Verslag Onderzoekstage
September 2008 – Maart 2009

Blood and semen corticosteroid levels in scrotal insulated *Bos indicus* bulls

Drs. Y.M. Roetert
Studentnr. 0461903

Stageplaats: University of Queensland, Australië
Begeleider ter plaatse: G.B. Boe-Hansen
Begeleider Nederland: P.L.A.M. Vos

Voorwoord

Onderzoekstages in het buitenland zijn niet altijd even gemakkelijk. Dit werd mij verteld toen ik het idee opperde dat ik graag in Australië mijn onderzoeksstage wilde doen. Maar het was ook zeker een goede mogelijkheid om wat meer van de wereld te zien en mijn Engels te verbeteren, en dat was waar mijn voorkeur naar uitging. Ik wilde meer dan de relatief kleine boerderijen in Nederland en de laboratoria in Utrecht. Ik wilde naar de andere kant van de wereld. En het is gelukt: na ongeveer 2 maanden van in het rond e-mailen, had ik dan toch een plaats gevonden, waar ze mij een stage konden aanbieden waar ik kon gaan ruiken aan het onderzoek doen en waar ik een eigen onderzoekje kon doen binnen een groter onderzoek. Na 2 maanden literatuuronderzoek, het opstellen van een onderzoeksplan en een aanvraag voor de ethische commissie, kon ik in september eindelijk vertrekken naar de Universiteit van Queensland, Brisbane, Australië.

Het onderzoek bleek veel omvattend en tijdens de 12 weken aan deze Universiteit heb ik mijn begeleidster, Gry Boe-Hansen dan ook wel eens met vraagtekens in mijn ogen aangekeken wanneer we dan “eindelijk gingen beginnen met het echte onderzoek”. Maar na 4 weken voor onderzoek, kwamen dan eindelijk de stieren voor het echte onderzoek en konden we beginnen. Onderweg heb ik verschillende hindernissen moeten nemen, maar na 12 weken leken de resultaten zo veelbelovend dat er over publicatie werd gesproken. Ook na overleg met mijn begeleider in Nederland, Peter Vos, leek het ons een mooie kans om te streven naar publicatie in een internationaal wetenschappelijk tijdschrift. Maar dit betekende ook dat er resultaten zullen moeten worden ge-her-analyseerd en er nog wijzigingen aan het verslag moeten worden gemaakt. Omdat er verschillende mensen betrokken zijn bij de analyse van de resultaten, laten deze nog even op zich wachten, maar met het oog op mijn studievoortgang, hebben Peter Vos en ik besloten om dit “voorlopige” verslag als eindverslag van mijn onderzoekstage in te leveren, zodat ik de volgende maand mee kan loten voor de co-schappen. Toch zullen wij blijven streven naar verbetering en uiteindelijk publicatie, zodra alle resultaten aanwezig zijn.

Mijn dank gaat uit naar mijn stagebegeleidster in Australië, Gry Boe-Hansen, en naar Nancy Phillips, voor de begeleiding in het praktische werk. Niet te vergeten Stephen Johnson, voor het opzetten van de laboratoriumtechnieken voor de corticosteroid-analyse en Michael McGowan, voor de algemene supervisie. En natuurlijk Peter Vos, voor het vertrouwen in mij en de hulp bij het tijdig afronden van dit project.

Hopelijk kan ik met dit verslag ook uw interesse wekken en bent u net zo benieuwd naar de uiteindelijke resultaten en de eventuele publicatie als wij.

Met vriendelijke groet,
Drs. Y.M. Roetert

Blood and semen corticosteroid levels in scrotal insulated *Bos indicus* bulls

Y.M. Roetert[1], G.B. Boe-Hansen[2], S.T. Anderson[3], N.J. Phillips[2], M.R. McGowan[2], P.L.A.M. Vos[1]

[1] *School of Veterinary Science, University of Utrecht, The Netherlands*
[2] *School of Veterinary Science, University of Queensland, St. Lucia/ Pinjarra Hills, Australia*
[3] *School of Biomedical Sciences, University of Queensland, St. Lucia, Australia*

September 2008 – March 2009

Abstract
Six *Bos indicus* bulls were used to determine if Scrotal Insulation (SI) has an effect on the corticosteroids in their blood and semen. SI was performed for 48 h using two layers of nappies around the scrotum. During SI, the Scrotal Skin Temperature (SST) was measured every two minutes. Using electroejaculation, every third day semen was collected from 19 d before until 14 d after SI. From 10 d before until 5 d after SI, pulse, respiration, rectal temperature and blood samples were collected every three days and during SI more frequent. Corticosteroids were determined using radioimmunoassay. Results were that the SI increased SST to a maximum of 39.2 °C in the SI (n=3) bulls, compared with a maximum of 37.8 °C in the controls (n=3). Maximum and minimal rectal temperature of the SI bulls were 37.8 ± 0.6 ºC and 38.8 ± 0.4 ºC compared with 38.6 ± 0.1 ºC and 39.5 ± 0.1 ºC in the controls. Sperm motility in the SI bulls decreased from 60-90% before to 20-30% 14 d after SI. Blood cortisol concentrations in the SI bulls increased to 3.7 ± 5.7 nmol/l compared with 1.8 ± 1.5 nmol/l in the control bulls, but these differences were particularly due to individual differences at certain timepoints. Blood cortisone fluctuated during the experiment, but gave no significant differences between the SI and control bulls. Average semen cortisol and cortisone levels didn't differ between SI and control bulls before and after the experiment.
This study showed that however SI increases the SST, there was neither an increase in body temperature nor in glucocorticoid concentrations in these bulls. From these results, the conclusion could be made that not corticosteroids, but the elevated local temperature plays the most important role in deterioration of semen quality during SI in these *Bos indicus* bulls.

Keywords: **bull; scrotum; insulation; corticosteroids; testicular temperature**

Introduction

A testicular temperature of 2-6°C below body temperature is essential for normal spermatogenesis in most mammals, including cattle, and is kept within these limits by complex physiological mechanisms involving the scrotum, testicular vascular cone and the testes themselves (Brito et al., 2004; Cook et al., 1994; Kastelic et al., 1996; 1997). An elevation of the testicular temperature results in increased metabolism and oxygen demand, due to testicular blood flow limitation the increased demand in oxygen cannot be meet, resulting in hypoxia, generation of reactive oxygen species and subsequently deterioration of semen quality (Brito et al., 2004).
Not only this direct pathway of heat can influence the reproductive performance of animals but elevated temperatures can also give a stress reaction in cattle with altered endocrine function which can lead to a decrease in fertility (Gwazdauskas, 1985). One hypothesis of this decrease in fertility is the rise in corticosteroids following stress. According to Möstl *et al.* (2002), not all kinds of stress will give an increase in corticosteroids, but research by Christison and Johnson (1972) confirmed that after the onset of hyperthermia, the cortisol levels in cows start to increase within a few hours and decline immediately after leaving the high temperature chamber and resume basic levels within a few days. During short-term stress, glucocorticoids improve fitness by mobilising energy and changing behaviour; however, during chronic stress with prolonged periods of high cortisol concentrations, the

level of glucocorticoids can affect individual fitness by inducing immuno-suppression, atrophy of tissues and decreased reproductive performance (Möstl et al., 2002; Michael and Cooke, 1994). Glucocorticoids can affect gonadal function by acting on the hypothalamus by decreasing the synthesis and release of GnRH, the anterior pituitary gland by decreasing the synthesis and release of LH and/or FSH, or the testis directly by modulating steroidogenesis (Michael and Cooke, 1994). This last, direct pathway is by binding of glucocorticoids to glucocorticoid receptors in Leydig cells, thus inhibiting the transcription of genes encoding testosterone biosynthetic enzymes and inducing apoptosis of Leydig cells. The reduced blood concentration of testosterone is accompanied by diminished libido and fertility (Hardy et al., 2005).
Several papers (Morris et al., 2003; Hardy et al., 2005) have established that access of glucocorticoids to their receptors within the Leydig cells is regulated by the local expression of the enzyme 11β- hydroxysteroid dehydrogenase (11β-HSD). Two distinct gene products exhibiting 11β-HSD activity have been identified in mammals:
the type 1 form (11β-HSD1) and the type 2 form (11β-HSD2). The conversion of active corticosterone and cortisol into the biologically inert 11-dehydrocorticosterone and cortisone, is catalysed by the oxoreductase11β-HSD1. 11β-HSD2 catalyses the conversion into inactive 11-dehydrocorticosterone and cortisone, essentially in a unidirectional mode (Michael and Cooke, 1994; Morris et al., 2003; Hardy et al., 2005).

