final report

Project code: B.AWW.0219

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Date published: 30 July 2016

PUBLISHED BY
Meat and Livestock Australia Limited
Locked Bag 1961
NORTH SYDNEY NSW 2059

Chemical sterilisation as an alternative to spaying of heifers

Meat & Livestock Australia acknowledges the matching funds provided by the Australian Government to support the research and development detailed in this publication.

Abstract

Alternatives to surgical sterilisation of female cattle may help improve animal welfare outcomes. The aim of this study was to investigate if administration of potential chemosterilants by transvaginal, ultrasound guided intraovarian injection offer an alternative to surgical sterilisation. *Bos indicus* heifers were treated with intraovarian injections of either saline (n = 10), $CaCl_2$ (n = 10), zinc gluconate (ZG; n = 10), or a combination of $CaCl_2$ and ZG (Ca + ZG; n = 10) and then exposed to a bull from 82 to 84 days after treatment. After treatment total ovarian mass at slaughter was less in heifers treated with $CaCL_2$ compared to the other treatments ($P<0.05$) but concentrations of haptoglobin, anti-Müllerian hormone and total oocyte counts did not differ ($P>0.150$). Pain responses were observed in heifers treated with ZG and ZG and $CaCl_2$ but not in heifers treated with saline or $CaCl_2$. Complete regression of one ovary was observed in 40% (4/10) of the heifers treated with $CaCl_2$. Pregnancies were recorded in ≥ 70% of heifers administered each treatment. Results suggest that the treatments used were not able to sterilise most *Bos indicus* heifers but treatment with $CaCl_2$ has the potential to cause complete ovarian atrophy without causing detectable pain.

Executive summary

Background

Speying of female cattle is used as a method of surgically sterilising female cattle that are surplus to breeding requirements. It is an important management tool that is used particularly on extensively managed beef cattle properties in northern Australia to improve economic returns and reduce mortality among female cattle that are surplus to breeding requirements. It helps prevent unwanted pregnancies and to manage stocking rates. Current techniques used to spay cattle are known to cause pain and can result in mortalities in a small percentage of cattle. Development of alternative methods of sterilisation may improve welfare outcomes. The aim of this study was to investigate the effectiveness of three potential methods of chemical sterilisation of *Bos indicus* heifers as an alternative to surgical sterilisation.

Experimental methods

Heifers were treated with intraovarian injections of either saline (n = 10, 3 mL), $CaCl_2$ (n = 10; 3 mL of a 20% solution in 95% ethanol), zinc gluconate (ZG; 3 mL, n = 2 or 1.5 mL, n = 8), or a combination of $CaCl_2$ and zinc gluconate ($CaCl_2$ + ZG; n = 10; 1.5 mL). Treatments were administered using ultrasound-guided, transvaginal needle puncture after administration of epidural anaesthesia using lignocaine. The volume of ZG administered was initially 3 mL in Replicate 1 but was reduced to 1.5 mL in subsequent replicates when pain responses were observed in association with treatments. Meloxicam (150 mg sc) was administered for pain relief within 30 min of treatment in heifers treated with ZG or ZG + $CaCl_2$.

Treatments were administered in 5 replicates, with each replicate containing 2 heifers from each treatment. Oestrous cycles were first synchronised by inserting an intravaginal progesterone releasing insert (CIDR-B) concurrently with injection of oestradiol benzoate (0.7 mg IM). Inserts were removed 8 days later and eCG (400 IU IM) and cloprostenol (0.5 mg IM) were administered. Oestradiol benzoate (0.7 mg IM) was administered 24 h later. Heifers were treated either 11 (Replicates 1 and 3) or 13 (Replicates 2, 4 and 5) days after removal of inserts. Heart rates of heifers were monitored on two occasions in the 7 days before treatment, within 4 h after treatment and again 24 h later. The potential aversion of heifers to enter the treatment area was measured by assessing the duration of time heifers took to enter the treatment area before intraovarian treatments were given and again 24 and 48 h after treatments. The behaviour of heifers were video recorded for up to 48 h after treatment. Behavioural responses following treatment were assessed by recording behaviours observed during a 90 minute observation period commencing 3 to 5 h after treatment and again 24 to 28 h after treatment. Blood samples were collected to measure changes in plasma concentrations of acute phase proteins fibrinogen and haptoglobin and the reproductive hormones anti-Müllerian hormone and progesterone. Heifers were placed with a bull 82 to 84 days after treatment and the bull was removed between 230 to 246 days after the start of treatment, depending on replicate. Heifers were slaughtered between 364 and 396 days after treatment, ovaries were recovered, weighed and the number of oocytes remaining in ovaries were estimated using histological examination. Pregnancy was diagnosed using transrectal ultrasonography at repeat intervals of about 4 to 6 weeks during

the mating period. When pregnancies were diagnosed, pregnancies were terminated by administration of cloprostenol (0.5 mg IM).

Results

Abnormal behaviours at the time of administering treatments and aversion to entering the treatment area were not detected suggesting that heifers initially tolerated treatments well. Between Days 0 and 7 days after treatment no significant differences between treatments were found in mean leukocyte counts and concentrations of haptoglobin. Mean concentrations of fibrinogen were greater in the heifers treated with both $CaCl_2$ and ZG compared to heifers treated with saline and $CaCl_2$ ($P < 0.05$). Mean heart rates did not differ significantly between treatments. A greater percentage of heifers treated with ZG compared to the saline treated heifers demonstrated abnormal lying behaviour, foot stamping and or kicking at the belly, more recumbent episodes and a shorter duration of recumbent episodes on the day of treatment which likely indicated agitation and the sensation of pain in heifers treated with ZG. Pain responses were not observed in any heifer 24 to 28 h after treatment as assessed by our failure to observe abnormal lying, foot stamping and kicking at the belly.

Survival curves for the intervals to detection of ovulation and conception after treatment did not differ between treatments ($P>0.45$). Pregnancies were recorded in ≥ 70% of heifers administered each treatment. Total ovarian mass at slaughter was significantly less in heifers treated with $CaCl_2$ compared to heifers in the other treatment groups ($P = 0.019$). In 40% (8/20) ovaries treated with $CaCl_2$ alone, ovarian weights at slaughter were <1 g while complete ovarian atrophy was found in 40% (4/10) of heifers treated with $CaCl_2$ alone. No significant differences in the total oocyte count or concentrations of AMH were found between treatments.

Results suggest that the treatments administered were not able to sterilise most *Bos indicus* heifers while pain responses were evident in animals treated with ZG and ZG and $CaCl_2$ but not $CaCl_2$ in ethanol. Complete atrophy of some ovaries treated with $CaCL_2$ in ethanol suggests that this treatment may have potential to sterilise heifers but further study is needed to determine if modification of the method of administration or dose administered can consistently cause ovarian atrophy. Treatment with ZG and the combination of ZG and $CaCL_2$ do not appear to offer potential as chemosterilants due to side effects of pain and failure to cause sterilisation or any significant reduction in total ovarian mass.

Implications

Transvaginal, ultrasound-guided administration of $CaCL_2$ in ethanol may potentially act as a chemosterilant without detectable pain responses in heifers provided that more consistent responses to treatment can be obtained.

Table of Contents

1 Background

Spaying of female cattle is used within the Northern Australian beef cattle industry to prevent unwanted pregnancies and improve economic returns from females that are surplus to breeding requirements (Niethe and Holmes 2008; McCosker *et al.* 2010). Sterilising some females can reduce mortalities of breeding cows, particularly where shortfalls in the available nutrition during pregnancy or lactation may result in severe energy deficits or increase the risk of dystocia (Pinner 2006). It can also help control stocking rates and improve sustainable land management (Jubb and Letchford 1997). Spaying of females is undertaken using either a flank approach, where ovaries are removed through the paralumbar fossa or a transvaginal approach, by which ovaries are removed by colpotomy using the dropped ovary technique (De Witte *et al.* 2006). Both methods are known to cause pain in cattle although the extent of pain responses can vary between the methods used (Petherick *et al.* 2011, 2013). Death rates with these methods of spaying vary between 0% and 5% (Habermehl 1993; Jubb *et al.* 2003; Pinner 2006; McCosker *et al.* 2010) but in spite of these disadvantages spaying of female cattle is widely used in northern Australia due to its effectiveness in sterilising cattle following a single intervention, relatively low cost and speed at which cattle can be processed. There is increasing community concern about welfare implications of some animal husbandry procedures (Petherick 2005) which may increase community demands for the adoption of other sterilisation techniques which have less of an adverse effect on animal welfare. Investigation into alternative means of sterilisation of female cattle could provide alternatives to spaying and provide better welfare outcomes for animals.

Chemical sterilization by intra-testicular injection of chemical agents has been attempted as an alternative to surgical castration in a variety of domestic and laboratory animal species (Kutzler and Wood 2006; Kutzler 2015). In males treatments have aimed at permanently arresting spermatogenesis, androgenesis or blocking the passage of spermatozoa during ejaculation (Bowen 2008). Effects following intraovarian injection of cattle have not been reported to the author's knowledge. Two potential chemosterilants that have been widely studied in male animals include zinc gluconate (ZG) or acetate and calcium chloride ($CaCl_2$). Zinc gluconate neutralized by arginine has been available for commercial use in dogs as an intratesticular sterilant (Oliveira *et al.* 2007; Soto *et al.* 2009) with side effects of pain and injection site reactions being rare (USFDA 1993; Levy *et al.* 2008). For example, injection site reactions in dogs requiring treatment were evident in <4% of cases (USFDA 1993; Levy *et al.* 2008). Zinc has also been effective in causing a significant reduction in the weight of the prostate gland when injected intraprostatically in rats indicating that it may be effective in reducing the size and function of more than one type of reproductive organ (Fahim *et al.* 1993). Calcium chloride in an aqueous or alcohol base has been used in a variety of species of male domestic animals in an attempt to induce sterilisation with variable results being obtained and with little in the way of overt signs of pain (Koger 1978; Ali *et al.* 1991; Jana *et al.* 2005; Jana and Samanta 2006, 2007; Baran *et al.* 2010; Jana and Samanta 2011; Ibrahim *et al.* 2016). No studies have, however, reported the effects of intraovarian administration of zinc containing compounds or $CaCl_2$ administered into the ovaries of cattle. Zinc or $CaCl_2$ when injected in small quantities into meat producing animals should also not present any harmful tissue residues and be low in cost. These substances could, therefore, offer the possibility of being a single use chemical sterilant in cattle and offer an alternative to surgical spaying.

Transvaginal ultrasound guided, ovarian needle puncture is a technique that is widely used and easily undertaken in cattle for the purposes of harvesting oocytes for use with in vitro fertilisation (Gibbons *et al.* 1994). This technique could be used to administer any potential intraovarian sterilant in a minimally invasive manner. It could offer a means of reducing mortalities and improving welfare outcomes compared with currently used speying techniques which induce some pain and livestock losses (Pinner 2006).

The aim of this study was to investigate the effectiveness of three potential chemosterilants to sterilise *Bos indicus* heifers after intraovarian administration. The hypothesis was that intraovarian administration of ZG, $CaCl_2$ in ethanol or the combination of both substances would induce sterilisation of *Bos indicus* cattle without having adverse effects on animal welfare or production.

2 Project objectives

The objectives of this project were:

1. To determine effectiveness of chemical sterilisation treatments on reproductive physiology, fertility and liveweights of female cattle.
2. To complete evaluation of histopathology and hormonal changes in female cattle.

3 Methodology

3.1 Approvals

The experimental procedures were approved by the James Cook University Animal Ethics Committee (Approval number: A1855).

3.2 Animals

Brahman heifers, 23 to 26 months of age (n=40) were obtained for the study from the James Cook University Tropical Veterinary Research Station, Fletcherview (latitude 19°53'4"S; longitude 146°10'43"E) located in the dry tropics, of Northern Queensland. Heifers were transported to, and maintained at the James Cook University campus (latitude 19^{o}19’37’’S, longitude 146^{o}45’27”E) for at least 3 weeks prior to commencing treatments and at least 2 weeks after completing treatments. Heifers were divided into 5 replicates of 8 animals, with 2 animals/treatment within each replicate (n = 10/treatment).

While at the James Cook University campus heifers were fed on a diet that consisted of ad libitum access to pasture hay, molasses supplemented with urea (8% by weight of urea) and whole cotton seed (1.0 kg/head/day). After completing treatments animals were returned to graze on mixtures of native perennial, native legume and exotic improved pasture at the Department of Agriculture, Fisheries and Forestry Swans Lagoon Research Station (latitude 20^{o}4’44’’ S, longitude 147^{o}13’27’’ E) for a period of 6 weeks before being returned to Fletcherview (Replicates 1 and 2) or to Fletcherview (Replicates 3 to 5) where they remained until the end of the study. Animals were assessed prior to commencing the study and found to be non-pregnant with a normal reproductive tract as determined using trans-rectal palpation and ultrasonography (Mylab 5; Medical Plus Australia Pty Ltd, Tullamarine, Vic).

