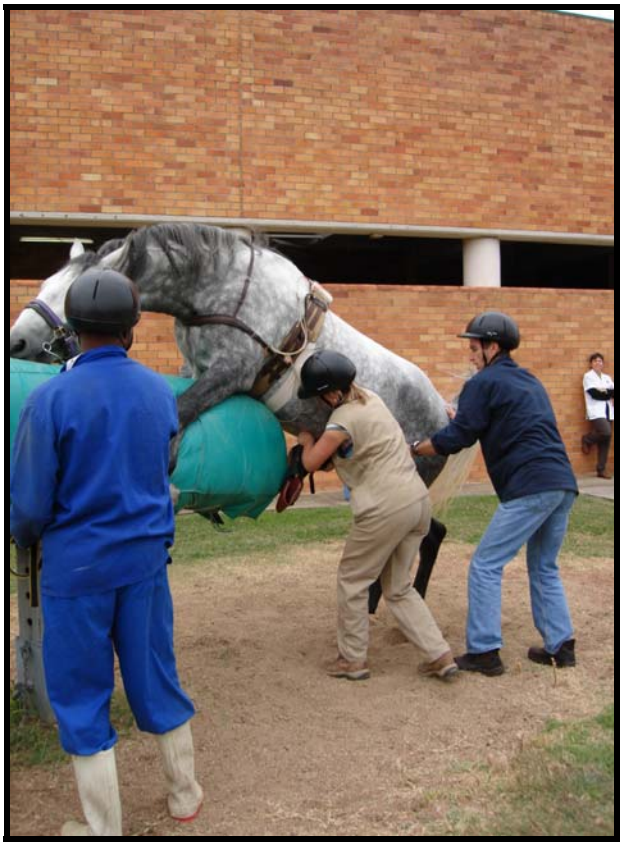
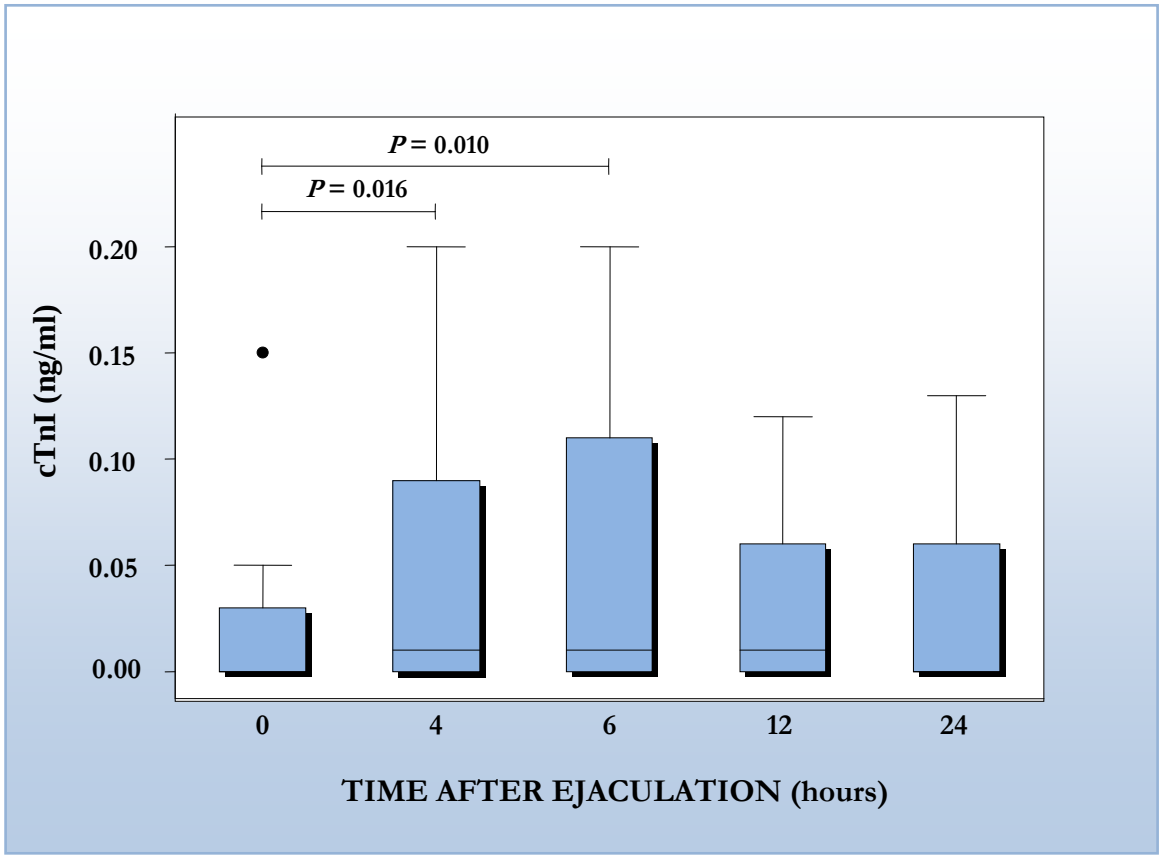