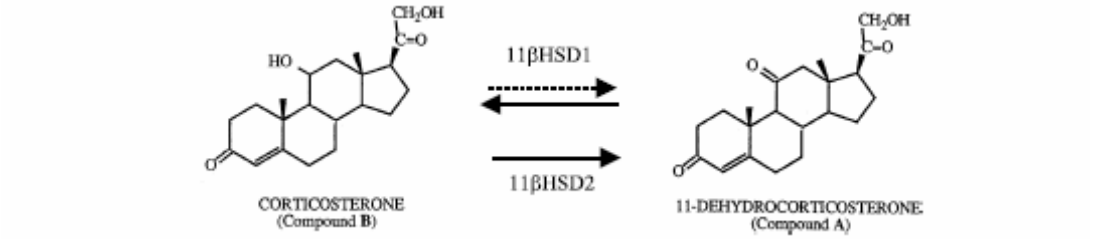

Figure 1. Biochemical reaction of metabolism of endogenous glucocorticoids by 11β-HSD1 and 11β-HSD2 (Morris, 2003)

Usually, glucocorticoids levels are measured in the plasma. However, blood cortisol levels are subject to a daily pattern in cattle (Lefcourt et al., 1993; Welsh et al., 1979). Previous studies have shown a correlation between the levels of cortisol in blood and seminal plasma of bulls (Graves and Eiler, 1979). In this paper it was suggested that cortisol in plasma would be rapidly transferred to semen to reach an equilibrium. J. Mayes (Mayes, unpublished research, 2008) found no significant differences between cortisol:cortisone ratios in ejaculates from 20 *Bos indicus* bulls collected early in the morning as compared to those collected in the afternoon. It could therefore be hypothesized that the cortisol concentrations in semen may be buffered against the changes in systemic steroid concentrations by local metabolism; wherefore corticosteroid levels in semen may not be influenced by circadian patterns to the same extend as plasma.

Insulating the scrotum of a bull, elevates scrotal and testicular temperature, presumably because heat radiation from the testicular vascular cone and possibly also the counter current heat exchange are impaired (Kastelic et al., 1996). These authors also suggest that there might be some difference in scrotal/testicular thermoregulation between different cattle breeds. This has been supported by Brito *et al.* (2004), who indicate that *Bos indicus* bulls have better body thermoregulation and slower and less pronounced decrease in semen quality, when exposed to high ambient temperatures, compared to *Bos taurus* and crossbred bulls. Better body thermoregulation could be due to a greater skin surface to body size ratio, type and quantity of sweat glands, number of epithelial layers of the skin, lower thermogenesis and usually a

smaller frame (Brito et al., 2004; Carvalho et al., 1995). Better testicular thermoregulation could be do to differences in morphology of the scrotum, testicular vascular cone and testicular volume in *Bos indicus* bulls (Brito et al., 2004). This could suggest that the heat stress achieved by scrotal insulation and the resulting increases in cortisol could be lower in *Bos indicus* cattle compared with other breeds.

The objective of the current study was to confirm that an elevation of the scrotal skin temperature (SST) obtained though scrotal insulation (SI) for 48h, cause testicular stress in *Bos indicus* bulls which is followed by an increase in corticosteroids, and an activation of 11β-hydroxysteroid dehydrogenase (11β-HSD) resulting in a shift in cortisol: cortisone ratio. The following variables were measured: (1) SST during SI, (2) corticosteroid levels (cortisol/cortisone ratio) in semen and blood indicative of 11β-HSD activity before, during and immediately after SI.

Materials and methods

Research protocols were approved by The University of Queensland Animal Ethics Committee, approval nr. SVS/355/08/NSRSF, chief investigator Gry Boe-Hansen.

Animals

The study was conducted from October to December 2008 at Pinjarra Hills, Queensland, Australia. A total of 6 *Bos indicus* bulls were transported from Belmont Research Station, Rockhampton to Pinjarra Hills, Brisbane. The bulls were 24 months old with an average weight of 393 kg (range 360 to 422 kg). The inclusion criteria were normal semen parameters (>70% normal sperm morphology), that they responded well to electro ejaculation (EE) and that they had a reasonable temperament. The first 10 days after their arrival the bulls were placed in a crush every two to three days as quietly as possible to make them familiar with handling in the crush. On day three they got treated with Cydectin® Pour-On (Fort Dodge Animal Health, Iowa, USA); on day 7 they received an Optimizer® Insecticidal Ear Tag (Y-Tex Corporation, Cody, USA) and on day 18 after arrival they were vaccinated against Tick Fever (Trivalent tick fever vaccine, Department of Primary Industries and Fisheries, QLD, AUS).
On day 30, they were distributed into the insulation (n=3) group and the control (n=3) group. This distribution was made by randomization after matching the animals based on weight and scrotal circumference.
Scrotal circumference was measured with a Reliabull® Scrotal Tape (Lane Manufacturing, Denver, USA). Scrotal shape was subjectively scored based on the scrotal conformation as described by Entwistle and Fordyce (2003). All bulls had a pendular scrotum.
During the experimental period, the bulls were kept on pasture. Mean weekly environmental temperatures ranged from 21.5 to 23.0ºC during the 6.5 weeks of the study.

Scrotal insulation

Thirty days after arrival, around noon, the bulls were subjected to scrotal insulation (SI) or included in the control group. The insulation material consisted of a double layer of nappies (Home Brand, Woolworths, Crawler nappies) and a layer of aluminium foil secured with Elastoplast® Vet (BSN Medical, UK) on the neck of the scrotum to minimize movement of the nappies. To protect the insulation material it was covered with one layer of Co-Plus® Flexible Cohesive Bandage (BSN Medical, UK).
The scrotal skin temperature was measured every 2 min. In all the bulls, one Thermochron® iButton® (model DS1922L, Maxim Integrated Products, Dallas Semiconductor, CA, USA)

was plastered on the caudal scrotal surface of the left testicle using Opsite◊ Flexigrid◊ (Smith & Nephew Medical Limited, UK) and covered with Elastoplast Water Resistant Plastic (Beiersdorf, AUS Ltd.). In the SI bulls, two other iButtons were placed on the inside of the insulation material; one in the area of the scrotal neck and one on the bottom of the scrotum. After an insulation period of 48 h, the insulation material and the iButtons were removed and the scrotum and testis palpated and scrotal circumference was measured.
The data from the iButtons were downloaded to a computer via a 1-Wire™ interface using an iButton probe and 1-Wire to USB port adapter (Maxim Integrated Products, Dallas Semiconductor, CA, USA).

Semen collection

Starting ten days after their arrival, semen was collected by EE every third day from 19 d before till 14 d after onset of SI. The bulls were placed in a crush for semen and blood collections. Rectal temperature (BDF, Handyplast China), respiration and pulse were also measured.
Semen was collected for cortisol measurement and semen evaluation. After flushing the prepuce with 50 ml physiological saline (Baxter, USA), a rectal examination was conducted to remove all the faeces from the rectum and to give a gentle massage to the ampullae and pelvic urethra. Then a 60 mm well lubricated rectal probe (Lane Manufacturing, Denver, USA) was inserted and stimulation was commenced at the lowest power setting with a Pulsator IV Auto Adjust™ (Lane Manufacturing, Denver, USA). The voltage was conducted in a rhythmic fashion progressively increasing until ejaculation. The semen was collected in a sterile 10 ml screw-capped conical tube (Sarstedt AG & Co., Nümbrecht, Germany), holding a collection handle with a disposable polystyrene vial (Lane Manufacturing, Denver, USA) over the penis during ejaculation.
A minimum of 4.5 ml was required per bull per collection. When the first ejaculate consisted of 4.0 ml semen or less, a second sample was collected immediately, following the same procedure as described above. When a minimum of 4.5 ml of semen had been collected, the probe was removed from the rectum. In some cases there was some blood on the probe, in which case another rectal examination to check for lesions in the rectal wall was conducted.

Semen evaluation

After semen collection, the semen sample was assessed for volume (ml), colour (Yellow or Skim-milk) and density (grade 1-4) and further under the microscope for mass activity (grade 1-5) and progressive motility (%) following the method as described by Entwistle and Fordyce (2003). Two samples were prepared for later sperm morphology assessment: one in a 1.5 ml Eppendorf tube (Sarstedt AG & Co., Nümbrecht, Germany) with 0.7 ml of Formalin Buffered Saline (FBS) and two smears with Nigrosin Eosin. A total of 0.5 ml of the raw semen was pipetted into a 1.2 ml Cryo tube (TPP®, Trasadingen, Switzerland) and placed into liquid nitrogen and stored at -80°C for later corticosteroid analysis.

Blood collections

Blood samples were collected in the morning after their arrival and from 10 days prior to SI every third day around noon, in order to establish the control levels of cortisol for the individual bull. After cleaning the tail with a paper towel and 70% ethanol, blood from the tail vein was collected using PrecisionGlide™ 0.9x38 mm Vacutainer Systems (Becton Dickinson, Plymouth, UK) in a 8 ml Vacuette® LH Lithium Heparin Sep tube (Greiner bio-one, Germany) or a 10 ml BD Vacutainer® LH tube (Becton Dickinson, Plymouth, UK). Twenty-four hour before, at the onset (0) and 6, 12, 18, 24, 36 and 48 h post initiation of SI blood samples were collected, continuing every third day at noon until six days after removal

of the insulation.
Each tube was labelled with bull identification and date, the colour of the blood sample was noted (light or dark), and the sample was stored on ice until all the samples were centrifuged after the last blood collection. The samples were centrifuged for 20 minutes at 2500 rpm at room temperature using a Damon IEC Model HN-SII Centrifuge, the plasma was pipetted off and immediately frozen at -20°C for later analysis.