3.3 Treatments

Heifers had their oestrous cycles synchronised by administration of an intravaginal progesterone releasing insert (CIDR®, Zoetis Australia, West Ryde, NSW) and administration of oestradiol benzoate (1 mg/500 kg IM, Cidirol, Genetics Australia, Bacchus Marsh, Vic) at the time of device insertion. Eight days later inserts were removed and heifers were treated with 0.5 mg IM of cloprostenol (Estroplan, Parnell Laboratories, Mascot NSW) and 400 IU SC of equine chorionic gonadotrophin (eCG; Folligon, Intervet Australia, Bendigo Vic). Twenty four hours later oestradiol benzoate (1 mg/500 kg IM) was administered to all heifers to induce a synchronised oestrus (Cavalieri *et al.* 2002). Heifers were then administered control and potential chemosterilants either 11 (Replicates 1, 3) or 13 days (Replicates 2, 4 and 5) after removing inserts. At the time of treatment heifers were restrained within a squeeze crush (Interrogator Pro-Chute; Leicht's, Goombungee, Qld) to restrict lateral movement and subjected to epidural anaesthesia by administering 3 to 4 mL of 2% lignocaine (Lignocaine 20, Ileum Veterinary Products, Smithfield, NSW) into the sacrococcygeal space.

Intraovarian injections of control and potential chemosterilants were then administered by transvaginal ultrasound-guided injection using a 7.5 MHz, micro-convex linear ultrasound probe (MyLab 5, MedicalPlus Australia Pty Ltd, Tullamarine, Vic) and a 19 g needle.

Treatments consisted of:

- Saline: 0.9% saline solution.
- $CaCl_2$: $CaCl_2 \cdot 2H_2O$ powder (10 g, Product number: C3306; Sigma Aldrich Corporation, Sydney) dissolved in 95% ethanol and made up to a volume of 50 mL (a 20% solution).
- ZG: 6.85 g of ZG (Catalogue number: 222820; MP Biomedicals Australia Pty Ltd, Seven Hills, NSW) dissolved in an aqueous solution containing 0.6 M L-arginine (product number: A8094, Sigma Aldrich Corporation, Sydney, NSW) buffer which was continually added to the dissolved solution until a pH of 7 was reached. The final volume was then adjusted with water to 50 mL, giving 19.18 mg/mL of zinc.
- Ca + ZG: $CaCl_2$ (10 g) plus ZG (6.85 g) dissolved in an aqueous solution containing arginine as for ZG (n = 10) with the solution adjusted to a pH of 7 and then the volume adjusted to 50 mL with water. Each solution was filtered through 20 µ filters and then stored ready for use.

A total of two animals from each group were enrolled in each replicate with the dates of intraovarian administration of treatments listed in Table 1.

Table 1. Date of administering intraovarian injections and heifer weights for each experimental replicate.

Replicate	Date	n	Weight (± SEM; kg)
1	9/12/13	8	285.4 ± 5.8
2	11/12/13	8	291.9 ± 6.9
3	17/2/14	8	295.4 ± 4.3
4	19/2/14	8	288.9 ± 6.9
5	3/4/14	8	315.3 ± 7.1

The volume of fluid administered into each ovary for the Saline and $CaCl_2$ treatments was 3 mL for all 5 replicates. The volume of ZG and the combination of $CaCl_2$ and ZG was 3 mL for Replicate 1 but was reduced to 1.5 mL per ovary for Replicates 2 to 5 when heifers treated in Replicate 1 showed signs of colic and intermittent recumbency commencing within 10 minutes of treatment. In response to these signs of pain in heifers treated with ZG or ZG plus $CaCl_2$ they were administered meloxicam (150 mg sc Metacam, Boehringer Ingelheim Pty Ltd, North Ryde, NSW) within 30 min of treatment (Replicate 1) or at the time of treatment (Replicates 2 to 5).

3.4 Blood sampling

Blood samples were collected from the coccygeal vein or artery on the day of administering intraovarian treatments (Day 0), and on Days 2 and 7 following treatment. Depending on replicate, from 39 days to 49 days after treatment on Day 0 samples were collected at repeat intervals of approximately 4 and 6 weeks throughout the remainder of the study for each replicate (Table A1).

From Days 0 to 7 blood samples were collected into plain evacuated tubes and tubes containing EDTA (Vacuetter® K2EDTA, Thermofisher, Scoresby Vic) and sodium citrate (BD Vacutainer®, BDM363095, Thermofisher, Scoresby Vic). All subsequent samples were collected into plain evacuated tubes (BD Vacutainer, BDM367895, Thermofisher, Scoresby Vic) for collection of serum. Blood collected into plain tubes was first allowed to clot before serum was separated after centrifugation. Serum was then stored frozen at -20^{o}C until the time of assay. Samples collected into tubes containing anticoagulants were stored at 4^{o}C until processing for haematology (EDTA) and fibrinogen (sodium citrate).

Concentrations of haptoglobin were determined in blood samples collected on Days 0 and 7 after treatment and concentrations of anti-Müllerian hormone (AMH) were determined in heifers between 215 and 222 days after treatment. Concentrations of progesterone in serum were determined using a radioimmunoassay kit (IBL Progesterone RIA, Abacus ALS, East Brisbane). The minimum detectable limit of the assay was 0.10 ng/mL and the intra and inter assay coefficients of variations for plasma pools of 1.0, 5.4 and 9.1 ng/mL were respectively, 10.2% and 11.0%; 13.2 and 11.0%, and 11.9 and 12.3%. Serum concentrations of

progesterone >1.5 ng/mL were used to indicate that ovulation had occurred (Oyedipe *et al.* 1986). Concentrations of AMH were determined using an ELISA kit (AMH Gen II ELISA two site immunoassay, Beckman Coulter). The limit of detection of the assay was 0.57 pmol/L. The ratios for observed to expected values for dilution parallelism with the standard curve for the assay was assessed using three serial dilutions (80%, 60%, 40%, 20%) of serum samples collected from intact heifers. The mean ± SEM observed/expected ratios (efficacy) were 101.5% ± 1.1. Concentration of AMH in serum from a steer were also found to be below the detection limit of the assay (0.05 pmol/L). Concentrations of haptoglobin in serum were determined using a quantitative colourmetric assay (Tridelta PHASE Haptoglobin Assay, cat. no. TP-801) on a Beckman Coulter AU680; Eckersall *et al.* (1999). The sensitivity of the assay was 0.005 mg/mL.

3.5 Aversion testing

Aversion of heifers to the treatment area was assessed by timing the entry of heifers to the treatment area. Heifers were allowed to enter a walk race that preceded the treatment area and restrained in the race 2 m behind the treatment area using a closed sliding gate. Aversion to entering the treatment area was assessed by measuring the interval of time between opening the sliding gate and when all 4 feet of heifers were located within the treatment area and by assessing the degree of assistance applied to enter the treatment area. Aversion testing was undertaken 5 and 7 days before applying treatments, on the day of treatment and 1 and 2 days after completing treatments. During aversion testing animals were assigned a score based on the degree of assistance applied to enter the treatment area (Table 2). Animals were accustomed to the race and treatment area by walking them through the facilities without applying any treatments on at least 10 occasions in the 2 weeks before intraovarian treatments were given. Treatments to synchronise oestrous cycles were conducted in a separate facility to the one used to apply intraovarian treatments.

Table 2. Behavioural score associated behaviour with pressure required to enter the treatment area.

Behavioural score	Description of pressure applied to initiate entry
1	No stimulus or force applied
2	If the animal did not move for 10 sec then voice and back slap at 1-sec intervals for 10 sec was applied until movement was initiated
3	If the animal did not move for 10 sec, voice and 1 inch PVC tube slap for 10 sec
4	Tail twist
5	Whatever reasonable force was necessary to move the animal into the crush

At the time of administering intraovarian treatments behaviour was assessed according to criteria outlined in Table 3.

Table 3. Criteria used to assess behaviour at the time of intraovarian administration of treatments.

Behaviour
No visible reaction
Vocalisation - audible bellow
Flank kicking, striking abdomen with hind foot
Mild struggling – moving forwards and backwards once only
Moderate struggling - struggling with movement of hind and front limbs, moving forward and backwards more than once.
Massive struggling - massive struggling that involved the whole body
Trembling - visible twitching and shaking of body.
Recumbency: front legs only
Recumbency: sternal
Kicking hind legs - Kicking backing gate during infusion or within 1 minute of infusion
Head turning
Head shaking
Jumping - lifting head and raising 2 front feet off the ground
Other

3.6 Heart rate monitoring and video recording of behaviour

Heifers were acclimatised to individual pens for restraint on two separate days before commencing heart rate monitoring. Heart rate monitoring then occurred on two occasions between 4 and 7 Days before conducting intraovarian treatments to further acclimatise heifers to the recording of heart rates. Data used for analysis of heart rates were recorded, within 2 h after administering treatments and again about 24 and 48 h after administering treatments. Heifers were restrained for up to an hour while heart rate monitoring was carried out. Heart rate was monitored using a Polar Equine 625x or RS400 heart rate monitor (Pursuit Performance, SA). Heifers were fitted with an Equine transmitter belt which encircled the thorax between the 5^{th} and 8^{th} ribs. Data were collected for a minimum of 30 minutes. Data collected from 10 to 30 minutes after commencing heart rate monitoring and heart rates recorded on Days 0, 1 and 2 following treatment were used for analyses.

After completion of treatments and heart rate monitoring all heifers within each replicate were confined to a yard with ad lib access to hay, water and molasses. Heifers were videotaped using a digital recorder and cameras (PAC-DVPIRVF700-2.8-10-GY, DAS, Loganholme Qld). Heifers were identified with numbers painted on each side to facilitate

identification of individual animals. Criteria used to assess the behaviour of heifers from recordings are outlined in Table 4. Heifers were video recorded for up to 48 h after treatment with the exception of when heifers were subjected to aversion testing and heart rate monitoring on the day after treatment. Behavioural responses following treatment were assessed by recording behaviours observed during a 90 minute observation period commencing 3 to 5 h after treatment. Between 24 to 28 h after treatment heifers were assessed for whether abnormal lying, foot stomping and/or kicking at the belly were observed.

Table 4. Criteria used to assess behaviour after intraovarian administration of treatments.

Behaviour	Description
Normal lying	Sternal recumbency with all legs folded under the body with head down or head up
Abnormal lying	Sternal or lateral recumbency with full or partial extension of the hind legs with the head up or down
Recumbent episodes	Number of times a heifer lay down fully during the observation period
Foot stamping and/or kicking at belly	Sudden flexion of hindlimb with toes touching or directed at the belly and/or lifting a hindlimb and forcefully placing it on the ground

3.7 Breeding

Heifers in each replicate were exposed to a bull 82 (Replicates 2 and 4) or 84 (Replicates 1, 3 and 5) days after treatment and depending on replicate, the bull was removed between 230 to 246 days after the start of treatment (Table A1). Pregnancy was diagnosed after visualisation of an embryo or foetus using transrectal ultrasonography. Video recordings of pregnancies were made and gestation length estimated following measurement of crown-rump length, biparietal diameter, or cross-section of the abdomen at the insertion of the umbilical cord depending on which parameter was best visualised in the embryo/foetus being assessed (Breukelman *et al.* 2004; DesCôteaux *et al.* 2009). Conception dates were then retrospectively determined from the calculated gestation lengths. When a pregnancy was diagnosed heifers were administered cloprostenol (0.5 mg IM) to terminate pregnancies, to determine if reconception occurred and to ensure non-pregnant tracts were recovered at the time of slaughter.

3.8 Ovarian histopathology and oocyte counts

At the time of slaughter ovaries were either placed on ice and returned to our laboratory for processing (Replicates 1 and 2) or processed at the time of slaughter (Replicates 3 to 5). Processing involved photographing of the reproductive tract, removal of ovaries, weighing each ovary and then fixation in Bouin's solution. Twenty four hours later the fixative was replaced with 10% neutral buffered formalin solution and stored until further processing. A

modification of the method used by Ireland *et al.* (2008) was used for the histological analysis of ovarian tissue and to obtain an estimate of the number of oocytes present within each ovary. In summary, ovaries were sectioned in half along their long axis at slaughter and after fixation one side was randomly selected for serial sectioning. After embedded in paraffin, at intervals of 8 µm, the 40th section was placed on a slide and stained with haematoxylin and eosin. Slides were examined and oocytes containing a cross section of the oocyte nucleus were counted. The total number of follicles per ovary was estimated by multiplying the total count by 80 (40 x 2; 40 corrects for counting follicles in the 40th section and 2 for counting only one half of the ovary).