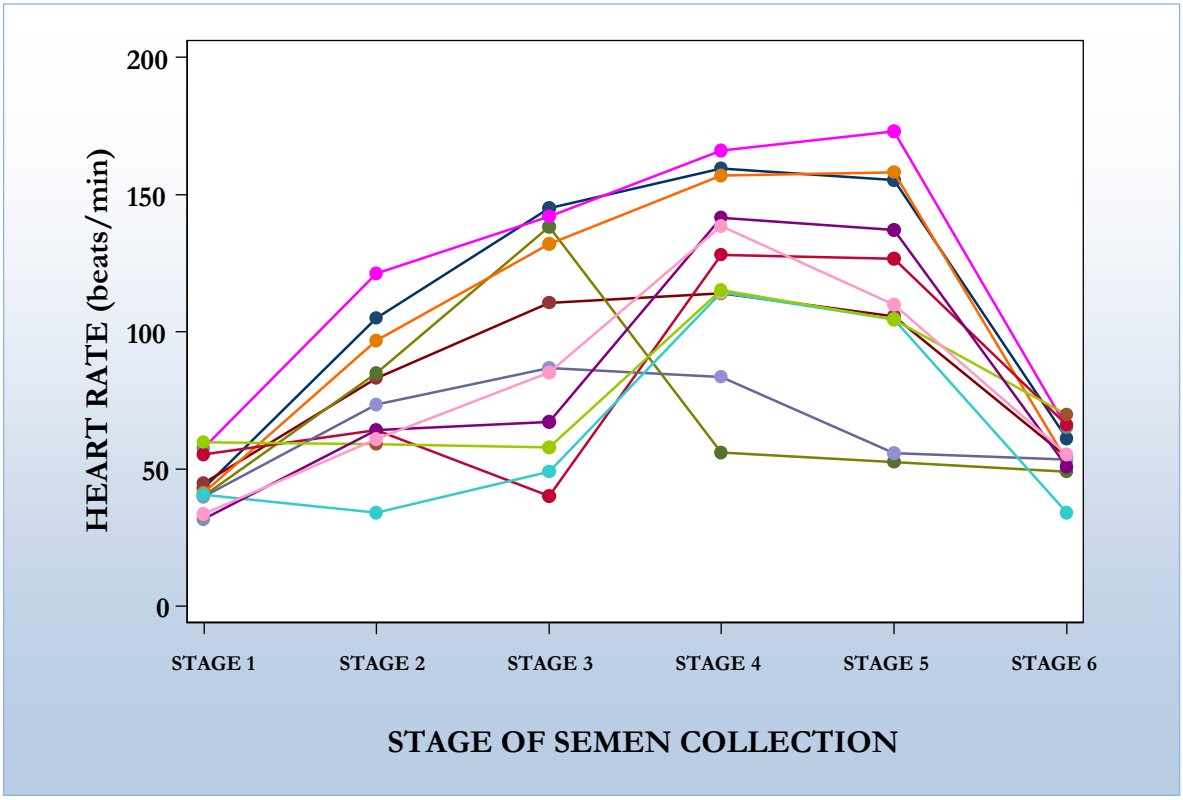