Corticosteroid analysis

Corticosteroids were determined using radioimmunoassay.
Steroids were extracted from 300 µl bloodplasma with 2 ml chloroform in 16x100 mm glass tubes. 400 µl raw semen was spun (Sigma Micro centrifuge, Quantum Scientific, AUS) for 5 minutes at 1200 rpm, stored on ice and 300 µl seminal plasma was pipetted off for extraction following the same procedure as for the bloodplasma. Tubes were placed on a multi tube vortex mixer (IKA-VIBRAX TYP VXR, Janke & Kunkel, Germany) for 8 minutes on 1200 rpm and placed in an ethanol/ dry-ice bath. When the plasma was frozen, the chloroform was decanted into 12x75 mm assay tubes. The tubes were placed under a gentle stream of air using a Multi-Blok Heater (Lab-Line Instruments Inc., USA) and when dried, the solvent was reconstituted with 300 µl RIA buffer (0.05M PBS containing 0.1% gelatine), vortexed, capped and stored at 4°C.
Stock solutions of cortisol and cortisone were diluted in RIA buffer to standards of 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.12, 6.25, 12.5, 50, 100 and 200 ng/ml**.** Standard tubes were run in triplicate and contained 50 µl standard and 50 µl buffer. Unknown tubes with samples from the bulls were run in duplicate and contained 50 µl extracted sample and 50 µl buffer.
Cortisol antibody was made up to a concentration of 1:4000, using Guildhay S020 (AssayPro, USA). Cortisone antibody, AB1299 (AssayPro, USA) diluted to 1:5000 in RIA buffer.
Tracers, [1,2,6,7-^{3}H]Cortisol and [1,2(n)-^{3}H]Cortisone (both GE Healthcare, UK), were diluted in RIA buffer until they reached a total of 10-11.000 counts.
After addition of 100 µl antiserum and 100 µl tracer, tubes were vortexed for 5 s and incubated at 4°C overnight.
The next day, the bound and unbound fractions were separated. 0.125 g Norit-A charcoal and 0.013g dextran were each prepared in 50 ml buffer without gelatine. When dissolved, they were pooled and stirred on ice for about 15 minutes. 0.5 ml was added to each assay tube and incubated on ice for 10 minutes, the tubes were centrifuged at 2500 rpm for 10 minutes at 4°C (Sorvall® Model RC 3B Plus, Kendro Laboratory Products) and the substrate was decanted into counting 6 ml polyethylene vials (PerkinElmer, USA). After addition of 1.5 ml IRGA-SAFE PLUS™ scintillant (PerkinElmer, USA), the vials were placed in the Liquid Scintillation Analyser (Model 1600 TR, Packard, AUS).
Counts measured by the Scintillation Analyser were converted to concentrations using the computer program AssayZap.

Environmental factors

From the arrival of the bulls until the end of the experiment, weather observations that were recorded every 30 minutes through the Bureau of Meteorology (Australian Government) at Archerfield Airport were collected from the internet. This weather station is situated 10 km in direct line from Pinjarra Hills. During the SI iButtons were placed in the yards, to register the local temperature every two minutes in order to compare local temperatures with the temperature measured in Archerfield.

Results

Scrotal Skin Temperature (SST)

There was a fluctuation in SST in both control and SI bulls during the study. In the control bulls the SST varied from 22.3 to 37.8°C, and in the SI bulls the SST varied from 32.0 to 39.2 °C, during the 48 h insulation period.

As shown in Figure 2, for all three SI bulls, the first two hours after onset of the SI, there was an increase in SST until the temperature had reached a plateau.. After two hours, the average SST was 37.6 ± 0.5°C in the SI bulls, compared with 34.5 ± 0.3°C in the control bulls during the following 24 h.

After about 26 h of insulation, the SST drop to an average of 36.2 ± 1.6°C in the SI bulls and 31.7 ± 1.7°C in the control bulls for the next 6.5 h. At the blood collection, 36 h after the onset of insulation, the iButton on one of the control bulls (#94) had lost contact and was removed. From around 32.5 h after onset of SI, a larger fluctuation occurred, with an average STT of 35.9 ± 1.2°C in the SI bulls and 30.7 ± 2.0°C in the control bulls.

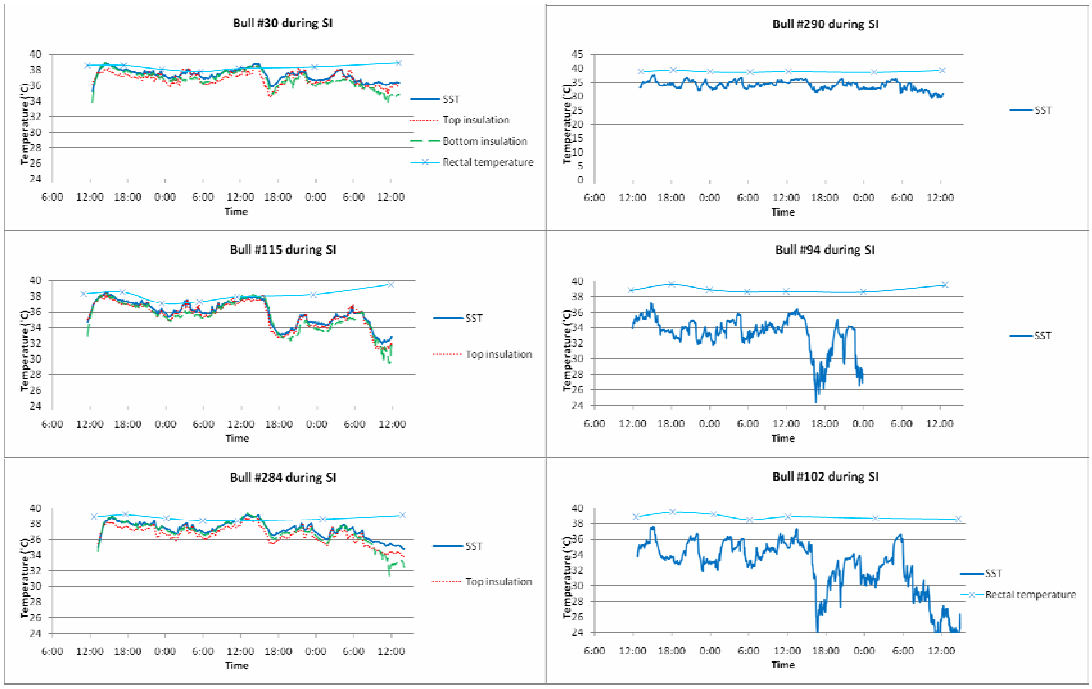

Figure 2: Scrotal Skin Temperature and rectal temperature during SI

The left column are bulls with insulated scrotum, the right column are control bulls. The matched pairs of bulls are on one line. Scrotal Insulation was performed for 48 hours from 12.00 day one. Scrotal Skin Temperature was measured every 2 minutes, rectal temperature every 6 to 12 hours.

Rectal temperature

The average rectal temperature from the SI bulls before SI was 38.8 ± 0.1ºC compared with an average of 39.0 ± 0.2ºC in the control bulls. As illustrated in Figure 3, at the onset of the SI the rectal temperatures for the SI and control animals were 38.6 ± 0.3ºC and 38.9 ± 0.1ºC, respectively. Six hours after the onset of SI, the mean rectal temperature of the SI bulls had increased to 38.8 ± 0.4ºC compared to 39.5 ± 0.1ºC in the control bulls. Twelve hours after the onset of the insulation, around midnight, the mean rectal temperature had dropped to an average of 38.0 ± 0.8ºC in the insulated and 39.0 ± 0.2ºC in the control animals. This drop continued to 37.8 ± 0.6ºC in the insulated and 38.6 ± 0.1ºC in the control animals at 18 h after the onset of SI. Then the temperatures started to increase to 38.2 ± 0.3ºC, 38.4 ± 0.2ºC and 39.2 ± 0.3ºC at 24, 36 and 48 h, respectively, after the start of the insulation in the SI animals.

The temperature in the control animals increased to 38.8 ± 0.1ºC, 38.7 ± 0.1ºC and 39.1 ± 0.5ºC at 24, 36 and 48 h, respectively.

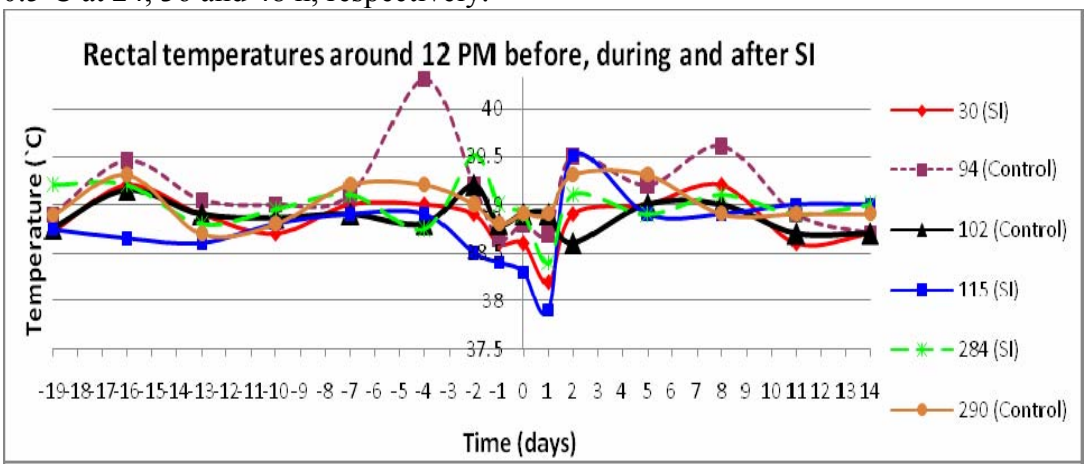

Figure 3: Rectal temperature before, during and after the SI period.
Rectal temperature was measured every third day around noon from 19 days before until 14 days after scrotal insulation in scrotal insulated (SI) and control bulls. Around the SI (day 0-2), rectal temperature was measured more frequent (day -2, -1, 0 and 1).