3.9 Statistical analyses

Repeated measures analysis of variance was used to assess mean heart rates and the area under the curve of heart rates, mean concentrations of haptoglobin and bodyweights over time. The model included the main effects of treatment, time, replicate and relevant interactions. Where Mauchly's Test of Sphericity indicated that the assumption of sphericity had been violated, probability values were obtained after degrees of freedom were corrected using the Greenhouse-Geisser statistic. A log rank test, using Kaplan-Meier survival curves was used to determine if there were differences in the survival distribution for the different treatments in relation to the interval to first ovulation (progesterone >1.5 ng/mL) and the estimated time of conception following the commencement of monitoring after treatment. Ovulation was assumed when concentrations of progesterone were first recorded to exceed 1.5 ng/mL after Day 7 following treatment. Analysis of variance was used to compare means with the model including treatment, replicate and the relevant interaction terms. Where significant effects were detected using ANOVA pairwise comparisons were conducted using Tukey's test. A Kruskal-Wallis one-way anlaysis of variance was used to compare the median duration of recumbency and the number of recumbent episodes between groups and median oocyte counts between treatments. Where significant effects of treatment were detected pairwise comparisons were conducted using a Mann-Whitney U test. Proportional responses were compared using a Pearson Chi-Square test with a Bonferroni correction applied to multiple comparisons. P-values <0.05 were considered significant and $0.05 < P < 0.10$ were regarded as a tendency. Weights of ovaries not detected at the time of slaughter were recorded as 0 g for the purposes of analyses.

4 Results

4.1 Data omissions

Data obtained during replicate one was retained for assessment of bodyweights, behaviour at the time of treatment, pregnancy, concentrations of progesterone, intervals to conception and ovarian characteristics as no significant effects of replicate were obtained for these variables. Data from replicate one was omitted from analyses of concentrations of haptoglobin, fibrinogen, total leukocyte counts and behaviour after the initial treatment due to potential confounding due to differences in dose rates administered to the animals treated with ZG in Replicate 1 and the extent of the pain responses observed after treatment in Replicate 1 compared to the other replicates.

4.2 Behaviour associated with treatment

4.2.1 Behaviour at treatment

Behaviour recorded at the time of administration was recorded as no visible reaction in 75% of animals that were treated with 25% of animals exhibiting mild struggling only. The percentage of animals exhibiting mild struggling at the time of treatment differed between treatments (20%, 2/10; 60%, 6/10; 0.0%, 0/10; 20%, 2/10; Saline, $CaCl_2$, ZG, ZG + $CaCl_2$, respectively; P = 0.017) with the percentage of animals exhibiting mild struggling at the time of injection being greater in the animals treated with $CaCl_2$ compared to those treated with ZG (P = 0.005).

Pain responses were evident within 15 minutes of administration of intraovarian treatments in animals in Replicate 1 that were treated with ZG and the combination of ZG + $CaCl_2$. Animals exhibited signs of colic which included flank kicking and intermittent recumbency. An immediate decision was made to reduce the subsequent volumes of intraovarian injections from 3 mL to 1.5 mL for animals treated with ZG and ZG + $CaCl_2$ and to administer the non-steroidal anti-inflammatory drug, meloxicam at the time of treatment to attempt to reduce the severity of pain responses with heifers treated in subsequent replicates.

4.2.2 Aversion testing and rectal temperatures

The time required for animals to enter the holding crate and the degree of assistance provided was not affected by treatment, day, replicate and no significant interactions were detected (P >0.10). Rectal temperatures were not significantly affected by treatment (P = 0.780), time (P = 0.121) and no significant interactions between treatment and time (P = 0.913) and treatment, time and replicate (P = 0.829) were found. Significant differences in rectal temperatures were, however, detected between replicates (P = 0.005; data not shown) and a significant interaction between replicate and day was detected (P <0.001). Mean rectal temperatures across treatment groups and replicates all fell within the normal range of rectal temperatures for cattle.

4.2.3 Heart rate monitoring

Mean heart rates were found to differ across days (P = 0.020) and between replicates (P = 0.002) but not between treatments (P = 0.378), and significant interactions included in the model were not detected (P > 0.10). Mean heart rates/min were greater on the day of treatment compared to 1 and 2 days after treatment (Figure 1).

4.2.4 Behaviour after treatment

Behavioural characteristics recorded in heifers during a 90 minute observation period after treatment are listed in Table 5. Abnormal lying or foot stamping or kicking at the belly was not observed in heifers treated with saline or $CaCl_2$. At least one of these behaviours was observed in 75% and 62.5% of heifers treated with ZG and the combination of ZG and $CaCl_2$, respectively. Heifers treated with ZG experienced more recumbent episodes compared to heifers treated with saline or $CaCl_2$. Measures of animal behaviour observed after treatment with ZG or ZG and $CaCl_2$ were similar. Differences were detected between replicates in the duration of recumbency with a shorter median duration observed in Replicate 4 (0, 95% CI: 0, 5.8) compared to Replicates 2 (20.2, 95% CI: 6.8, 50.8) and 5 (19.5, 95% CI: 7.2, 31.3 Heavy rain was noted as falling at the time of videoing Replicate 4.

Abnormal lying, foot stamping or kicking at the belly were not detected 20 to 24 hours after treatment in any treatment group.

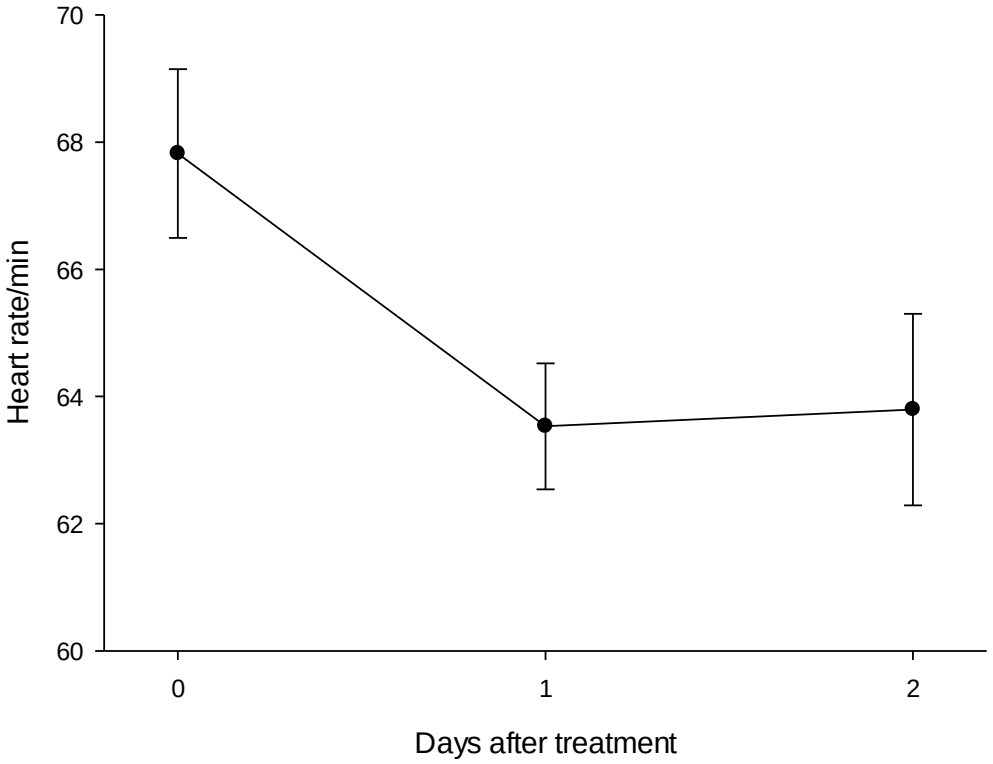

Figure 1. Mean heart rates for all treatments groups combined between the time of treatment (Day 0) and up to 2 days after treatment.

Table 5. Characteristics of animal behaviour observed in heifers during a 90 minute observation period on the first day after treatment.

Variable	Saline	$CaCl_2$	ZG	$CaCl_2$ + ZG	P
Abnormal lying (%)	0.0^{A}	0.0^{A}	50.0^{B}	25.0^{AB}	0.029
Foot stamping and/or kicking at belly (%)	0.0^{A}	0.0^{A}	75.0^{B}	50.0^{AB}	0.001
Recumbency* (min)	0.0 (0, 20.4)	3.5 (0, 15.4)	21.2 (8.6, 48.2)	14.5 (0, 48.1)	0.109
Recumbent episodes*	0.0^{A} (0-0.81)	0.5^{AC} (0-1.2)	5.5^{B} (2.3-9.7)	1.5^{BC} (0.4-5.9)	0.010
Duration of recumbency/episode (min)	24.5 ± 4.8^{A}	12.5 ± 5.2^{AB}	4.2 ± 0.8^{B}	8.5 ± 2.5^{B}	0.005

*Median (95% C.I.).

[ab] differ P<0.05.

4.3 Haematology, acute phase proteins and anti-Müllerian hormone

4.3.1 Haematology

Mean total leukocyte counts between Days 0 and 7 following administration of treatments did not differ between treatments (P = 0.679), replicates (P = 0.561), days (P = 0.530) and no significant interactions were detected.

4.3.2 Acute phase proteins

Concentrations of haptoglobin obtained on Days 0 and 7 in heifers in Replicates 2 to 5 are illustrated in Figure 2. Mean concentrations of haptoglobin did not differ between treatments (P = 0.194), over time (P <0.110) and no significant interactions were detected (P > 0.05). Differences between replicates were found (P = 0.002) with concentrations of haptoglobin being greater within replicates 2 (73.1 ± 2.8 mg/dL), 3 (66.8 ± 4.2 mg/dL) and 4 (67.8 ± 2.2 mg/dL) compared to 5 (55.6 ± 2.1 mg/dL). Concentrations of fibrinogen obtained between Days 0 and 7 in heifers in Replicates 2 to 5 are illustrated in Figure 3. Mean concentrations of fibrinogen differed between treatments (P = 0.002), replicates (P = 0.011) and over time (P <0.001) with no significant interactions detected (P > 0.05). Concentrations of fibrinogen were greater on Days 2 and 7 compared to Day 0 but similar between Days 2 and 7. Mean concentrations of fibrinogen were greater in the heifers treated with both $CaCl_2$ and ZG compared to heifers treated with saline and $CaCl_2$. Mean concentrations of fibrinogen were greater in the Replicate 5 heifers compared to the Replicate 2 heifers (3.52 ± 0.23 g/L versus 2.53 ± 0.13 g/L, respectively; P< 0.05).

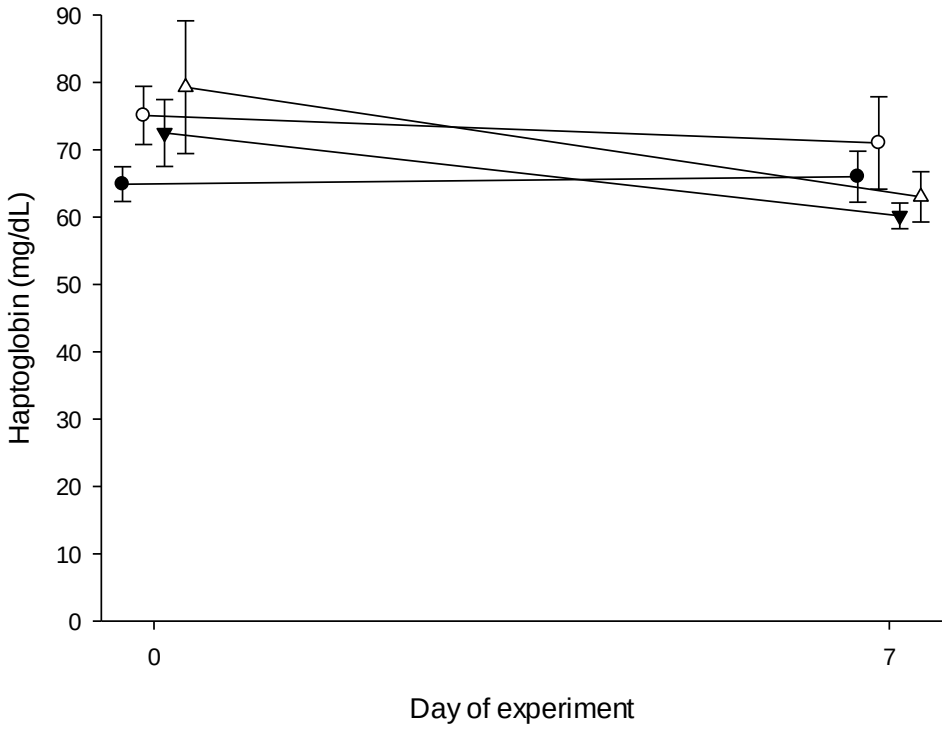

Figure 2. Concentrations of haptoglobin in plasma of heifers on Day 0 and 7 days after intraovarian administration of either saline (●), $CaCl_2$ (O), zinc gluconate (▼) or $CaCl_2$ and zinc gluconate (△).