Figure 2.7 Box and whisker plots of changes in cardiac troponin I concentration.



The horizontal line in each box represents the median value. Boxes represent the interquartile range (IQR [25th to 75th percentile]). Whiskers extend to the upper adjacent value (largest value ≤75th percentile + 1.5 x IQR). Outliers are plotted as (•). Cardiac troponin I values that differed significantly (P<0.05) are indicated.

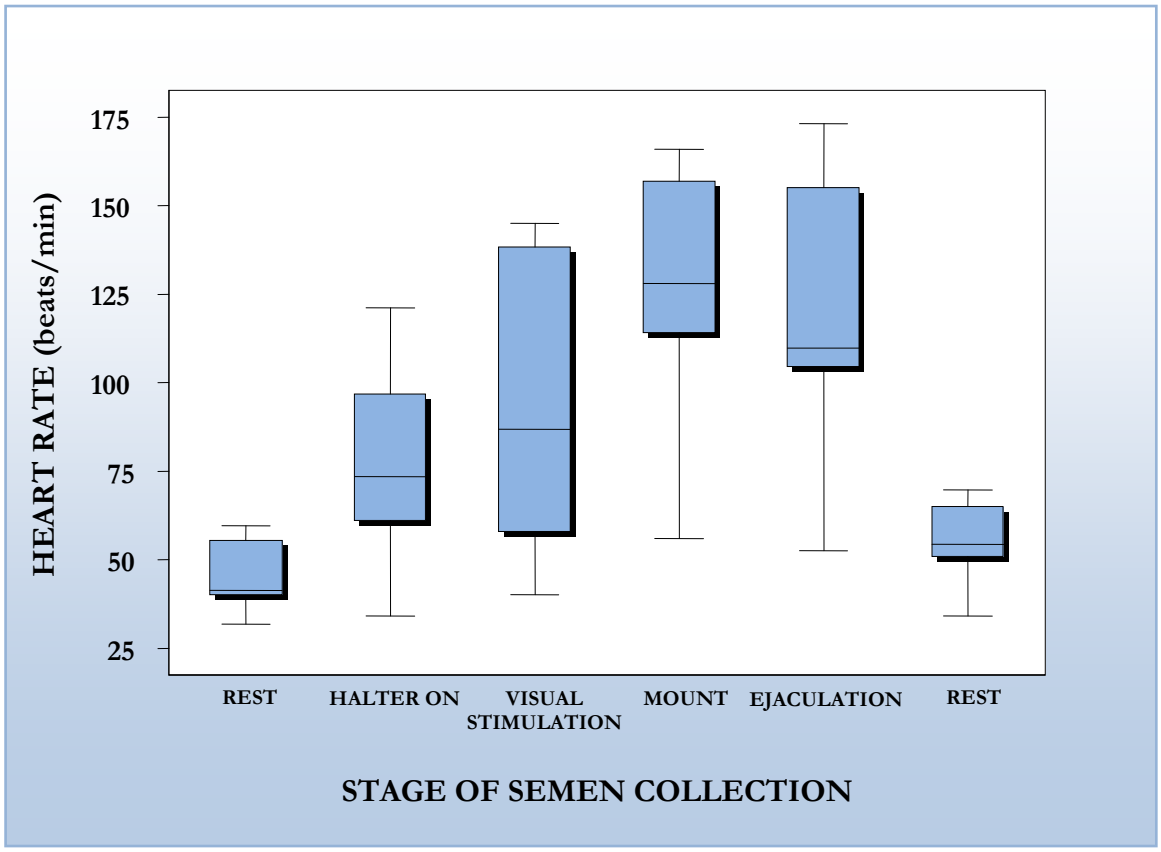
Figure 2.8 Average heart rate using cardiac telemetry at different stages of semen collection.



Different stages of semen collection were identified and included: at rest inside the stable prior to semen collection (stage 1); in a holding area, prior to entering the collecting area, but still out of sight of the phantom (stage 2); entering the collecting area with visual stimulation by a mare in oestrus (stage 3); successful mounting until dismount of the phantom (stage 4); ejaculation (stage 5) and at rest in the stable post-semen collection (stage 6).

Each colour represents a different stallion:
Stallion 1:
Stallion 2:
Stallion 3:
Stallion 4:
Stallion 5:
Stallion 6:
Stallion 7:
Stallion 8:
Stallion 9:
Stallion 10:
Stallion 11:

Figure 2.9 Box and whisker plots of average heart rate using cardiac telemetry during different stages of semen collection.



The horizontal line in each box represents the median. Boxes represent the interquartile range (IQR [25th to 75th percentile]). Whiskers extend to the upper (largest value ≤75th percentile + 1.5 × IQR) and lower adjacent value (smallest value ≥25th percentile - 1.5 × IQR). Different stages of semen collection were identified and included: at rest inside the stable prior to semen collection (stage 1); in a holding area, prior to entering the collecting area, but still out of sight of the phantom (stage 2); entering the collecting area with visual stimulation by a mare in oestrus (stage 3); successful mounting until dismount of the phantom (stage 4); ejaculation (stage 5) and at rest in the stable post-semen collection (stage 6).