Semen

Before SI, the variation in sperm motility (%) was 60 to 90% (average 76.2%) over all bulls. Immediately after SI (48 h after onset), the sperm motility of the insulated bulls was decreased by 10 to 20%. The motility continued to decrease to 20-30% 14 d after SI. The average motility of the samples taken 2 d until 14 d after SI was 45.7% in the SI bulls, compared with 75.3% in the control bulls.
The mass activity (grade) showed a similar pattern; before SI the average grade in all the bulls was 3.2 while it decreased to an average of 1.7, 2.3 and 1.7 in the SI bulls 8, 11 and 14 d after insulation, respectively. The control bulls had an average of 3.0, 3.3 and 3.3 during the same period.

Corticosteroids

Blood

Blood samples 19, 10, 7, 4 and 1 d before and at the onset of SI were collected in 8 ml Vacuette® LH Lithium Heparin Sep tubes by tail bleeding. The samples taken at 6, 12, 18, 24, 36 and 48 h post initiation of SI were collected in 10 ml BD Vacutainer® LH tubes. Two of the samples 36 h after onset of SI (#284 and #94) were collected by jugular bleeding because the tail bleeding proved unsuccessful after repeated attempts. The other samples were all collected by tail bleeding.
Blood cortisol levels the morning after arrival were between 2.0 and 15.0 nmol/l (5.5 and 41.5 ng/ml), with an average of 6.8 ± 4.4 nmol/l. The the average blood cortisol concentration in the samples collected 10 days before SI, was 2.6 ± 1.9 nmol/l.
As shown in Figure 4, there were some fluctuations in cortisol levels during the insulation. During the SI the average cortisol level of the SI bulls was 3.7 ± 5.7 nmol/l compared to 1.8 ± 1.5 nmol/l in the control bulls, with some individual peaks during the SI period.
Figure 5 shows results from the samples collected at noon to avoid daily fluctuations. Three days after the SI was removed, the mean concentrations were 1.6 ± 0.8 nmol/l in the SI bulls and 1.6 ± 0.7 nmol/l in the control bulls. Five days after removal of SI, there was an increase in cortisol concentrations to 3.1 ± 2.8 nmol/l in the SI bulls and a decrease to 0.7 ± 0.7 nmol/l in the control bulls.

Blood cortisone levels fluctuated during the experiment as shown in Figure 6.
The day after arrival the averages were 0.4 ± 0.2 nmol/l but they dropped to an average of 0.2 ± 0.2 nmol/l in the days before onset of SI. During the insulation period, the average cortisone concentrations were 0.3 ± 0.2 nmol/l for the SI bulls and 0.2 ± 0.2 nmol/l for the control bulls.
After SI…. (continue)
As formerly mentioned for the cortisol concentrations, the cortisone concentrations could also be influenced by daily fluctuations. To exclude this fluctuations, Figure 7 shows cortisone concentrations from the samples taken at noon.
The cortisol/ cortisone ratio shows much variation as shown in Figure 8.
Most of the ratios were between 0.1 and 20, with peaks up to 108.6, especially in the SI period in either the SI as the control bulls.

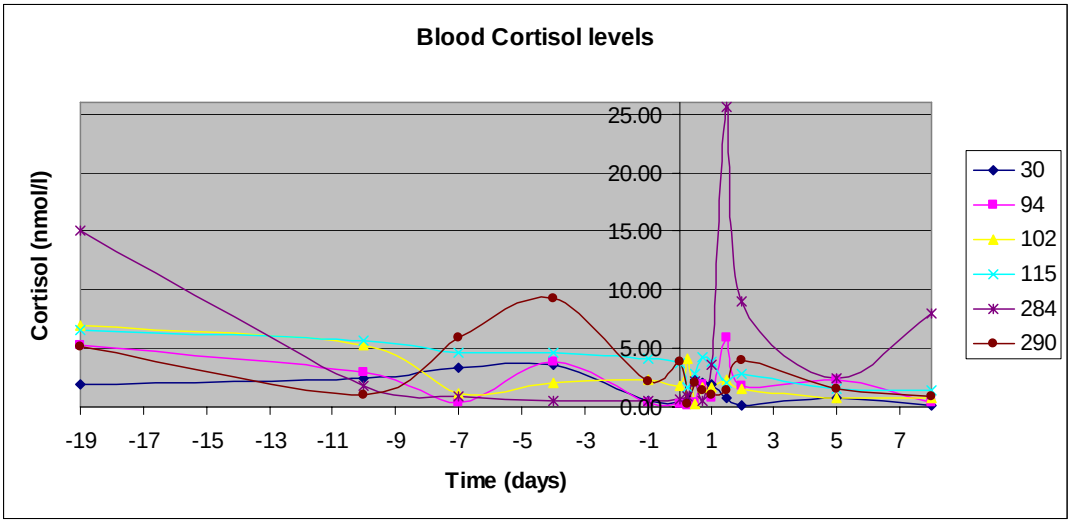

Figure 4: Blood cortisol concentrations before, during and after SI
Blood for cortisol measurement was collected the morning after arrival and then every third day around noon from 10 days before until 8 days after scrotal insulation in scrotal insulated (SI) and control bulls. (Bull #30, #115 and #284 were SI; bull #94, #102 and #290 were control.) Twenty-four hour before, at the onset (0) and 6, 12, 18, 24, 36 and 48 h post initiation of SI blood samples were collected more frequent.

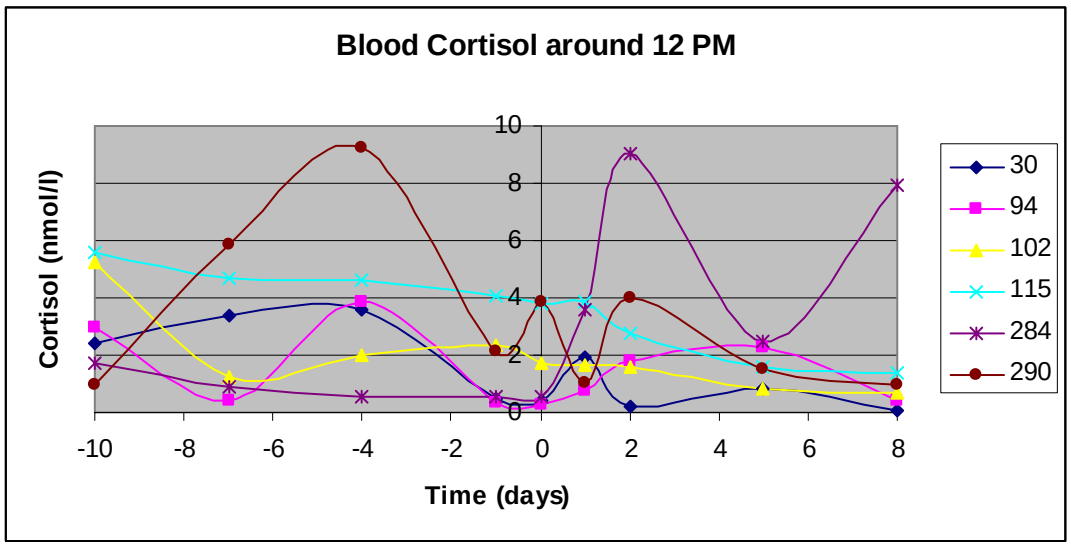

Figure 5: Blood cortisol concentrations around 12 PM before, during and after SI
By collecting blood samples every third day around noon, there is tried to overcome the problem of daily fluctuations.

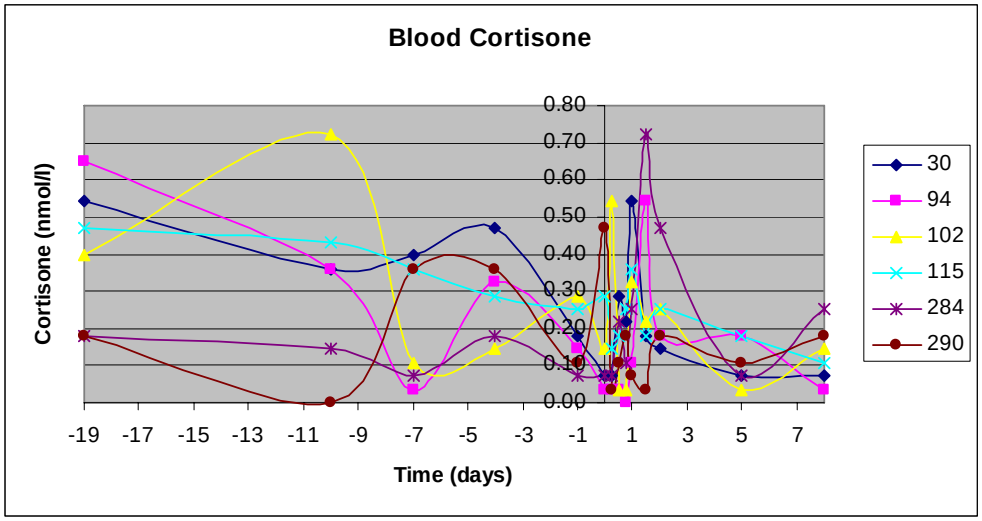

Figure 6: Blood cortisone concentrations before, during and after SI
Blood for cortisone measurement was collected the morning after arrival and then every third day around noon from 10 days before until 8 days after scrotal insulation in scrotal insulated (SI) and control bulls. (Bull #30, #115 and #284 were SI; bull #94, #102 and #290 were control.) Twenty-four hour before, at the onset (0) and 6, 12, 18, 24, 36 and 48 h post initiation of SI blood samples were collected more frequent.