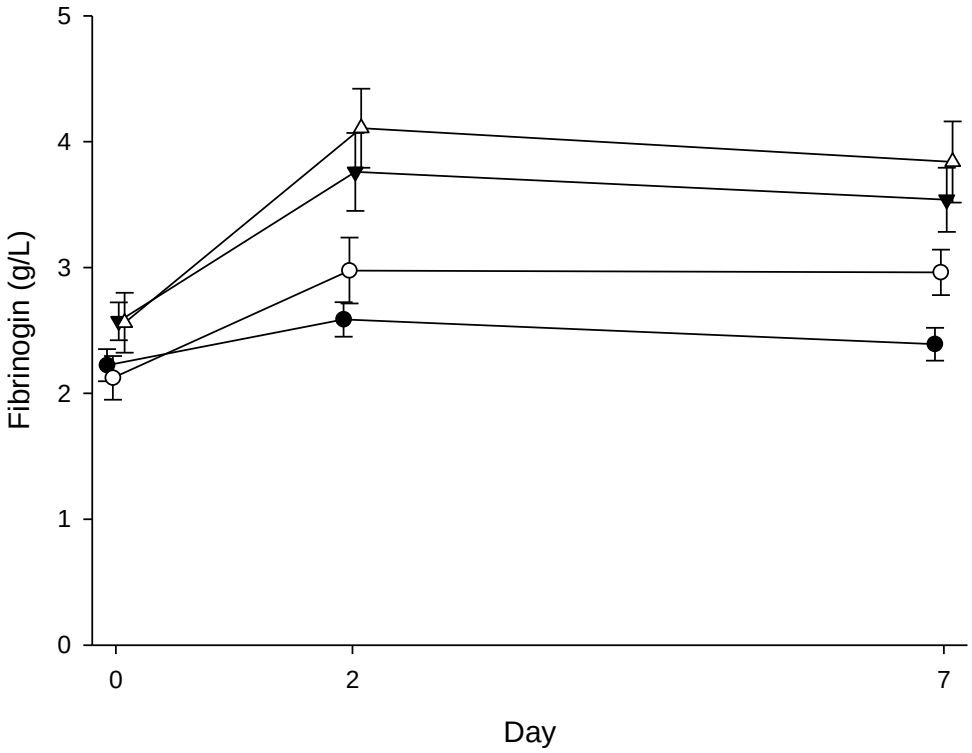

Figure 3. Mean concentrations of fibrinogen in heifers treated with intraovarian injections of saline (●), $CaCl_2$ (O), zinc gluconate (▼) or $CaCl_2$ and zinc gluconate (△).

4.3.3 Anti-Müllerian hormone

Concentrations of anti-Müllerian hormone between 215 and 222 days after treatment did not differ between treatments (P = 0.356; Table 6) or replicates (0.578). Concentrations of AMH were positively associated with total oocyte count (Figure 4a; R^2 = 0.14; P = 0.016) and with total ovarian mass (r^2 = 0.52; P < 0.001; Figure 4b).

Table 6. Total ovarian mass and oocyte count in ovaries recovered from heifers and concentrations of anti-Müllerian hormone in heifers treated with intraovarian injections of saline, calcium chloride ($CaCl_2$), zinc gluconate (ZG) and zinc gluconate plus calcium chloride ($CaCl_2$ + ZG).

Variable	Saline	$CaCl_2$	ZG	$CaCl_2$ + ZG	P
Total ovarian mass (g)	12.8 ± 1.0^{A}	8.56 ± 1.1^{B}	11.9 ± 1.0^{AB}	11.9 ± 0.99^{AB}	0.033
Total oocyte count (median x 10^3; 95% CI)	2.64 (0-19.47)	1.88 (0.547-5.37)	3.52 (2.26-7.77)	4.88 (3.29-10.34)	0.178
Anti-Müllerian hormone (pmol/L)	7.56 ± 1.23	4.87 ± 1.37	7.70 ± 1.24	8.16 ± 1.56	0.321

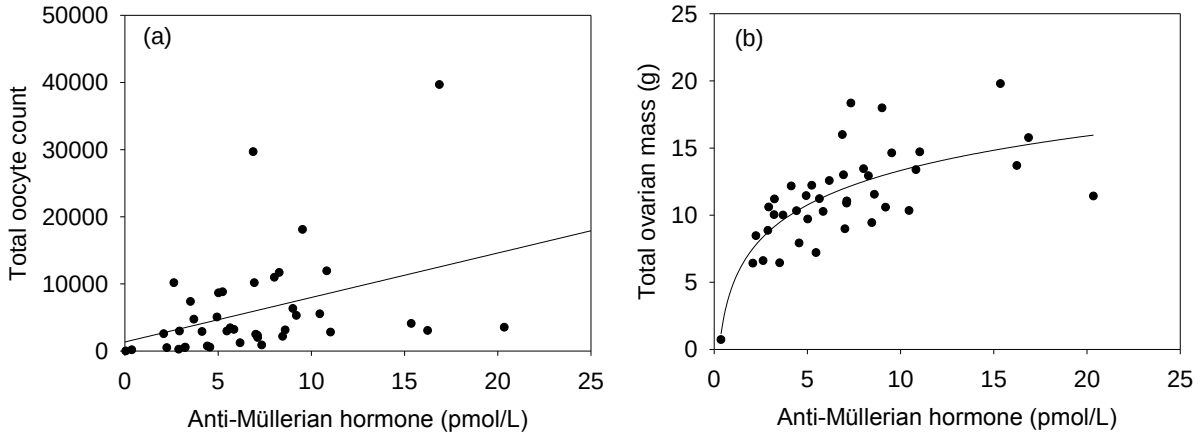

Figure 4. Relationship between serum concentration of anti-Müllerian hormone and (a) total oocyte count (r^2 = 0.140; P = 0.016) and, (b) total ovarian mass (r^2 = 0.55; P < 0.001). Total oocyte count = 1351 + 662.2*[AMH]. Total ovarian mass (g) = 1554 *[AMH]^0.0024 -1549.

4.4 Intervals to ovulation and pregnancy

Ovulation was detected in every heifer during the monitoring period after treatments were applied. No significant differences in the survival distributions of the intervals to detection of ovulation (P = 0.475) and conception after treatment (P = 0.479) were detected (Figure 5). No significant differences were found in the mean interval to ovulation, first and second conception, pregnancy rates and the percentage of heifers that were detected to conceive twice within the period of monitoring (Table 7).

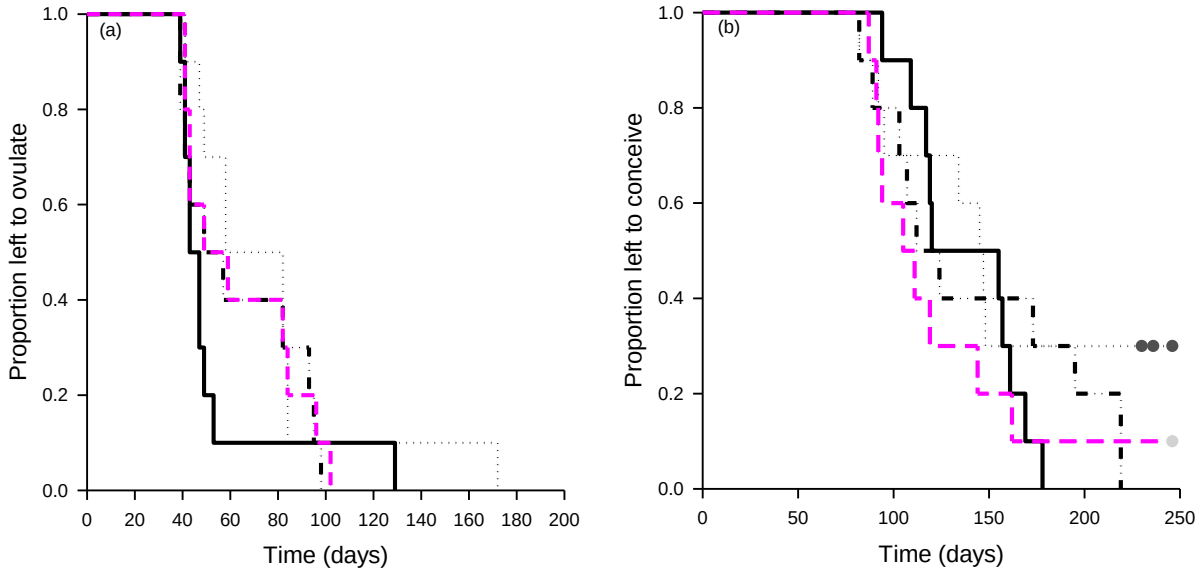

Figure 5. Kaplan-Meier survival curve for the, (a) interval to ovulation and (b) the interval to first conception after treatment (Saline: —; $CaCl_2$: …; ZG: - -, $CaCl_2$ + ZG ---; ● censored).

Table 7. Intervals to ovulation, pregnancy and pregnancy outcomes in heifers administered different intraovarian treatments.

Variable	Treatment				P
	Control	$CaCl_2$	Zinc	$CaCl_2$ + Zn	
n	10	10	10	10	
Interval to ovulation (days)	53.2 ± 8.5	75.7 ± 11.9	63.6 ± 8.0	64.0 ± 7.7	0.406
Interval to first conception (days)	137.9 ± 9.2	120.4 ± 11.1	142.3 ± 17.0	111.7 ± 8.7	0.263
Interval to second conception (days)	193.7 ± 11.3	178.3 ± 13.4	187.4 ± 14.3	171.4 ± 10.4	0.606
Pregnancy rate (%)	100	70	100	90	0.083
Conceived twice (%)	70	70	60	70	0.952

4.5 Changes in body weight

Mean body weights over time did not differ between replicates (P =0.121), treatments (P =0.613) and there was no significant treatment by time interaction (P = 0.990). Body weights did, however, increase over time (P < 0.001; Figure 6).

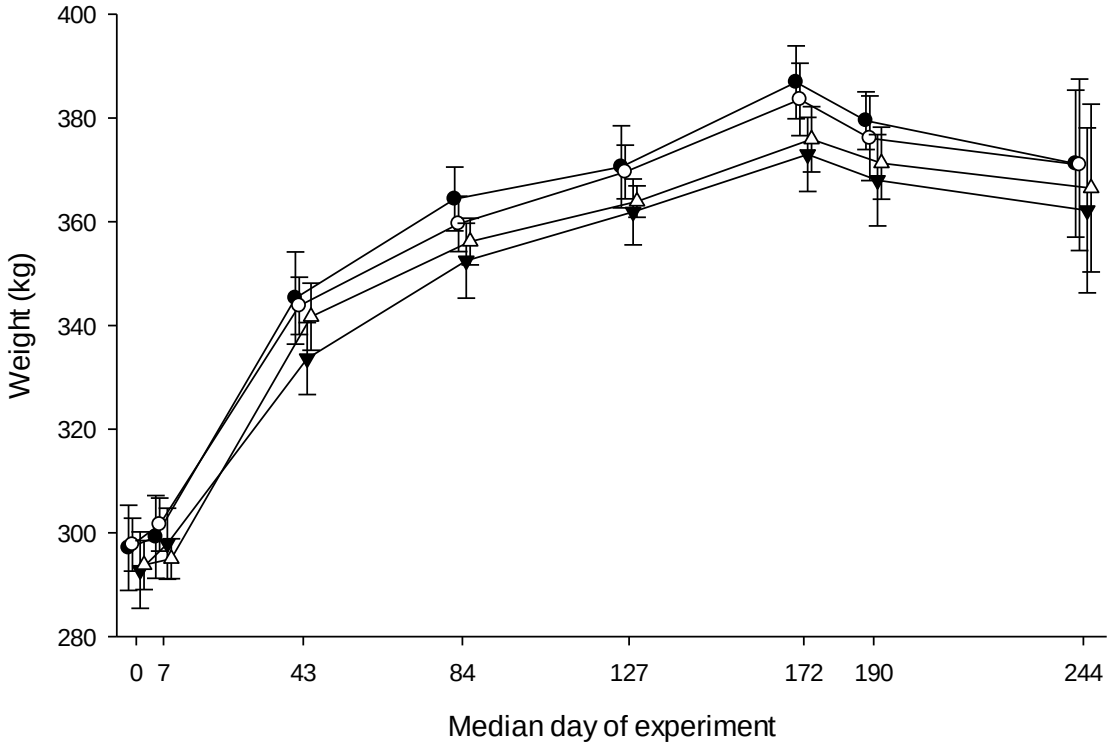

Figure 6. Body weights (Mean ± SEM) of heifers during after intraovarian administration of either saline (●), $CaCl_2$ (O), zinc gluconate (▼) or $CaCl_2$ and zinc gluconate (△).