Table 2.1 Cardiac troponin I concentration measured at different intervals.

Stallion	Cardiac troponin I concentration (ng/ml)				
	0h	4h post-SC	6h post-SC	12h post-SC	24h post-SC
1	0.01	0.01	0.01	0	0
2	0.01	0.01	0.02	0.01	0.01
3	0	0	0	0.03	0.01
4	0	0.02	0.14	0	0
6	0.03	0.09	0.11	0.11	0.1
7	0	0.02	0.01	0.01	0
8	0	0.01	0.01	0	0
9	0	0	0	0	0
12	0.15	0.2	0.2	0.12	0.13
13	0.05	0.09	0.09	0.06	0.06
14	0	0	0	0	0

0h, prior to semen collection
4h post-SC, 4 hours post-semen collection
6h post-SC, 6 hours post-semen collection
12h post-SC, 12 hours post-semen collection
24h post-SC, 24 hours post-semen collection

Table 2.2 Minimum, maximum, median and interquartile range of different cardiac troponin I concentrations and predictors measured.

Variable	Minimum	Maximum	Median	IQR
cTnI pre-SC (ng/ml)	0.001	0.15	0.001	0.001-0.03
cTnI 4 hours (ng/ml)	0.001	0.2	0.01	0.001-0.09
cTnI 6 hours (ng/ml)	0.001	0.2	0.01	0.001-011
cTnI 12 hours (ng/ml)	0.001	0.12	0.01	0.001-0.06
cTnI 24 hours (ng/ml)	0.001	0.13	0.001	0.001-0.06
Age (years)	3	24	8	6-17
Weight (kg)	385	700	503	467-550
FS ejaculation (%)	21	58	34.5	29-50
AUC HR	158	901	404	259-595
Max HR (bpm)	152	228	178	161-200
Ave HR stage 1 (bpm)	32	60	41	40-55
Ave HR stage 2 (bpm)	34	121	73	61-97
Ave HR stage 3 (bpm)	40	145	87	58-138
Ave HR stage 4 (bpm)	56	166	128	114-157
Ave HR stage 5 (bpm)	52	173	110	104-155
Ave HR stage 6 (bpm)	34	70	54	51-65

Summary statistics of different cTnI concentrations and variables measured: cTnI pre-SC, cTnI pre-semen collection; cTnI 4 hours, cTnI 4 hours post-semen collection; cTnI 6 hours, cTnI 6 hours post-semen collection; cTnI 12 hours, cTnI 12 hours post-semen collection; cTnI 24 hours, cTnI 24 hours post-semen collection; FS ejaculation, fractional shortening in the immediate post-ejaculation period; AUC HR, area under the heart rate curve; Max HR, maximum heart rate; Ave HR stage 1, average heart rate stage 1 of semen collection; stage 2, stage 2 of semen collection; stage 3, stage 3 of semen collection; stage 4, stage 4 of semen collection; stage 5, stage 5 of semen collection; stage 6, stage 6 of semen collection and IQR, interquartile range.

Table 2.3 Summary of haematological parameters obtained (Part 1).

Stallion	Hb g/l	RCC x 10^{12}/l	HT %	MCV fl	MCH g/dl cells	MCHC g/dl cells	RDW %	Plt C x 10^9/l
1	164	8.75	44	50.5	18.7	37.1	18.7	210
2	158	8.92	43	48.7	17.7	36.4	18	176
3	150	8.91	41	45.9	16.9	36.7	17.7	214
4	171	9.73	45	46.6	17.5	36	17.9	180
5	157	8.35	43	51.5	18.8	36.5	18.3	125
6	123	7	34	48.2	17.5	36.4	18.6	140
7	149	8.68	40	46.4	17.2	37	18.1	136
8	136	8.85	37	41.5	15.3	37	17.9	166
9	165	9.08	45	49.5	18.5	36.7	19	153
10	163	10.24	44	43.3	16.3	368	20	167
11	113	7.55	32	42.2	16.3	35.5	19.1	167

Hb, haemoglobin
RCC, red cell count
HT, haematocrit
MCV, mean corpuscular volume
MCH, mean corpuscular haemoglobin
MCHC, mean corpuscular haemoglobin concentration
RDW, red cell distribution width

Table 2.4 Summary of haematological parameters obtained (Part 2).