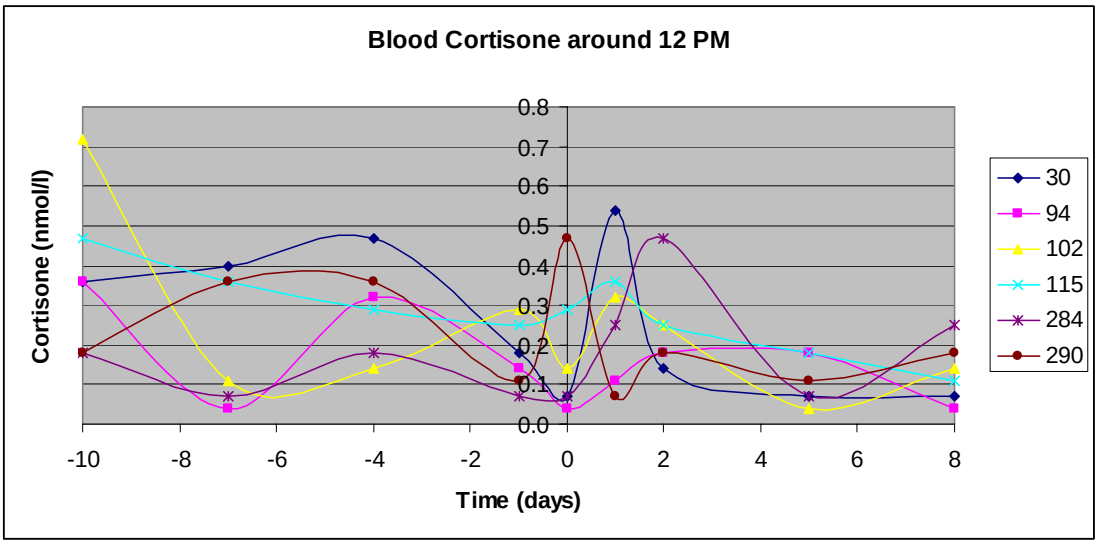

Figure 7: Blood cortisone concentrations around 12 PM before, during and after SI
By collecting blood samples every third day around noon, there is tried to overcome the problem of daily fluctuations.

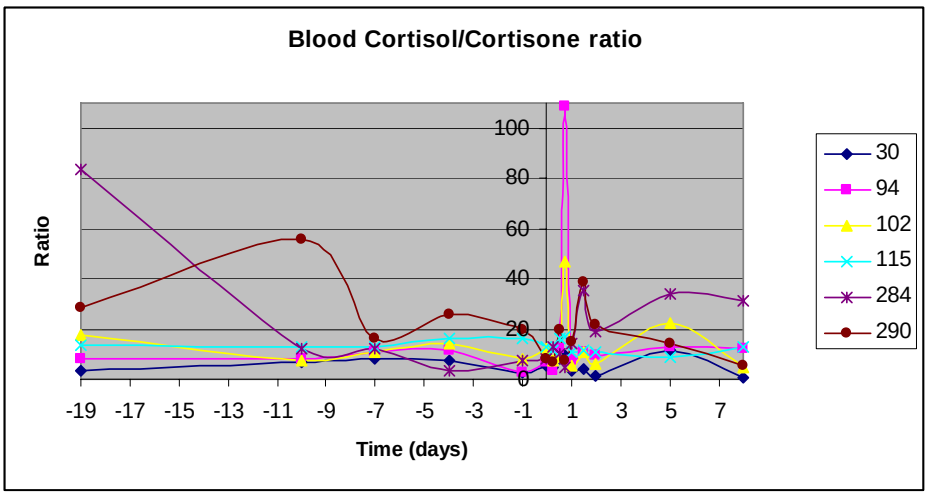

Figure 8: Blood cortisol/ cortisone ratio before, during and after SI
Blood for cortisol and cortisone measurement was collected the morning after arrival and then every third day around noon from 10 days before until 8 days after scrotal insulation in scrotal insulated (SI) and control bulls. (Bull #30, #115 and #284 were SI; bull #94, #102 and #290 were control.) Twenty-four hour before, at the onset (0) and 6, 12, 18, 24, 36 and 48 h post initiation of SI blood samples were collected more frequent.

Semen

As shown in Figure 9, cortisol concentrations in semen varied from 1.9 to 2.4 nmol/l. Average cortisol concentrations of all the samples before SI were 1.7 ± 0.4 nmol/l for the SI and 1.4 ± 0.3 nmol/l for the control bulls. The average concentrations of all samples collected after SI were 1.4 ± 0.3 nmol/l and 1.2 ± 0.4 nmol/l for the SI and control bulls, respectively.
The cortisone levels showed fluctuations between 0.2 and 0.8 nmol/l as illustrated in Figure 10. Average cortisone levels from all samples taken before SI were 0.42 ± 0.1 nmol/l for both the SI and the control bulls. The samples after SI gave cortisone concentrations of 0.36 ± 0.2 nmol/l for both groups.
As shown in Figure 11, the cortisol/cortisone ratio varied from 1.7 to 8.7. The average ratio before SI was 4.4 ± 1.5 in the SI bulls and 3.9 ± 1.8 nmol/l in the controls. After SI, the ratios were 4.4 ± 1.5 and 3.7 ± 1.1 nmol/l for SI and control bulls, respectively.

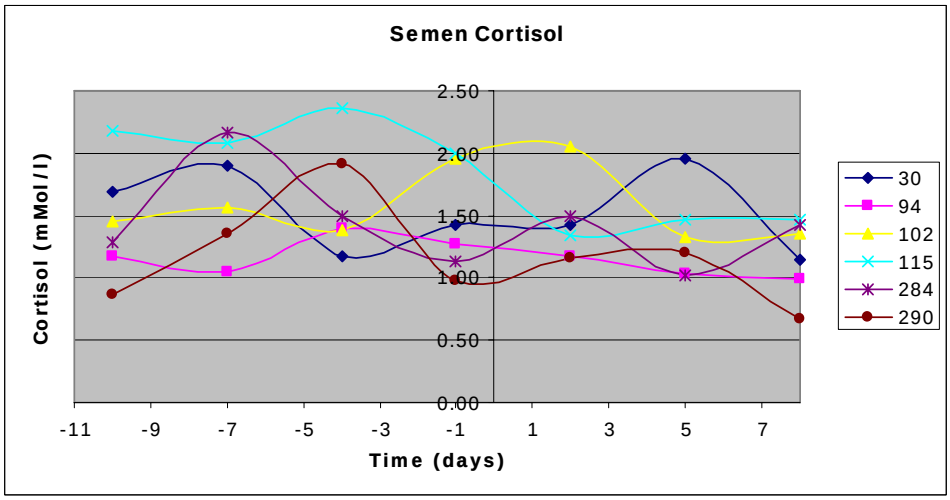

Figure 9: Semen cortisol concentrations before and after SI.
Semen for cortisol measurement was collected every third day around noon from 10 days before until 8 days after scrotal insulation in scrotal insulated (SI) and control bulls. (Bull #30, #115 and #284 were SI; bull #94, #102 and #290 were control.)

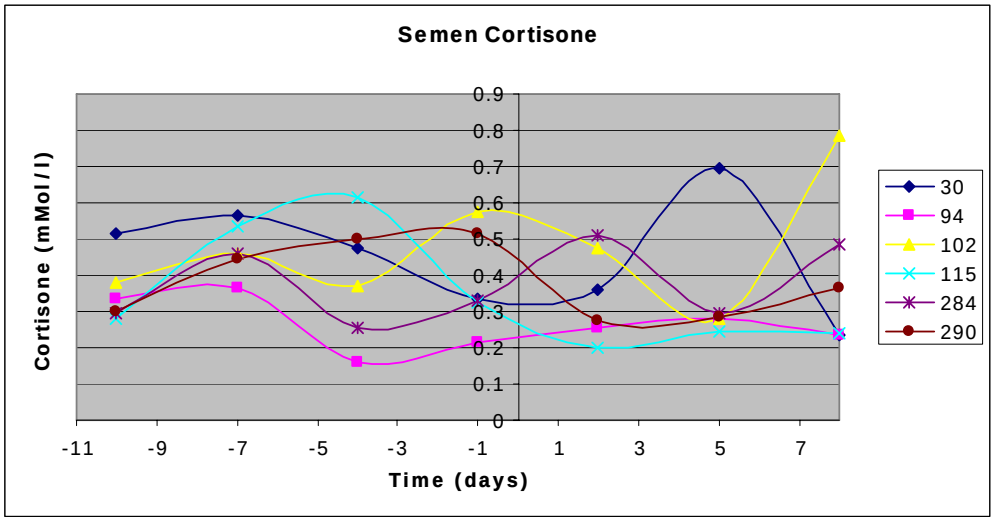

Figure 10: Semen cortisone concentrations before and after SI.
Semen for cortisone measurement was collected every third day around noon from 10 days before until 8 days after scrotal insulation in scrotal insulated (SI) and control bulls. (Bull #30, #115 and #284 were SI; bull #94, #102 and #290 were control.)