4.6 Ovarian characteristics at slaughter

Characteristics of ovaries recovered at slaughter from heifers from each treatment group are listed in Table 6. One ovary was not located at the time of slaughter in 40% (4/10) of the heifers treated with $CaCl_2$. An ovarian mass of 0 g was recorded for these ovaries. In two other heifers treated with $CaCl_2$ an ovarian remnant was found in one and very small ovaries with a combined weight of 0.72 g were located in another. Pregnancy was not diagnosed during the course of the study in the heifer with bilaterally small ovaries. In 35% (7/20) of ovaries treated with $CaCl_2$ in ethanol, ovaries were either missing or had a mass at slaughter <1 g. Total ovarian mass was significantly less in heifers treated with $CaCl_2$ than heifers treated with saline (Table 6). No significant differences in the total oocyte count was found between treatments (Table 6). Total oocyte count ranged between 160 and 39,680. Examples of pairs of ovaries derived from heifers treated with saline or $CaCl_2$ are shown in Figure 7 while images of reproductive tracts recovered from heifers treated with intraovarian injections of $CaCl_2$ dissolved in ethanol in which complete ovarian atrophy of one ovary occurred are shown in Figure 8.

Histopathological examination of ovaries revealed the presence of mineralisation and osseous metaplasia in 50% (8/16) of the ovaries that were recovered from heifers treated with $CaCl_2$ in ethanol and none of the ovaries treated with any of the other treatments. Other specific pathological changes in ovaries treated with Saline, ZG and ZG and $CaCl_2$ were not found. Examples of mineralisation and osseous metaplasia observed in the ovarian medulla of heifers treated with $CaCl_2$ in ethanol are shown in Figure 9.

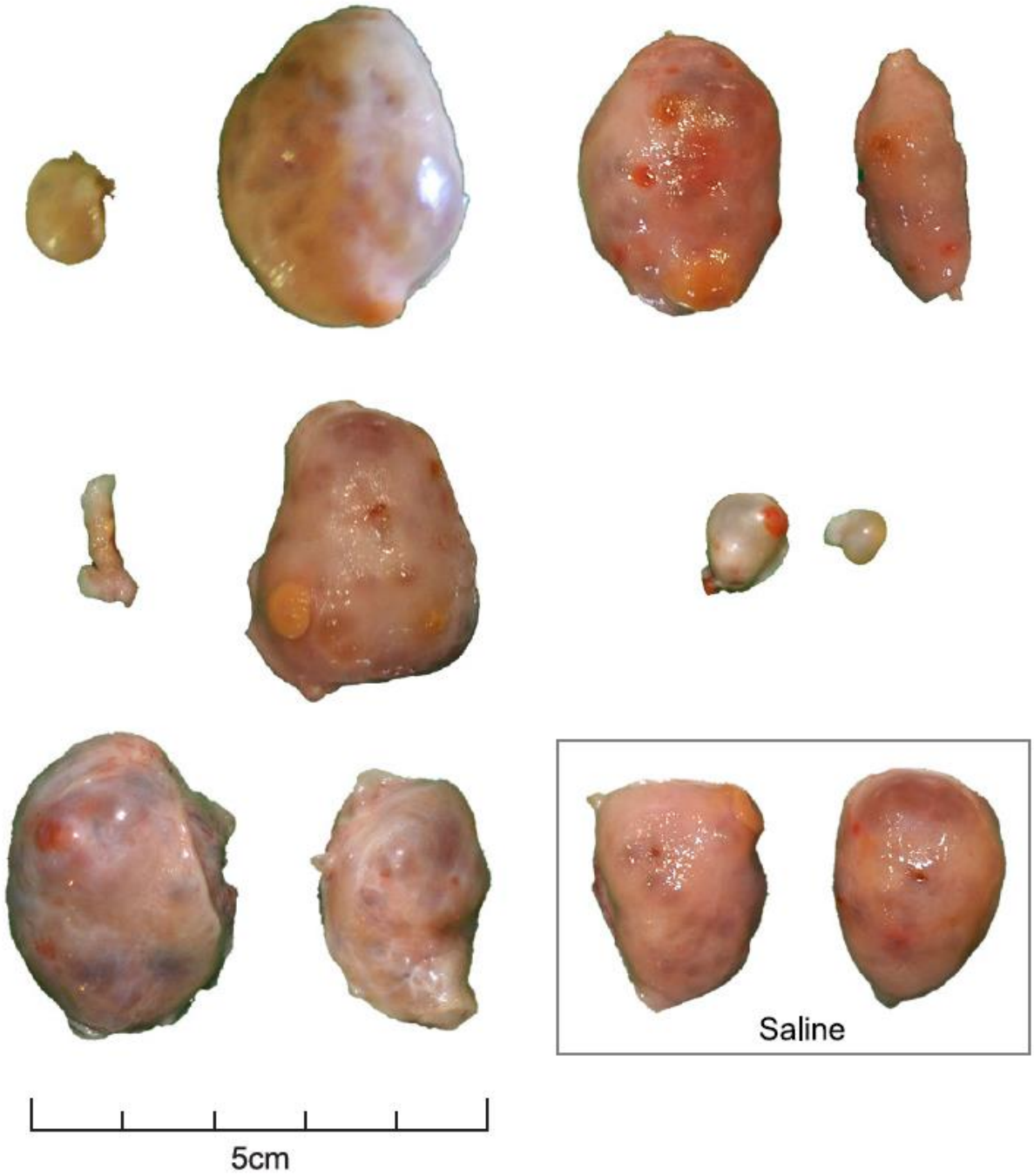

Figure 7. Examples of pairs of ovaries derived from heifers treated with intraovarian injections of saline or $CaCl_2$ dissolved in ethanol.

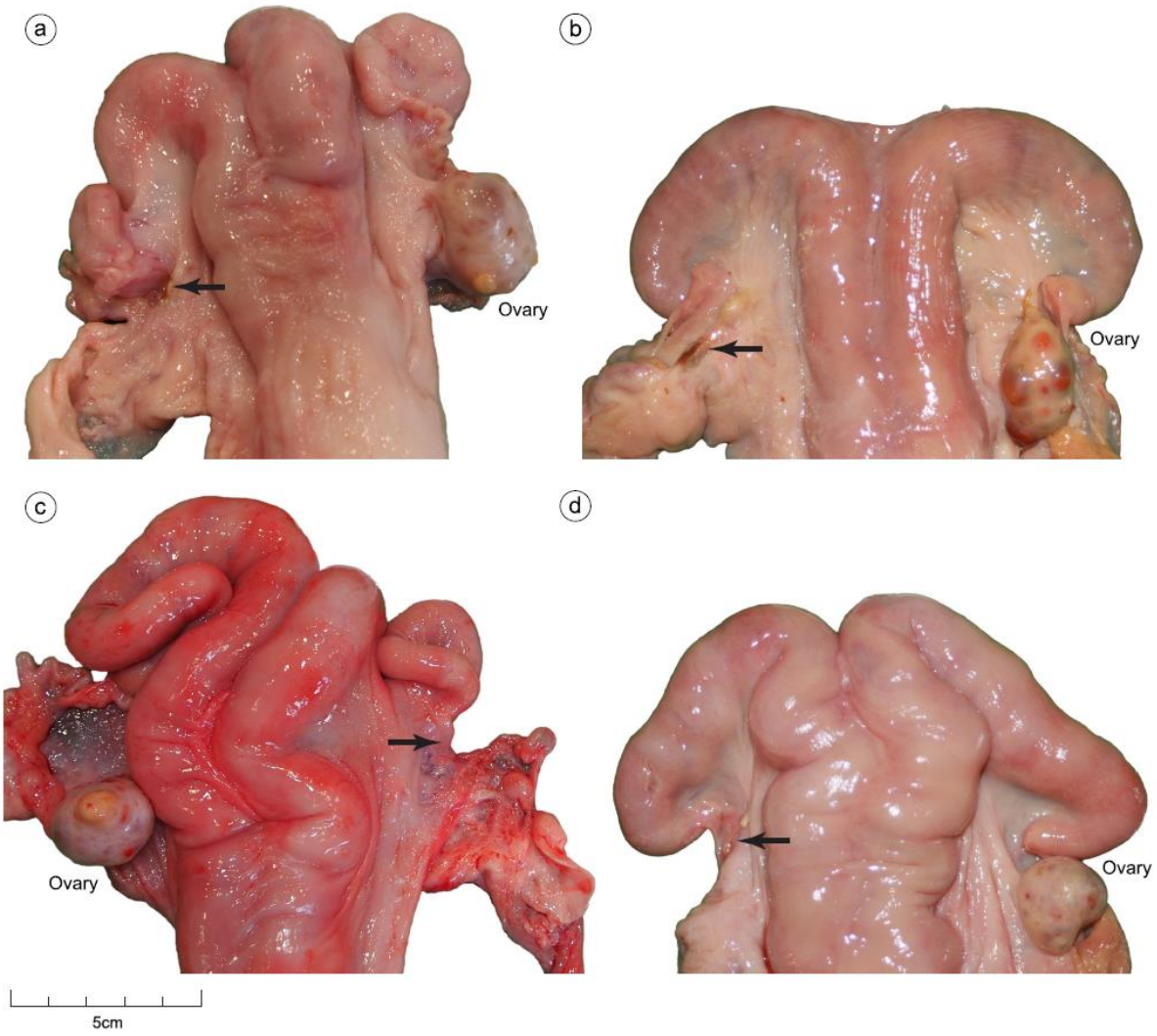

Figure 8. Examples of reproductive tracts recovered from heifers treated with intraovarian injections of $CaCl_2$ dissolved in ethanol in which complete ovarian atrophy of one ovary occurred (→).

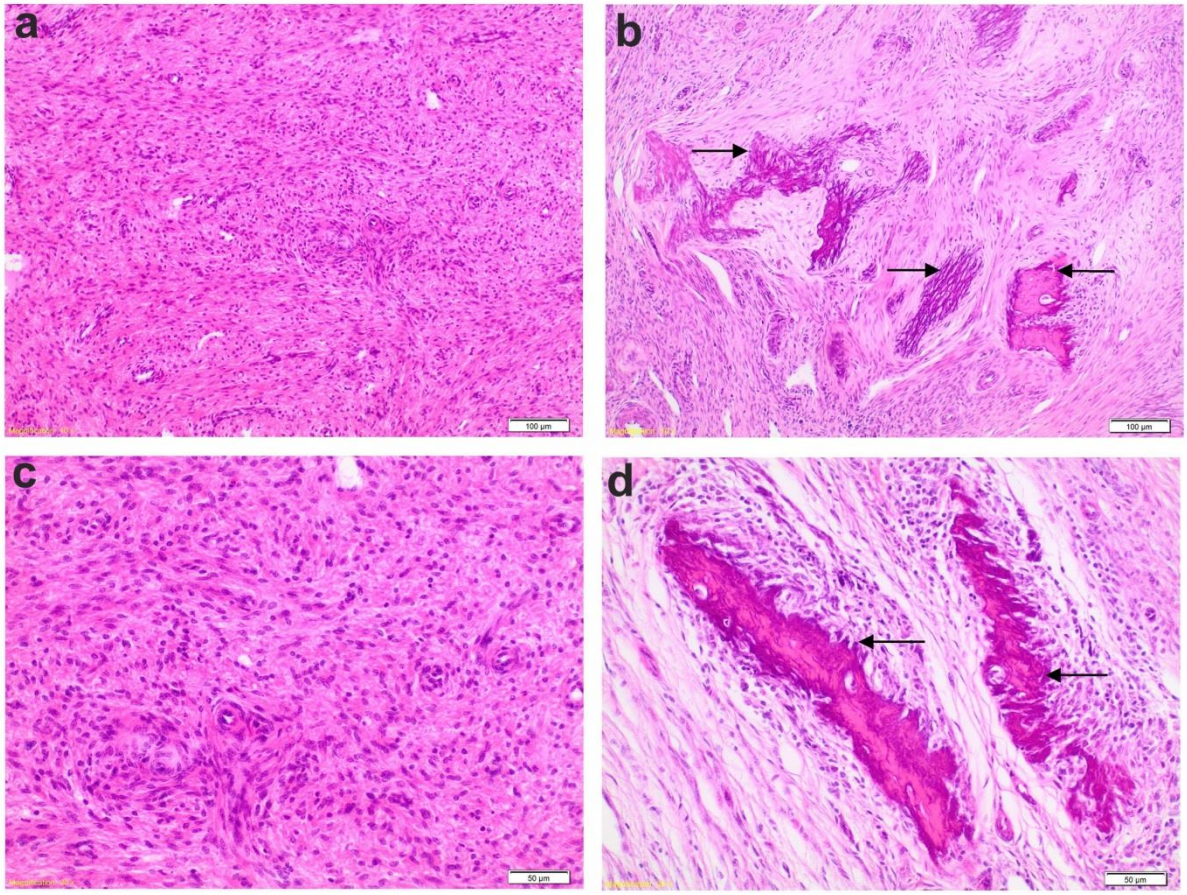

Figure 9. Histological sections of ovarian medullary stroma from heifers treated with Saline (a, c) or $CaCl_2$ in ethanol (b, d). Areas of mineralisation (➔) and osseous metaplasia (🡐) are shown.