Stallion	WCC x10^9/l	Ab N(total) x 10^9/l	Ab Nmat x 10^9/l	Ab Nimm x 10^9/l	Ab Lymph x 10^9/l	Ab Mono x 10^9/l	Ab Eos x 10^9/l	Ab Baso x 10^9/l
1	8.43	4.99	4.99	0	2.97	0.24	0.23	0.02
2	8.92	6.24	6.24	0	1.96	0.71	0	0
3	10.76	7.83	7.83	0	2.11	0.46	0.18	0.17
4	9.38	5.88	5.88	0	3.03	0.26	0.08	0.14
5	10.72	6.65	6.65	0	3.22	0.43	0.43	0
6	6.45	4.26	4	0.26	2.06	0.13	0	0
7	6.29	3.52	3.52	0	2.52	0.25	0	0
8	8.72	7.39	7.39	0	1.18	0.07	0.05	0.03
9	6.54	4.19	4.19	0	2.09	0.13	0.13	0
10	8.16	4.9	4.9	0	2.77	0.08	0.41	0
11	11.74	8.81	8.81	0	2	0.82	0.12	0

WCC, white cell count
AbN(total), absolute total neutrophil count
AbNmat, absolute mature neutrophil count
AbNimm, absolute immature neutrophil count
AbLymph, absolute lymphocyte count
AbMono, absolute monocyte count
AbEos, absolute eosinophil count
AbBaso, absolute basophil count

Table 2.5 Echocardiographic measurements at rest prior to semen collection and fractional shortening post-semen collection.

Stallion	LA (cm)	LVIDd (cm)	LVFWd (cm)	IVSd (cm)	LVIDs (cm)	LVFWs (cm)	IVSs (cm)	FS (%)	FS post-SC (%)	HR post-SC FS (bpm)
1	11.26	14.15	2.73	3.57	10.29	2.86	5.34	27	50	96
2	13.31	9.97	3.29	2.28	5.88	4.17	3.73	40	58	72
3	12.19	8.02	2.73	2.34	5.52	2.68	3.9	31.35	50	54
4	10.4	9.94	1.83	2.6	6.46	3.28	6.46	35	58	90
6	11.82	8.68	2.89	3.09	5.5	4.73	4.63	36.67	NA	NA
7	12.8	12.35	2.51	2.12	8.01	3.09	4.15	35	29	68
8	11.43	11.38	4.24	2.22	7.04	5.5	4.24	38.14	22	48
9	11.2	7.91	2.12	2.51	4.92	3.18	4.05	37.6	29	36
12	14.48	11.38	2.12	3.28	5.11	4.92	5.21	55	21	65
13	14.24	12.87	2.61	3.26	6.81	4.2	5.69	47.1	30	99
14	11.95	9.1	3.78	2.6	6.5	4.96	4.14	28.6	39	NA
Mean	12.28	10.52	2.80	2.72	6.55	3.96	4.69	37.41	38.60	69.78
SD	1.30	2.06	0.73	0.50	1.54	0.98	0.87	8.03	14.36	18.23

LA, left atrium diameter
LVIDd, left ventricular internal diameter in diastole
LVFWd, left ventricular free wall in diastole
IVSd, interventricular septum in diastole
LVIDs, left ventricular internal diameter in systole
LVFWs, left ventricular free wall in systole
IVSs, interventricular septum in systole
FS, fractional shortening
FS post-SC, fractional shortening post-semen collection
HR post-SC FS, heart rate at time of post-semen collection FS measurement
bpm, beats per minute
SD, standard deviation
NA, not available

Table 2.6 Statistical significance of associations between measured predictors and increased cardiac troponin I concentration (outcomes).