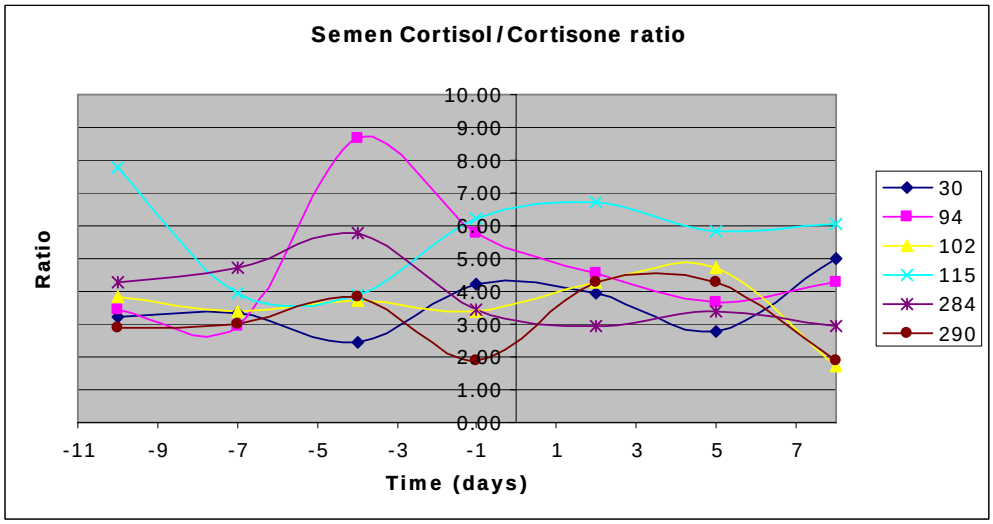

Figure 11: Semen cortisol/ cortisone ratio before and after SI.
Semen for cortisol and cortisone measurement was collected every third day around noon from 10 days before until 8 days after scrotal insulation in scrotal insulated (SI) and control bulls. (Bull #30, #115 and #284 were SI; bull #94, #102 and #290 were control.)

Environmental factors

Average weekly temperatures during the experiment ranged from 21.5 ºC to 23.0ºC
The first 24 h of insulation, the average temperature was 25.4ºC and reached 33.0ºC 25 h after the onset of SI at Archerfield Weather station. The local temperature in the yards measured by the iButtons was 38.0ºC at the same time.
Twenty-eight hours after the onset of SI a thunderstorm with 33 mm of rain in 6 hours was recorded. The temperature dropped to a minimum of 20.2ºC in Archerfield 35 h after the onset of SI and the temperature detected by the iButtons decreased to 20.7ºC at the same time. The next twelve hours, between 36 to 48 h of SI, were cooler with an average temperature of 18.6ºC measured at Archerfield station.

At the end of the insulation period (between 43 to 48 h after the onset of SI) the bulls escaped the yards. At 48 h the bulls were found running with a mixed herd: from calves to heifers around puberty and pregnant cows. The bulls were mustered and leaded into the crush, were the protocol was followed without any change.
At 8 days after the onset of SI, the bulls escaped their yards again. They were mustered and leaded into the crush as 6 days before.

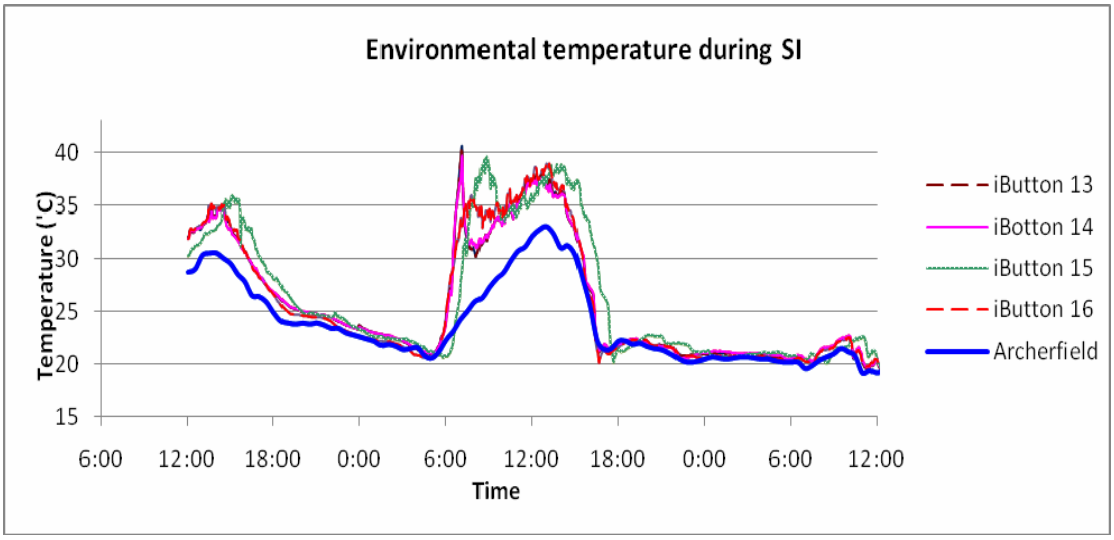

Figure 12: Environmental temperature during SI
Environmental Temperature during the 48 hours of Scrotal Insulation, starting 12.00 day one. Local iButton temperatures were measured every 2 minutes; 30 minute observations from Archerfield Weather Station were downloaded from the internet.

Discussion

Scrotal Skin Temperature (SST)

In the present study was choosen to measure the SST continuously during the insulation period. The reason for choosing this, was the fact that during a pilot study prior to this study with continuous SST measurement (Boe-Hansen, unpublished), it was found that in *Bos taurus* bulls, there was an average SST at the middle of the scrotum of 34.7°C without insulation with an average environmental temperature of 24.0°C. In this unpublished study, the SST reached an average of 37.3 °C during a 24 h SI period. This SST was measured with the same iButtons as used in current study with a mean environmental temperature of 19.9°C. The temperature graphs in the pilot study showed high variations in SST especially without SI, most of them influenced by bull behaviour especially the standing and laying patterns. The SST increased when the bulls were lying down and decreased when they were standing.
In previous published articles, scrotal temperatures were only measured immediately before and immediately after SI or changing environmental temperature. In one of these articles a SST of 28.8 ± 0.4°C after 48 h of insulation at the top of the scrotum was measured using infrared temperature thermograms (Kastelic et al., 1996). The same research group measured during a later study a top SST of 32.4°C ± 0.5°C at an environmental temperature of 25°C (Kastelic et al., 1997). Another experiment detected a scrotal surface temperature of 33.4 ± 0.8°C with a body temperature of 39.0 ± 0.2°C. The scrotal temperature was measured with a thermocouple into a fold of the scrotal skin in *Bos indicus* bulls; the environmental temperature was not described (Brito et al., 2004). Due to the extreme scrotal surface temperatures that were detected in the unpublished mentioned pilot study, which were not described in the literature, the decision to measure STT continuously was supported.

As shown in Figure 1, there was most of the time less than 0.5°C variation between the temperatures recorded through the iButtons placed on the skin compared with the ones in the insulation material. It was therefore chosen to only describe the temperatures recorded by the iButtons on the scrotal skins, because these were directly comparable with the SST measured in the control bulls. In current study, there was no support for the formerly described higher temperatures at the top of the scrotum compared with those at the bottom (Cook et al., 1994; Brito et al., 2004), because there were continuous changes in which of the three iButtons detected the highest temperature as shown in Figure 2.

As shown in Figure 2, immediately after the onset of SI, the SST started to increase. After about two hours the temperature had reached a plateau. This could be a result of not immediate activation of the thermoregulation mechanisms. The thermoregulation mechanisms were possibly activated when a certain temperature had been reached. This could be mechanisms that try to release heat from other parts of the body, to decrease the increased SST. This could be performed by heat release from sweat glands and a lower thermoregulation as previously described (Brito et al., 2004; Carvalho et al., 1995).
The SST of the SI bulls was much higher than the SST in the control bulls. The maximum difference in SST of the control bulls was 15.5°C, while these difference in the SI bulls was 7.2°C; this result showed that the insulation material worked as a good isolator and definitely gave an increase in SST.
After 26 h of SI, the graphs showed an immediate reaction of the thunderstorm on the SST. It seemed that the rain decreased the SST of the SI and the control bulls. The decrease in SST might followed a decrease in whole skin temperature because of the cooling water or could be a result of decreased isolation capacity of the SI material. The decreased isolation capacity of the insulation material could be a result of the water weakening the material. The decrease in SST was smaller in two of the bulls, one control and one SI bull. It is possible that these bulls were not standing or lying in the rain, but no behavioural observations were recorded during this period to support this.
After the rain, the SST started to increase; this could be due to drying of the body and testicular skin and the covering material. About 2 hours later, there was another drop in SST, this could be a result of the lower environmental temperature during the night as formerly described (Brito et al., 2004; Kastelic et al., 1996; 1997). In the morning, there was an increase in SST, possibly following the increase in environmental temperature. About 42 h after the onset of SI, the SST decreased again. There are no observations to support this behaviour, but probably the bulls escaped the yard around this timepoint and their SST decreased because of the increased movements of the scrotum.