5 Discussion

5.1 Overall findings

In order to sterilise female cattle in Northern Australia that are surplus to breeding requirements the ideal treatment would be effective after a single intervention, not have an adverse effect on animal welfare or leave any undesirable tissue residues, be inexpensive and enable animals to be processed in as short a time as possible. In this study intraovarian administration of ZG, $CaCl_2$ or the combination of both substances failed to prevent pregnancy in most heifers in this study and, therefore could not be recommended as methods of sterilising female cattle when administered in the manner conducted in this study. Treatment with $CaCl_2$ in ethanol did, however, cause ovarian atrophy with 35% of ovaries treated being either undetectable or weighing less than 1 g. It also was not associated with any adverse measures of animal welfare compared to control animals. Treatment with ZG, either alone or with $CaCl_2$, were associated with behaviours that were consistent with the sensation of pain and had no significant effect on ovarian mass after treatment. These results suggests that $CaCl_2$ in an ethanol base does have the potential to cause ovarian atrophy and sterilise female cattle but further study will be needed to improve

the consistency of responses obtained. Zinc gluconate alone or when combined with $CaCl_2$ appear to be unsatisfactory potential treatments for the sterilisation of female cattle.

5.2 Ovarian atrophy and mechanism of action

In this study administration of $CaCl_2$ significantly reduced the paired weight of ovaries compared to heifers treated with saline. Failure to cause ovarian atrophy in every ovary that was treated could perhaps be due to not adjusting the dose for ovarian size, escape of fluid out of the ovary at, or after injection, variable distribution of the agent within the ovary or unknown factors. A dose-dependent reduction in testicular mass has been observed after treatment with $CaCl_2$ in bucks (Jana *et al.* 2005), rats (Jana *et al.* 2002; Jana and Samanta 2006), dogs (Jana and Samanta 2007; Leoci *et al.* 2014a) and cats (Jana and Samanta 2011). In cases of hepatocellular tumours in humans treated with ethanol complete tumoral destruction has been reported in be about 90% of cases where tumour diameters are less than 3 cm but only 30% when tumour size exceeds 5 cm indicating that the size of a mass or ovary could affect responses to treatment which include ethanol (Rougier *et al.* 2007). In this study the centre of the ovary, within the ovarian medulla, was targeted for administration of chemicals. This is a region that consists of abundant fibrous tissue (Eurell and Frappier 2013), which at times offered resistance to administration of chemicals and potentially forced the escape of fluid from the ovary in some cases. Interference with tissue distribution of injected ethanol by fibrous tissue has also been reported in humans (Ohnishi *et al.* 1998) which may suggest that the fibrous composition of the ovarian medulla could also impede tissue distribution of chemicals. Oocytes are distributed only within the region of the cortex of the ovary so perhaps administration should focus on more peripheral administration of any potential intraovarian chemosterilant or multiple sites around the ovarian cortex. Given the potential for $CaCl_2$ to lead to tissue necrosis targeting of regions such as the oviduct may also offer some potential for inducing sterilisation. Future studies should, therefore, examine the effect of increasing the dose and different sites of administration within ovaries and administering $CaCl_2$ in alcohol in the region of the oviduct to determine if responses to treatments can be more consistent in inducing ovarian atrophy and sterilisation.

In this study no significant reduction in ovarian mass was found in heifers treated with Zn plus $CaCl_2$ compared to the saline treated heifers. This may be due to the 50% reduction in the dose of $CaCl_2$ administered compared to the heifers treated with $CaCl_2$ only or the use of an aqueous instead of an alcohol base. In dogs intratesticular administration of $CaCl_2$ in 95% ethanol resulted in more dogs being azoospermic and lower concentrations of testosterone in serum twelve months after treatment compared to treatment with $CaCl_2$ in a 1% lidocaine solution (Leoci *et al.* 2014b). Percutaneous intralesional administration of ethanol has been used to treat a range of tumour types (Ellman *et al.* 1981; Livraghi *et al.* 1988; Bas *et al.* 2001), cysts (Bean 1981; Gan *et al.* 2005) and even hydatid cysts in humans (Filice *et al.* 1990; Filippou *et al.* 2007). Moreover, pain responses in association with treatment do not feature prominently in these reports. The local necrotic effect of ethanol alone would appear to compliment the action of $CaCl_2$ in causing tissue necrosis so improvements in responses in dogs with inclusion in an alcohol rather than an aqueous base is perhaps expected. We can also not rule out the possibility that ethanol rather than $CaCl_2$ caused ovarian atrophy in some heifers in this study.

The pathogenesis of tissue injury following administration of $CaCl_2$ has been suggested to be associated with ischaemic necrosis produced by cellular dehydration, protein

denaturation and osmotic injury inducing vascular thrombosis and occlusion which in turn leads to coagulative necrosis and tissue fibrosis (Koger 1977; Lee *et al.* 1995). Coagulation necrosis following administration of $CaCl_2$ could explain the atrophy of ovaries observed in some heifers in this study. In male dogs and goats treated with intratesticular injections of $CaCl_2$ significant reductions in enzymes associated with androgen synthesis have been recorded along with local infiltration of leucocytes, necrosis of interstitial tissue and seminiferous tubules, germ cell depletion and fibrosis (Jana *et al.* 2005; Jana and Samanta 2007, 2011) confirming the local destruction of testicular tissue that occurs in males after administration. Elevated testicular concentrations of thiobarbituric acid reactive substances and conjugated dienes have also been recorded in males treated with $CaCl_2$ indicating that free radical production and lipid peroxidation may also play a role in tissue injury after administration of $CaCl_2$ (Jana and Samanta 2007). The results of this study do not elucidate the mechanism of action of $CaCl_2$ but support its potential for inducing ovarian atrophy in heifers.

Pathological changes observed within ovaries across treatments were difficult to detect and quantify. Small areas of mineralisation and osseous metaplasia were observed in 50% of ovaries that were recovered at slaughter and treated with $CaCl_2$ but no specific pathological lesions could be identified in ovaries treated with ZG or ZG and $CaCl_2$. Osseous metaplasia and mineralisation were likely associated with the induction of tissue necrosis. The reason for the absence of observing this change in ovaries infused with ZG and $CaCl_2$ is uncertain but may be due to failure to induce tissue necrosis which may in turn be related to differences in the dose of $CaCl_2$ administered, the aqueous base of the solution used or an interaction between ZG and $CaCl_2$ that rendered the treatment less effective.

The median number of oocytes were also not affected by any treatment in this study suggesting that a specific toxic effect on oocytes could not be detected. This may suggest that tissue destruction through, for example, coagulation necrosis may be the main mechanism operating to cause ovarian atrophy rather than specifically a toxic effect on oocytes. Oocyte counts were numerically but not significantly less in heifers treated with $CaCl_2$ compared to other treatments. Variation in ovarian responses to treatment with $CaCl_2$, large variation in oocyte numbers between animals, the method used for counting oocytes and a lack of statistical power may also have contributed to our failure to detect a reduction in oocyte counts within heifers treated with $CaCl_2$. Intratesticular administration of ZG or zinc acetate in males has been associated with germ cell depletion, vacuolation of Sertoli cells and interstitial fibrosis (Oliveira *et al.* 2007; Fagundes *et al.* 2014; Cavalieri *et al.* 2015). Our failure to demonstrate pathological changes associated with the intraovarian administration of ZG and confirmation of pregnancies in every animal treated with ZG alone suggests that oocytes are much more resistant to toxic effects of zinc compared to germ cells and Sertoli cells within testicular tissue.

5.3 Animal behaviour

Treatment with ZG either alone or in combination with $CaCl_2$ in an aqueous base induced changes in behaviour that were consistent with the sensation of pain. These behaviours included signs of colic soon after treatment, abnormal lying events, foot stamping and/or kicking at belly and more episodes of recumbency. Abnormal leg movements (Molony *et al.* 1995; Fisher *et al.* 2001), postures (Molony *et al.* 1995), restlessness (Morisse *et al.* 1995; Thuer *et al.* 2007) and reduced time lying (Gonzalez *et al.* 2010) have been attributed to pain

in cattle subjected to castration or dehorning. More frequent standing and sitting postures would suggest that animals were more restless and not content when sitting or standing while an increased frequency of abnormal lying and signs of colic are consistent with an animals attempt to alleviate pain in response to visceral pain (Molony *et al.* 1995). It has been suggested that foot stomping or kicking at the belly could be an attempt to escape from or remove a nocioceptive stimulus (Robertson *et al.* 1994). Significant differences in the median duration of lying were not detected but it is likely that a longer period of monitoring was needed to detect differences and rain at the time of filming may have affected the duration of lying in some replicates. Behavioural responses following treatment with ZG and $CaCl_2$ tended to be intermediate between responses obtained in the Saline and $CaCl_2$ only treated heifers and the heifers treated only with ZG (Table 5) which may suggest that pain responses were moderated by the mixing of $CaCL_2$ and ZG. The detection of abnormal behaviours in animals treated with ZG, compared to the control and $CaCl_2$ treated animals and behavioural responses that are consistent with other known painful procedures in cattle supports the conclusion that treatment with ZG induced pain following administration of ZG which was also not prevented by administration of the non-steroidal anti-inflammatory medication, meloxicam.

Pain responses in heifers treated with ZG and ZG and $CaCl_2$ may have been due to outflow of fluid out of the ovary. Intraovarian administration of water to abattoir specimens conducted by the author before the study commenced indicated that during administration of water the ovary initially increased in volume but when the volume of fluid exceeded between 1 and 2 mL the ovary became distended with fluid and excess fluid escaped and flowed along the ovarian pedicle. Diffusion away from sites of injection has also been observed when treating hepatic tumours with ethanol in humans (Lee *et al.* 1995). This movement of fluid may have exposed sensory nerve endings to ZG resulting in pain responses in some heifers. In heifers treated with $CaCl_2$ in alcohol either the solution was inherently ineffective in stimulating nerve endings or the alcohol component desensitised areas of contact. A lack of overt pain responses have been associated with intracystic administration of ethanol in humans (Gan *et al.* 2005) and intratesticular administration of $CaCl_2$ in ethanol in dogs (Jana and Samanta 2007; Leoci *et al.* 2014b).

5.4 Heart rates

Mean heart rates on all of the days on which heart rates were monitored fell within normal reference values for cattle and did not differ between treatments. They were greater on the day of treatment compared to 1 and 2 days after treatment and differed between replicates. Differences between the day of treatment and subsequent days may indicate that an increase in sympathetic activity occurred on the day of treatment which may have been due to the perception of pain or other stressors associated with the intravaginal and/or intraovarian injection process and/or inadequate acclimatisation of heifers to the treatment and monitoring regimes. Differences between replicates could have been due to changes in environmental conditions, temperament and/or differences in heifers to acclimatise to the treatment and recording regime. Use of heart rates can be problematic in the assessment of pain due to the potential for interfering variables such as activity, external stimuli, and temperament. Changes in successive cardiac interbeat intervals, otherwise known as heart rate variability (HRV) has been used in humans and a variety of domestic animals, including cattle to provides a more sensitive measure of responses to stressors and may have offered more information on the physiological stress responses associated with treatment (von

Borell *et al.* 2007; Kovacs *et al.* 2014). Further studies involving more detailed examination of heart rate variability will be needed to better elucidate physiological changes associated with the treatments applied in this study.

5.5 Inflammatory markers

No significant effects of treatment were observed within 7 days of treatment in total white cell counts or concentrations of haptoglobin. Mean concentrations of fibrinogen increased significantly after Day 0 in every treatment group but mean values still remained within normal reference ranges for cattle (2 to 7 g/L; Sutton and Hobman 1975). This may indicate that each treatment may have been associated with induction of mild inflammation. Greater concentrations of fibrinogen in the heifers treated with ZG and $CaCl_2$ combined, compared to treatment with Saline or $CaCl_2$, suggests that the combination treatment may have induced a greater local inflammatory reaction as serum concentrations of acute phase proteins such as fibrinogen and haptoglobin can be used as non-specific indicators of the severity of an inflammatory response in cattle (Tóthová *et al.* 2013). This is also supported by a relative absence of gross pathological changes associated with reproductive tracts at the time of slaughter. Perhaps more frequent sampling of cattle would be needed to detect any acute inflammatory responses with haptoglobin as heifers were only sampled on Days 0 and 7 in this study. Elevated concentrations of haptoglobin were found 4 days after flank speying of heifers (Petherick *et al.* 2011) which may suggest that earlier sampling after treatment may be needed to detect changes in concentrations of haptoglobin. Overall our failure to induce significant changes in total white cell counts or concentrations of haptoglobin or fibrinogen above normal reference values suggest that any inflammatory response following treatment was likely mild.