Predictor	*P*-values at different outcomes		
	1	2	3
Age	0.053	0.109	0.121
Weight	0.081	0.285	0.282
FS-ejaculation	0.149	0.954	0.875
FS-2 hours	0.771	0.845	0.631
AUCHR	0.761	0.478	0.452
HR_{max}	0.498	0.165	0.116
Ave HR Stage 1	0.109	0.249	0.183
Ave HR Stage 2	0.577	0.277	0.283
Ave HR Stage 3	0.596	0.794	0.802
Ave HR Stage 4	0.273	0.172	0.132
Ave HR Stage 5	0.195	0.108	0.083
Ave HR Stage 6	0.083	0.224	0.104

FS-ejaculation, fractional shortening immediately following ejaculation; FS-2 hours, fractional shortening 2 hours following semen collection; AUCHR, area under curve for heart rate; HR_{max}, maximum heart rate; Ave HR, average heart rate; Stage 1, the stallion was at rest in the stable; stage 2, in a holding area, prior to entering the collecting area, but still out of sight of the phantom; stage 3, entering the collecting area with visual stimulation by a mare in oestrus; stage 4, successful mounting until dismount of the phantom; stage 5, ejaculation; stage 6, at rest in the stable post-semen collection; 1, change in cardiac troponin I (cTnI) concentration from pre- to 4 hours post-semen collection; 2, change in cTnI concentration from pre- to 6 hours post-semen collection and 3, cTnI concentration at 6 hours post-semen collection.

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Appendices

Appendix 1: Weekly schedule for data collection

University of Pretoria

Department of Companion Animal Clinical Studies
Private bag X04 Onderstepoort 0110 Republic of South Africa
Tel 012-529-8000 Fax 012-529-8300
http://www.up.ac.za
Faculty of Veterinary Science

	Monday		Tuesday		Wednesday				Thursday		Friday	
	S1 (D1)	S2 (D1)	S1 (D2)	S2 (D2)	S1 (D3)	S2 (D3)	S3 (D1)	S4 (D1)	S3 (D2)	S4 (D2)	S3 (D3)	S4 (D3)
08h00			CE/T1	CE/T1	CE	CE			CE/T1	CE/T1	CE	CE
09h00			ECG on						ECG on			
10h00	ETA	ETA	SC/FS		T5				SC/FS		T5	
11h00	CE/ CBC	CE/ CBC	ECG off	ECG on			ETA	ETA	ECG off	ECG on		
12h00			FS	SC/FS	ETD	ETD/ T5	CE/ CBC	CE/ CBC	FS	SC/FS	ETD	ETD/ T5
13h00	Echo						Echo					
14h00		Echo	T2	FS				Echo	T2	FS		
15h00												
16h00			T3	T2					T3	T2		
17h00												
18h00				T3						T3		
19h00												
20h00												
21h00												
22h00			T4						T4			
23h00												
00h00				T4						T4		
01h00												
02h00												
03h00												
04h00												
05h00												
06h00												
07h00												

ETA - estimated time of arrival; CE - clinical examination; ETD - estimated time of departure; CBC - complete blood count; FS - fractional shortening; SC - semen collection; T1-5 - cTnI samples; ECG - telemetric electrocardiogram; Echo - echocardiographic examination; S - stallion

Appendix 2: Data collection sheet

Stallion: _____
Date D1: ___

University of Pretoria

Department of Companion Animal Clinical Studies
Private bag X04 Onderstepoort 0110 Republic of South Africa
Tel 012-529-8000 Fax 012-529-8300
http://www.up.ac.za
Faculty of Veterinary Science

Day 1:
Clinical exam and cardiac auscultation at presentation:

CBC:

Hb		80.00 - 140.00
RCC		5.50 - 9.50
HT		0.24 - 0.44
MCV		36.00 - 49.00
MCH		
MCHC		33.00 -37.00
RDW		
WCC		6.00 -12.00
Ab N(total)		
AbNmat		3.54 - 7.08
AbNimm		0.00 - 0.24
AbLympho		1.80 - 3.60
AbMono		0.00 - 0.72
AbEos		
AbBaso		0.00 - 0.12
Plt C		200.00 – 600.00

Day 2:
Clinical examination on the morning following admission:

HR:	RR:	B:	CRT:	MM:

Telemetric ECG:

cTnI concentrations:

T1:	**T2:**	**T3:**
T4:	**T5:**	

Fractional shortening:

5 min after semen collection	2 hrs after semen collection

Scientific proceeding associated with this dissertation

VILJOEN, A., SAULEZ, M.N., CARSTENS, A., SCHULMAN, M., THOMPSON, P.N. Changes in serum cardiac troponin I concentration after semen collection in stallions. In *Proceedings of the 29th Forum of the American College of Veterinary Internal Medicine*, Anaheim CA. 2010; 778.