The environmental temperatures measured with the iButtons during the day seemed to be a slightly higher than the temperatures measured at Archerfield Weather Station; this was probably because the iButtons were exposed to the direct sun and the weather station protected their sensors against this. iButtons 13 and 14 seemed to peak in the morning; these two iButtons were placed underneath a roof, the low morning sun did however reach them and did probably increase the temperature. All the iButtons reached higher temperatures in the afternoon than measured in Archerfield; this could be due to the fact that the material of the iButtons is more sensible for warming up than the materials used for temperature registration in Archerfield or that the temperature in Pinjarra Hills was higher than at the Weather Station.

Rectal temperature

When looking at the results, it looked that the SI bulls in this research were able to lower their rectal temperature while their SST started to increase. Unfortunately, there seemed to be no

literature reviews about the progression of the body temperature during SI. During the SI, the SST reached temperatures 0.1°C below the body temperature in two bulls 24 h after the onset of SI. These temperatures were outside the range earlier estimated to be necessary for normal spermatogenesis (Brito et al., 2004; Cook et al., 1994; Kastelic et al., 1996; 1997). Following the patterns illustrated in Figure 2, it seemed that the body temperature decreased shortly after the onset of SI while the SST increased; this could probably be due to a mechanism that tries to release heat from the testes (Brito et al., 2004; Cook et al., 1994; Kastelic et al., 1996). Probably heat loss by the skin surface and sweat glands have played a role in controlling the SST and not only the, during SI impaired, mechanisms of heat release by the scrotal surface and the testicular vascular cone.

The drop in body temperature during the night could be a result of the daily pattern (Piccione et al., 2003) or could follow the decreased environmental temperature. However, there is little literature available about the daily body temperature rhythm in cattle, Piccione *et al*. (2003) described that, the body temperature should peak at midnight. Results of the current study however show lower rectal temperatures in the evening in the SI bulls. Based on the fact that the rectal temperature is decreased at noon (12 h after onset of SI) compared with their rectal temperature before and after SI and the rectal temperature of the control bulls, it could suggested that there was a decrease in body temperature as result of the SI. There could be though about two reasons for the decreased rectal temperature during SI in these bulls: (1) a decrease in body temperature is a normal physiological response to SI, or (2) the decrease measured in this research could be due to a better thermoregulatory mechanism of the *Bos indicus* breed. This better thermoregulatory mechanism in the *Bos indicus* could be due to a greater skin surface to body size ratio, type and quantity of sweat glands, number of epithelial layers of the skin, lower thermogenesis and usually a smaller frame (Brito et al., 2004; Carvalho et al., 1995). To distinguish between these two, there should be done a research in which the progress of the rectal temperature between *Bos indicus* and *Bos Taurus* bulls during SI is compared.

Following the fact that there was no increase in rectal temperature during SI it appears that SI did not result in a general heat stress response in the bulls. The body temperature was not measured frequently enough to make a conclusion about what happened more than 18 h after the onset of SI. The rectal temperature seemed to increase slightly, but stayed below the rectal temperature of the control bulls until 36 h after the onset of SI. At the end of the SI, the rectal temperature was above the temperature of the control bulls and the temperature before SI. There could be made two hypotheses to support an increase in rectal temperature during SI; 1. The scrotal temperature is so high that the bull is not longer able to release enough heat to maintain his body temperature; or 2. There was a raise in body temperature caused by the increased body activity during the escaping, with no relation to the SST. This last hypothesis is supported by our present findings that the rectal temperature from two of the control bulls also increased during this period.

Together with the observation that not only the SST of the SI bulls, but also the SST of the control bulls decreased, might could make the conclusion that there should be some external factor that not only influenced the SST , but also the rectal temperature of all the bulls at this timepoint.

To make some definite conclusions about what happens with the body temperature during SI, the body temperature should be measured more frequent, maybe even continuously, during other SI studies. It would be preferable to minimize environmental stimuli to exclude external influences on the rectal temperature during SI.

Semen

Semen evaluation was not a point of main interest in this research, following the decrease in motility, there could be made the conclusion that there were damaging effects as a result of

scrotal insulation on spermatogenesis. Prolonged periods with a SST above 36°C obtained through SI result in an effect on spermatogenesis, which could be detected as early as 2 days after the termination of the SI. This confirms results found in earlier studies (Brito et al., 2004; Gwazdauskas, 1985; Cook et al., 1994; Kastelic et al., 1996).

Corticosteroids

The results showed that corticosteroids, and especially cortisol concentrations in the blood, were fluctuating between … and …. Nmol/l. These fluctuations were most visible during the SI period, but it is important to consider that cortisol excretion follow a daily pattern. Following literature, this daily pattern could give high fluctuations in cortisol concentrations around sunrise, but with less variation in the afternoon (Lefcourt et al., 1993; Welsh et al., 1979). By collecting blood samples at about the same time of day (12 PM) at each collection, there is tried to overcome this problem before and after the SI. Following this cortisol concentrations around noon as illustrated in Figure 5, it looks that the concentrations before and after SI didn't fluctuate a lot except from some individual differences.

To avoid missing an elevation in cortisol levels that could be performed through higher temperatures of the scrotum, there was a more frequent sampling during the SI. Former research has described that an elevation in cortisol levels starts within a few hours after the onset of hyperthermia, peak within a few hours and decline immediately the hyperthermia is stoped and resume basic levels within a few days (Christison and Johnson, 1972; Gwazdauskas, 1985). The more frequent sampling in the first 12 h of the SI was designed to enable us to detect the possible peak. The frequency of 6 hours was chosen following practical considerations, because the housing conditions of the bulls, including the bias of handling stress didn't allow a jugular catheter. Obtaining frequent blood samples from the tail vein could damage the tail and it took some time to do all the samplings. To overcome the problem of the daily pattern, it was decided to compare the samples taken at midnight with samples taken at midnight the next day, 36 h after onset of SI

There seems to be a slight increase in cortisol levels in the SI bulls between 6 and 12 h after the onset of SI, but it's uncertain if this was the result of the SI or the daily pattern, because also one of the control bulls had this increase and the other two control bulls had this slightly increase 6 h later.

When comparing the cortisol concentrations in blood samples taken at 12 and 36 h, there could made the suggestion that there is no significant increase in cortisol in the SI bulls between 12 and 36 h after onset of SI. The sampling of 18 h is not comparable with another sample, but following the daily pattern, the samples taken in the morning should have higher cortisol concentrations than the ones taken in the afternoon. This was the case in five of the bulls, hence this increase was probably a result of the daily pattern and not a result of the SI. As formerly described, cortisol levels can increase as a reaction of stress (Möstl and Palme, 2002). To make conclusions about what kind of stress gave the stress reaction in this research it is important to consider what handling could give a stress reaction in this bulls with a following increase in cortisol levels.. EE could influence the serum concentrations of glucocorticosteroids (Falk et al., 2001; Welsh and Johnson, 1981). To minimise this influence, the blood samples were taken before the EE and EE was performed in the same manner every time. Also the more frequent blood sampling around the SI, within 24 hours after EE, should not have contributed, because following earlier studies, increased corticosteroid levels due to EE should be declined to normal levels within 45 minutes and 4 hours after EE (Falk et al., 2001; Welsh and Johnson, 1981). This means that the influence of the EE on the serum corticosteroid levels in this research could be ignored. Another influence on the basal cortisol level is the temperament of the animal (Curley et al., 2008; Stahringer et al., 1990). Former

mentioned studies described that the basal cortisol levels of cattle depends on their reaction on handling. The reaction on handling was scored in three grades, from tame to wild. To avoid unacceptable differences in basal cortisol levels in this research, the bulls were selected based on comparable temperament prior to the initiation of the study. To exclude the influence of the handling procedure on the corticosteroid levels, control and SI bulls were handled the same throughout the study.
During the research, the bulls escaped their yards twice: at 2 d and at 8 d after onset of SI. The freedom and the fact that they were caught and brought back to the crush, could stress them and result in an increase in cortisol concentrations. Especially bull #284 had higher cortisol concentrations 2 d and 8 d after onset of SI concentrations. The increased cortisol concentration of bull #284 at 2 d could be due to the increased SST, but it could also be affected by the escape and subsequent increase in activity and perhaps stress. Bull #284 seemed to be the most dominant bulls in the group, and this might explained the higher cortisol response on acute stressors 19 d before and 2 and 8 d post SI in this bull (Solano et al., 2004). The higher cortisol concentrations of bull #290 at 7 and 4 d before SI, might be explained by the fact that this bull was incorporative in the crush. After ½ h struggling, on 7 d before SI it was decided to collect blood and semen in the race and on 4 d before SI, an electric cattle prodder was used to get him in the crush. The same prodder was used in bull #102 at 1 and 2 d before SI, but following this results, he had no increased cortisol, what gives the indication that the raise in cortisol isn't by definition caused by the use of the prodder itselves. A difference between these bulls could be that in bull #290, there was ½ h of struggling, while the prodder in bull #102 was used almost immediately to get the bull in the crush, but there could also be some variation between animals with respect to response to pain stimuli.
As shown in Figure 4 and 6, there were two peaks in cortisol and cortisone in the blood sampled 36 h after the onset of SI; one for bull #94 and one for bull #284. This could be due to the fact that this blood samples were collected by jugular bleeding because the tail bleeding proved unsuccessful after repeated attempts.
These results could indicate that external stress factors are more important for the increased cortisol levels than the increased SST following SI.