5.6 Anti-Müllerian hormone

Both the total oocyte count and concentrations of AMH were not significantly affected by treatment. This may reflect a general failure of treatments and the inconsistent effect of $CaCl_2$ treatment alone to reduce ovarian mass to the point where differences in AMH could be detected. Anti-Müllerian hormone is synthesised by ovarian granulosa cells with secretion beginning at the primary follicle stage with synthesis ceasing in the latter stages of antral follicular growth (8 to 10 mm in humans; Visser *et al.* 2006). Strong positive correlations have been found between concentrations of AMH in serum and the number of small growing follicles in women (Durlinger *et al.* 2002), mice and cattle (Ireland *et al.* 2008). A weak positive correlation was found in this study between total oocyte count and concentrations of AMH in serum but a stronger positive correlation was found between total ovarian mass and concentrations of AMH. Anti-Müllerian hormone is secreted from young growing follicles but AMH expression was not observed in primordial follicles (Bezard *et al.* 1987; Weenen *et al.* 2004) while it is decreased or absent in atretic follicles (Durlinger *et al.* 2002). In this study we did not differentiate between the types of ovarian follicles, for example, primordial, growing and atretic follicles, when performing oocyte counts which could account for the weak association between total oocyte count and concentrations of AMH observed in this study. Greater correlations ($r = 0.88$) were recorded when comparing antral follicle counts with concentrations of AMH when oocyte counts were determined using transrectal ultrasonography in cattle ($r = 0.88$; (Ireland *et al.* 2008) and the number of growing follicles were determined in the ovaries of mice, using histological examination of the ovaries ($r =$

0.86; (Kevenaar *et al.* 2006). These results are consistent with the secretion of AMH by growing follicles (Ireland *et al.* 2008).

The maximum total oocyte count obtained (160 and 39,680) was less than the average number of healthy and atretic follicles reported by (Ireland *et al.* 2008) in the ovaries of dairy cows with low (88,960) or high (8,291,85) antral follicle counts as determined with ultrasonography before slaughter and less than the mean number of preantral follicles measured in *Bos indicus* heifers (76,851; Silva-Santos *et al.* 2011). Considerable variation was observed between individuals which has also been recorded in other studies that have examined oocyte numbers within the ovaries of cattle (Erickson 1966; Ireland *et al.* 2008; Silva-Santos *et al.* 2011). Factors that may have contributed to lower oocyte counts in ovaries in this study could include variation in methodologies used for counting oocytes, differences between breeds, ages, (Silva-Santos *et al.* 2011) and nutrition during prenatal life (Mossa *et al.* 2009).

5.7 Limitations of the study

This study was the first known attempt to sterilise female cattle by infusing $CaCl_2$ and or ZG. Statistical power was kept to a minimum in case undesirable side effects such as pain occurred in response to treatment. Use of relatively few animals, variability in the effects of treatments and within saline treated control animals, particularly in regards to oocyte counts would have contributed to our failure to detect differences in total oocyte counts and concentrations of AMH in serum. A longer period of monitoring of animal behaviour would have helped to provide more detailed analysis of behaviour while use of heart rates alone may have been insufficiently sensitive measure to detect the behavioural changes that occurred in some animals that were consistent with the sensation of significant pain.

5.8 Success in meeting the objectives

The objectives of the project were met although the development of a treatment that is applicable to the livestock industry requires further study.

1. To determine effectiveness of chemical sterilisation treatments on reproductive physiology, fertility and liveweights of female cattle.

   Each of the treatments used failed to stop heifers ovulating as elevated concentrations of progesterone were detected in every heifer during the monitoring period after treatments were applied. No significant differences in the survival distributions of the intervals to detection of ovulation and conception after treatment were detected and pregnancy was detected in at least 70% of heifers within each treatment group. These findings suggest that treatments used in this study, when used in accordance with the dose rates and method of administration used in this study, would be unable to sterilise the majority of animals that were treated and would be unable to be used as an alternative to surgical sterilisation.

2. To complete evaluation of histopathology and hormonal changes in female cattle.

   Examination of ovaries at slaughter demonstrated that total ovarian mass was significantly less in heifers treated with $CaCl_2$ compared to heifers treated with saline, ZG and ZG and $CaCl_2$. Histopathological examination failed to demonstrate any significant differences in oocyte counts and this was reflected in serum concentrations

of AMH being similar between treatments. Pathological changes were difficult to detect in ovaries subjected to each treatment apart from some areas of mineralisation in ovaries infused with $CaCl_2$. Ovarian atrophy in some heifers treated with $CaCl_2$ raises the prospect that treatment with $CaCl_2$ in ethanol is a potential chemosterilant but modification of dose rate or method of infusion maybe needed to increase the probability of sterilising treated females.

6 Conclusions/recommendations

6.1 General conclusions

Intraovarian treatment with $CaCl_2$ may offer potential as a chemosterilant in *Bos indicus* heifers as a reduction in ovarian mass to <1 g was observed in 40% of treated ovaries and overt evidence of pain was not detected when observing animal behaviour after treatment compared to saline treated control animals. Further studies will be needed to determine if adjustments in dose or administration technique or concentration could result in more consistent responses to treatment. Treatment with ZG and the combination of ZG and $CaCl_2$ did not affect ovarian mass compared to administration of saline and was associated with behavioural changes that were indicative of pain and, therefore, appear to offer no potential for use as intraovarian chemosterilants in cattle.

6.2 Future research

Given that administration of $CaCl_2$ in 95% ethanol resulted in complete ovarian atrophy in some heifers further study should be undertaken to investigate whether adjustments in dose and site of administration could result in more consistent effects of treatment or if administration at other sites such as the oviduct, could induce sterilisation. Effects of any potential chemosterilant on cattle of varying ages and at different stages of the oestrous cycle would also require further study.

Given the potential for ethanol alone to cause tissue necrosis treatment with ethanol alone could be attempted in comparison with $CaCl_2$ dissolved within ethanol.

Additional study is needed to determine if a lack of overt signs of pain observed in this study is consistent with any modification of the treatment method and whether any side effects are detected when treatments are extended to larger numbers of animals. If a treatment regime can be developed that consistently induces a high rate of sterilisation then further studies will be needed to determine when animals are rendered sterile after treatment, what meat witholding times may be applicable and to develop standard operating procedures, training and extension to help ensure safe and effective implementation of the technique.

Heart rates were significantly greater on the day of treatment compared to 1 and 2 days after treatment. Additional studies could include a non-treated control group to rule out the possibility that intraovarian injection of saline contributed to this elevation in heart rate that was seen on the day of treatment.

Further work will also be needed to modify equipment with a view of simplifying treatment procedures and reducing the costs of equipment needed to administer treatments.

7 Key messages

The results of this study suggest that at the doses administered in this study that intraovarian administration of saline, $CaCl_2$ in 95% ethanol, ZG or ZG plus $CaCl_2$ in an aqueous base are not able to sterilise most heifers. Moreover intraovarian administration of ZG either alone or in combination with $CaCl_2$ causes an unacceptable degree of pain.

A 20% solution of $CaCl_2$ in 95% ethanol was able to cause complete or nearly complete ovarian atrophy in 40% of ovaries treated without causing behavioural responses that were consistent with pain. No mortalities were recorded and liveweight changes over time were similar to saline treated control heifers. These results suggests that that this treatment could potentially be used as an intraovarian chemosterilant in *Bos indicus* heifers without having an adverse effect on animal welfare provided that with further study more consistent responses to treatment can be obtained.

8 Bibliography

Ali, M, Selem, M, Makady, F, SH, S (1991) Calcium chloride castration in 370 donkeys (an experimental study). *Assiut Vet Med J* **25**, 196-202.

Baran, A, Ozdas, O, Gulcubuk, A, Hamzaoglu, A, Tonguk, M (2010) Pilot study: intratesticular injection induces sterility in male cats. , 4th International Symposium on Non-Surgical Methods of Pet Population Control.' Dallas, Texas, USA, April 8–10, 2010. Available at https://www.acc-d.org/docs/default-source/4th-symosium/baran_poster.pdf?sfvrsn=2

Bas, T, Aparisi, F, Bas, JL (2001) Efficacy and safety of ethanol injections in 18 cases of vertebral hemangioma: a mean follow-up of 2 years. *Spine* **26**, 1577-82.

Bean, WJ (1981) Renal cysts: treatment with alcohol. *Radiology* **138**, 329-331.

Bezard, J, Vigier, B, Tran, D, Mauleon, P, Josso, N (1987) Immunocytochemical study of anti-Mullerian hormone in sheep ovarian follicles during fetal and post-natal development. *J Reprod Fertil* **80**, 509-516.

Bowen, RA (2008) Male contraceptive technology for nonhuman male mammals. *Anim Reprod Sci* **105**, 139-43.

Breukelman, SP, Reinders, JMC, Jonker, FH, de Ruigh, L, Kaal, LMTE, van Wagtendonk-de Leeuw, AM, Vos, PLAM, Dieleman, SJ, Beckers, JF, Perenyi, Z, Taverne, MAM (2004) Fetometry and fetal heart rates between Day 35 and 108 in bovine pregnancies resulting from transfer of either MOET, IVP-co-culture or IVP-SOF embryos. *Theriogenology* **61**, 867-882.

Cavalieri, J, Coleman, C, Rodrigues, H, Macmillan, KL, Fitzpatrick, LA (2002) The effect of timing of administration of oestradiol benzoate on characteristics of oestrus, timing of ovulation and fertility in Bos indicus heifers synchronised with a progesterone releasing intravaginal insert. *Aust Vet J* **80**, 217-223.

Cavalieri, J, Wang, M, Johnson, L (2015) Chemical sterilisation of Bos indicus bull calves following intratesticular injection of zinc acetate: Effects on semen quality and testicular changes. *Anim Reprod Sci* **156**, 23-33.

De Witte, K, Jubb, T, Letchford, P (2006) 'The dropped ovary technique for spaying cattle.' Australian Cattle Veterinarians, Eight Mile Plains, Australia.

DesCôteaux, L, Gnemmi, G, Colloton, J (2009) Ultrasonography of the Bovine Female Genital Tract. *Vet Clin N Am-Food A* **25**, 733-752.

Durlinger, AL, Visser, JA, Themmen, AP (2002) Regulation of ovarian function: the role of anti-Mullerian hormone. *Reproduction* **124**, 601-9.

Eckersall, PD, Duthie, S, Safi, S, Moffatt, D, Horadagoda, NU, Doyle, S, Parton, R, Bennett, D, Fitzpatrick, JL (1999) An automated biochemical assay for haptoglobin: Prevention of interference from albumin. *Comp Haematol Int* **9**, 117-124.

Ellman, BA, Parkhill, BJ, Curry, TS, 3rd, Marcus, PB, Peters, PC (1981) Ablation of renal tumors with absolute ethanol: a new technique. *Radiology* **141**, 619-26.

Erickson, BH (1966) Development and Senescence of the Postnatal Bovine Ovary. *J Anim Sci* **25**, 800-805.

Eurell, JA, Frappier, BL (2013) 'Dellmann's textbook of veterinary histology.' (John Wiley & Sons:

Fagundes, AK, Oliveira, EC, Tenorio, BM, Melo, CC, Nery, LT, Santos, FA, Alves, LC, Douglas, RH, Silva, VA, Jr. (2014) Injection of a chemical castration agent, zinc gluconate, into the testes of cats results in the impairment of spermatogenesis: A potentially irreversible contraceptive approach for this species? *Theriogenology* **81**, 230-236.

Fahim, MS, Wang, M, Sutcu, MF, Fahim, Z (1993) Zinc arginine, a 5α-reductase inhibitor, reduces rat ventral prostate weight and DNA without affecting testicular function. *Andrologia* **25**, 369-375.

Filice, C, Pirola, F, Brunetti, E, Dughetti, S, Strosselli, M, Foglieni, CS (1990) A new therapeutic approach for hydatid liver cysts. Aspiration and alcohol injection under sonographic guidance. *Gastroenterology* **98**, 1366-1368.

Filippou, D, Tselepis, D, Filippou, G, Papadopoulos, V (2007) Advances in liver echinococcosis: diagnosis and treatment. *Clin Gastroenterol Hepatol* **5**, 152-9.

Fisher, AD, Knight, TW, Cosgrove, GP, Death, AF, Anderson, CB, Duganzich, DM, Matthews, LR (2001) Effects of surgical or banding castration on stress responses and behaviour of bulls. *Aust Vet J* **79**, 279-284.

Gan, SI, Thompson, CC, Lauwers, GY, Bounds, BC, Brugge, WR (2005) Ethanol lavage of pancreatic cystic lesions: initial pilot study. *Gastrointest Endosc* **61**, 746-52.

Gibbons, JR, Beal, WE, Krisher, RL, Faber, EG, Pearson, RE, Gwazdauskas, FC (1994) Effects of once- versus twice-weekly transvaginal follicular aspiration on bovine oocyte recovery and embryo development. *Theriogenology* **42**, 405-19.