There are no publications available about cortisone measurement in blood of adult cattle. This means that there is not much known about the physiology or standard concentrations of this hormone. However there is no support for the suggestion that cortisone is excreted following a daily pattern, when looking at the results, with the many fluctuations in the more frequent sampling period that seems to follow the increases in cortisol levels, there might could made the suggestion that cortisone levels follow the same (daily) pattern as cortisol.

Figure 7, were the cortisone levels in blood samples taken around noon are illustrated, showed that there are some peaks in cortisone levels. The SI bulls #30 and #115 showed an increase in cortisone levels 1 d after onset of SI, but also control bull #102 showed this increase. Bull #284 showed an increase in cortisone 2 d after onset of SI. Because either one of the control and the SI bulls show this increase, there could be made no conclusion about if there is an influence of the SI on the cortisone levels.

The cortisone concentrations seem to follow the cortisol concentrations: when cortisol increases, cortisone also increases. This could be affected by the activity of the enzyme 11b-HSD, which converts the cortisol rapidly to cortisone.

The blood cortisol/cortisone ratio shows a few peaks, especially during periods with very high cortisol levels; this could indicate that when cortisol peaks, 11β-HSD was delayed activated to convert cortisol into the inactive cortisone.

Cortisol levels in the semen seemed to show fewer fluctuations than the levels in the blood. This could be due to the fact that the samples are all taken around 12 PM, so are buffered against possible daily fluctuations. The fact that all samples were collected around 12 PM, there could be made no suggestions whether semen cortisol concentrations follow a daily pattern or not as suggested in the introduction (Mayes, unpubliced). When comparing the semen cortisol with the blood cortisol concentrations at 12 PM, it is not possible to determine if semen cortisol followed blood cortisol as previously described (Graves and Eiler, 1979). Cortisone levels in the semen appear more stable than in the blood. Cortisone levels follow cortisol levels in the semen what could indicate a local 11β-HSD activity. This could be supported by the fact that the cortisol/ cortisone ratios showed not many fluctuations. However it seems that corticosteroid concentrations are more influenced by environmental factors than by the SI, it is not possible to make the conclusion that increased cortisol levels didn't influence the semen quality in this study. To make sure that the semen quality is only affected by elevated local temperature and not by the effect of glucocorticoids on the testosterone concentrations, future studies could be looked at the testosterone concentrations during SI, or environmental stress factors should be minimised. A decrease in environmental stress factors could be performed by minimizing differences in environmental temperatures, wind and the influence of rain by the use of climate chambers, and by making it impossible to escape by using adequate fencing. The effects of handling are already tried to minimize in this research by handling all bulls the same, but could be improved by making the animals even more easy to get them in the crush or by housing them under conditions that samples could be taken without moving them (reward in the crush or anchored in small space).

Conclusion

This study showed that, however SI increases the SST, there was neither an increase in body temperature nor in glucocorticoid concentrations in these bulls. Because there was no increase in glucocorticoids following SI and the decrease in semen quality was very clear, the conclusion could be made that not corticosteroids, but the elevated local temperature plays the most important role in deterioration of semen quality during SI.

Acknowledgements

Financial support was provided by the University of Queensland New Staff Research Start-Up Fund (NSRSF) with Gry Boe-Hansen as chief investigator. Appreciation is expressed to T. Connelly, farm manager at Pinjarra Hills and R. Young, farm manager at Belmont Research Station.

References

Boe-Hansen, G. (2008) Personal communication, University of Queensland, Brisbane, Australia.

Brito, L.F.C., Silva, A.E.D.F., Barbose, R.T. and Kastelic, J.P. (2004) Testicular thermoregulation in *Bos indicus*, crossbred and *Bos taurus* bulls: relationship with scrotal, testicular vascular cone and testicular morphology, and effects on semen quality and sperm production *Theriogenology* **61**; 511-528.

Carvalho, F.A., Lammoglia, M.A., Simoes, M.J. and Randel, R.D. (1995) Breed affects thermoregulation and epithelial morphology in imported and native cattle subjected to heat stress. *Journal of Animal Science* **73**; 3570-3573.

Christison, G.I. and Johnson, H.D. (1972) Cortisol Turnover in Heat-Stressed Cows. *Journal of Animal Science* **35**; 1005-1010.

Cook, R.B., Coulter, G.H. and Kastelic, J.P. (1994) The testicular vascular cone, scrotal thermoregulation, and their relationship to sperm production and seminal quality in beef bulls. *Theriogenology* **41;** 653-671.

Curley Jr., K.O., Neuendorff, D.A., Lewis, A.W., Cleere J.J., Welsh Jr., T.H. and Randel, R.D. (2008) Functional characteristics of the bovine hypothalamic–pituitary–adrenal axis vary with temperament. *Hormones and Behavior* **53**; 20-27.

Entwistle, K. and Fordyce, G. (2003) **Evaluating and Reporting Bull Fertility**, published by the Australian Association of Cattle Veterinarians, Indooroopilly, Australia.

Falk, A.J., Waldner, C.L., Cotter, B.S., Gudmundson, J. and Barth, A.D. (2001) Effects of epidural lidocaine anesthesia on bulls during electroejaculation. *Canadian Veterinary Journal* **42**; 116-120.

Graves, C.N. and Eiler, H. (1979) Cortisol content of semen and the effect of exogenous cortisol on the concentration of cortisol and minerals (Ca, Mg, K and Na) in semen and blood plasma of bulls. *Biology of Reproduction* **21**; 1225-1229.

Gwazdauskas, F.C. (1985) Effects of Climate on Reproduction in Cattle. *Journal of Dairy Science* **68**; 1568-1578.

Hardy, M.P., Gao, H., Dong, Q., Ge, R., Wang, Q., Chai, W.R., Feng, X. and Sottas, C. (2005) Stress hormone and male reproductive function. *Cell Tissue Res* **322**; 147–153.

Kastelic, J.P., Cook, R.B., Coulter, G.H. and Saacke, R.G. (1996) Insulating the scrotal neck affects semen quality and scrotal/ testicular temperatures in the bull. *Theriogenology* **45**; 935-942.

Kastelic, J.P., Cook, R.B. and Coulter, G.H. (1997) Contribution of the scrotum, testes, and testicular artery to scrotal/testicular thermoregulation in bulls at two ambient temperatures. *Animal Reproduction Science* **45**; 255-261.

Lefcourt, A.M., Bitman, J., Kahl, S. and Wood, D.L. (1993) Circadian and Ultradian Rhythms of Peripheral Cortisol Concentrations in Lactating Dairy Cows. *Journal of Dairy Science* **76**; 2607-2612.

Mayes, J. (2008) Personal communication, University of Queensland, Brisbane, Australia.

Michael, A.E. and Cooke, B.A. (1994) A working hypothesis for the regulation of steroidogenesis and germ cell development in the gonads by glucocorticoids and 11β-hydroxysteroid dehydrogenase (11βHSD). *Molecular and Cellular Endocrinology* **100**; 55-63.

Morris, D.J., Brem, A.S., Ge, R., Jellinck, H., Sakai, R.R. and Hardy, M.P. (2003) The functional roles of 11b-HSD1: vascular tissue, testis and brain. *Molecular and Cellular Endocrinology* **203**; 1-12.

Möstl, E. and Palme, R. (2002) Hormones as indicators of stress. *Domestic Animal Endocrinology* **23**; 67–74.

Piccione, G., Caola, G. and Refinetti, R. (2003) Daily and estrous rhythmicity of body temperature in domestic cattle. *BMC Physiology* 3: 7.

Solano, J., Galindo, F., Orihuela, A. and Galina, C.S. (2004) The effect of social rank on the physiological response during repeated stressful handling in Zebu cattle (Bos indicus). *Physiology & Behavior* **82**; 679–683

Stahringer, R.C., Randel, R.D. and Neuendorff, D.A. (1990) Effects of naloxone and animal temperament on serum luteinizing hormone and cortisol concentrations in seasonally anestrous Brahman heifers. *Theriogenology* **34**; 393–406.

Welsh, T.H., Randel, R.D. and Johnson, B.H. (1979) Temporal relationships among peripheral blood concentrations of corticosteroids, luteinising hormone and testosterone in bulls. *Theriogenology* **12**; 169-179.

Welsh Jr, T.H. and Johnson, B.H. (1981) Influence of electroejaculation on peripheral blood concentrations of corticosteroids, progesterone, LH and testosterone in bulls. *Archives Andrology* **7**; 245-250.