Gonzalez, LA, Schwartzkopf-Genswein, KS, Caulkett, NA, Janzen, E, McAllister, TA, Fierheller, E, Schaefer, AL, Haley, DB, Stookey, JM, Hendrick, S (2010) Pain mitigation after band castration of beef calves and its effects on performance, behavior, Escherichia coli, and salivary cortisol. *J Anim Sci* **88**, 802-10.

Habermehl, N (1993) Heifer ovariectomy using the Willis spay instrument: Technique, morbidity and mortality. *Can Vet J* **34**, 664-667.

Ibrahim, A, Ali, MM, Abou-Khalil, NS, Ali, MF (2016) Evaluation of chemical castration with calcium chloride versus surgical castration in donkeys: testosterone as an endpoint marker. *BMC Vet Res* **12**, 46.

Ireland, JL, Scheetz, D, Jimenez-Krassel, F, Themmen, AP, Ward, F, Lonergan, P, Smith, GW, Perez, GI, Evans, AC, Ireland, JJ (2008) Antral follicle count reliably predicts number of morphologically healthy oocytes and follicles in ovaries of young adult cattle. *Biol Reprod* **79**, 1219-1225.

Jana, K, Samanta, PK (2006) Evaluation of single intratesticular injection of calcium chloride for nonsurgical sterilization in adult albino rats. *Contraception* **73**, 289-300.

Jana, K, Samanta, PK (2007) Sterilization of male stray dogs with a single intratesticular injection of calcium chloride: a dose-dependent study. *Contraception* **75**, 390-400.

Jana, K, Samanta, PK (2011) Clinical evaluation of non-surgical sterilization of male cats with single intra-testicular injection of calcium chloride. *BMC Vet Res* **7**, 39.

Jana, K, Samanta, PK, Ghosh, D (2002) Dose-dependent response to an intratesticular injection of calcium chloride for induction of chemosterilization in adult albino rats. *Vet Res Commun* **26**,

Jana, K, Samanta, PK, Ghosh, D (2005) Evaluation of single intratesticular injection of calcium chloride for nonsurgical sterilization of male Black Bengal goats (Capra hircus): a dose-dependent study. *Anim Reprod Sci* **86**, 89-108.

Jubb, T, Letchford, P (1997) Cattle spaying and electroimmobilisers—Their use in extensive beef cattle herds in the Kimberley region, Proc. Australian Cattle Veterinarians Annual Conference, Brisbane, Australia, pp. 31-35.

Jubb, TF, Fordyce, G, Bolam, MJ, Hadden, DJ, Cooper, NJ, Whyte, TR, Fitzpatrick, LA, Hill, F, D'Occhio, MJ (2003) Trial introduction of the Willis dropped ovary technique for spaying cattle in northern Australia. *Aust Vet J* **81**, 66-70.

Kevenaar, ME, Meerasahib, MF, Kramer, P, van de Lang-Born, BM, de Jong, FH, Groome, NP, Themmen, AP, Visser, JA (2006) Serum anti-mullerian hormone levels reflect the size of the primordial follicle pool in mice. *Endocrinology* **147**, 3228-34.

Koger, LM (1977) Calcium chloride, practical necrotizing agent. *Bovine Pract* **12**, 118-119.

Koger, LM (1978) Calcium chloride castration. *Mod Vet Pract* **59**, 119-121.

Kovacs, L, Jurkovich, V, Bakony, M, Szenci, O, Poti, P, Tozser, J (2014) Welfare implication of measuring heart rate and heart rate variability in dairy cattle: literature review and conclusions for future research. *Animal* **8**, 316-30.

Kutzler, M, Wood, A (2006) Non-surgical methods of contraception and sterilization. *Theriogenology* **66**, 514-25.

Kutzler, MA (2015) Intratesticular and intraepididymal injections to sterilize male cats: From calcium chloride to zinc gluconate and beyond. *J Fel Med Surg* **17**, 772-776.

Lee, MJ, Mueller, PR, Dawson, SL, Gazelle, SG, Hahn, PF, Goldberg, MA, Boland, GW (1995) Percutaneous ethanol injection for the treatment of hepatic tumors: indications, mechanism of action, technique, and efficacy. *Am J Roentgenol* **164**, 215-20.

Leoci, R, Aiudi, G, Silvestre, F, Lissner, E, Lacalandra, G (2014a) A dose-finding, long-term study on the use of calcium chloride in saline solution as a method of non-surgical sterilization in dogs: Evaluation of the most effective concentration with the lowest risk. *Acta Vet Scand* **56**, 63.

Leoci, R, Aiudi, G, Silvestre, F, Lissner, EA, Lacalandra, GM (2014b) Alcohol diluent provides the optimal formulation for calcium chloride non-surgical sterilization in dogs. *Acta Vet Scand* **56**, 62.

Levy, JK, Crawford, PC, Appel, LD, Clifford, EL (2008) Comparison of intratesticular injection of zinc gluconate versus surgical castration to sterilize male dogs. *Am J Vet Res* **69**, 140-143.

Livraghi, T, Salmi, A, Bolondi, L, Marin, G, Arienti, V, Monti, F, Vettori, C (1988) Small hepatocellular carcinoma: percutaneous alcohol injection--results in 23 patients. *Radiology* **168**, 313-317.

McCosker, K, Letchford, P, Petherick, JC, Meyer, D, McGowan, M (2010) Morbidity, mortality and body weight gain of surgically spayed, yearling Brahman heifers. *Aust Vet J* **88**, 497-503.

Molony, V, Kent, JE, Robertson, IS (1995) Assessment of acute and chronic pain after different methods of castration of calves. *Appl Anim Behav Sci* **46**, 33-48.

Morisse, JP, Cotte, JP, Huonnic, D (1995) Effect of Dehorning on Behavior and Plasma-Cortisol Responses in Young Calves. *Appl Anim Behav Sci* **43**, 239-247.

Mossa, F, Kenny, D, Jimenez-Krassel, F, Smith, GW, Berry, D, Butler, S, Fair, T, Lonergan, P, Ireland, JJ, Evans, AC (2009) Undernutrition of Heifers During the First Trimester of Pregnancy Diminishes Size of the Ovarian Reserve in Female Offspring. *Biol Reprod* **81**, 135.

Niethe, GE, Holmes, WE (2008) Modelled female sale options demonstrate improved profitability in northern beef herds. *Aust Vet J* **86**, 458-464.

Ohnishi, K, Yoshioka, H, Ito, S, Fujiwara, K (1998) Prospective randomized controlled trial comparing percutaneous acetic acid injection and percutaneous ethanol injection for small hepatocellular carcinoma. *Hepatology* **27**, 67-72.

Oliveira, ECS, Moura, MR, Silva, VA, Peixoto, CA, Saraiva, KLA, de Sa, MJC, Douglas, RH, Marques, AD (2007) Intratesticular injection of a zinc-based solution as a contraceptive for dogs. *Theriogenology* **68**, 137-145.

Oyedipe, EO, Voh, AA, Marire, BN, Pathiraja, N (1986) Plasma progesterone concentrations during the oestrous cycle and following fertile and non-fertile inseminations of Zebu heifers. *Brit Vet J* **142**, 41-46.

Petherick, J (2005) Animal welfare issues associated with extensive livestock production: The northern Australian beef cattle industry. *Appl Anim Behav Sci* **92**, 211-234.

Petherick, J, McCosker, K, Mayer, D, Letchford, P, McGowan, M (2011) Preliminary investigation of some physiological responses of Bos Indicus heifers to surgical spaying. *Aust Vet J* **89**, 131-137.

Petherick, J, McCosker, K, Mayer, D, Letchford, P, McGowan, M (2013) Evaluation of the impacts of spaying by either the dropped ovary technique or ovariectomy via flank laparotomy on the welfare of beef heifers and cows. *J Anim Sci* **91**, 382-394.

Pinner, KK (2006) Lack of animal welfare assessment regarding trans-vaginal spaying of heifers. *Can Vet J* **47**, 266.

Robertson, IS, Kent, JE, Molony, V (1994) Effect of different methods of castration on behaviour and plasma cortisol in calves of three ages. *Res Vet Sci* **56**, 8-17.

Rougier, P, Mitry, E, Barbare, J-C, Taieb, J (2007) Hepatocellular carcinoma (HCC): an update, Seminars in Oncology. Available at http://www.seminoncol.org/article/S0093-7754(07)00024-3/abstract

Silva-Santos, KC, Santos, GM, Siloto, LS, Hertel, MF, Andrade, ER, Rubin, MI, Sturion, L, Melo-Sterza, FA, Seneda, MM (2011) Estimate of the population of preantral follicles in the ovaries of Bos taurus indicus and Bos taurus taurus cattle. *Theriogenology* **76**, 1051-7.

Soto, FR, Viana, WG, Mucciolo, GC, Hosomi, FY, Vannucchi, CI, Mazzei, CP, Eyherabide, AR, de Fatima Lucio, C, Dias, RA, de Azevedo, SS (2009) Evaluation of efficacy and safety of zinc gluconate associated with dimethyl sulphoxide for sexually mature canine males chemical neutering. *Reprod Domest Anim* **44**, 927-31.

Sutton, RH, Hobman, B (1975) The value of plasma fibrinogen estimations in cattle: A comparison with total leucocyte and neutrophil counts. *NZ Vet J* **23**, 21-27.

Thuer, S, Mellema, S, Doherr, MG, Wechsler, B, Nuss, K, Steiner, A (2007) Effect of local anaesthesia on short- and long-term pain induced by two bloodless castration methods in calves. *Vet J* **173**, 333-42.

Tóthová, C, Nagy, O, Kovac, G (Ed. S Janciauskiene (2013) The use of acute phase proteins as biomarkers of diseases in cattle and swine. In: Acute Phase Proteins, (Ed.) Janciauskiene, S, InTech Available from: http://www.intechopen.com/books/acute-phase-proteins/the-use-of-acute-phase-proteins-as-biomarkers-of-diseases-in-cattle-and-swine

USFDA (1993) Freedom of Information summary. Neutersol injectable solution for dogs (zinc gluconate neutralized by arginine). Intratesticular injection for chemical sterilization in 3 to 10 month old male dogs. NADA: 141-217 Available from: http://www.fda.gov/downloads/AnimalVeterinary/Products/ApprovedAnimalDrugProducts/FOIADrugSummaries/ucm118024.pdf

Visser, JA, de Jong, FH, Laven, JSE, Themmen, APN (2006) Anti-Müllerian hormone: a new marker for ovarian function. *Reproduction* **131**, 1-9.

von Borell, E, Langbein, J, Després, G, Hansen, S, Leterrier, C, Marchant-Forde, J, Marchant-Forde, R, Minero, M, Mohr, E, Prunier, A (2007) Heart rate variability as a measure of autonomic regulation of cardiac activity for assessing stress and welfare in farm animals—a review. *Physiol Behav* **92**, 293-316.

Weenen, C, Laven, JS, Von Bergh, AR, Cranfield, M, Groome, NP, Visser, JA, Kramer, P, Fauser, BC, Themmen, AP (2004) Anti-Mullerian hormone expression pattern in the human ovary: potential implications for initial and cyclic follicle recruitment. *Mol Hum Reprod* **10**, 77-83.

9 Appendix

9.1 Outline of experimental timetable

Table A1. Treatment and monitoring days in relation to the day intraovarian treatments were administered for each replicate.

Activity	Replicate					Median day
	1	2	3	4	5	
Insert intravaginal progesterone releasing inserts	-19	-21	-19	-21	-21	-21
Remove inserts, administer cloprostenol IM, eCG sc	-11	-13	-11	-13	-13	-13
Administer oestradiol benzoate	-10	-12	-10	-12	-12	-12
Hear rate monitoring, Aversion testing	-5	-7	-5	-7	-7	-7
Heart rate monitoring, aversion testing	-4	-6	-4	-6	-6	-6
Weigh, aversion testing, treatment, heart rate monitoring	0	0	0	0	0	0
Aversion testing, heart rate monitoring	1	1	1	1	1	1
Aversion testing, heart rate monitoring, BS	2	2	2	2	2	2
Weigh, BS	7	7	7	7	7	7
Weigh, BS, US	43	41	49	47	39	43
BS, US	58	56	59	57	53	57
Weigh, BS, US, Introduce bulls	84	82	84	82	84	84
BS, US	95	93	98	96	102	96
Weigh, US, BS	119	117	129	127	131	127
US, BS	129	127	147	145	145	145
Weigh, US, BS	154	152	176	174	172	172
US, BS	168	166	190	188	187	187

Activity	Replicate					Median day
	1	2	3	4	5	
Weigh, US, BS	199	197	217	215	222	215
Bull removed, US, BS	217	215	232	230	246	230
Final US	287	285	281	279	294	285
Weigh	365	363	394	392	395	392
Slaughter	366	364	395	393	396	393

US = transrectal ultrasound examination

BS = Blood sample

9.2 Project outputs

Abstract: Cavalieri, J (2016) Chemical sterilisation of animals: A review of the use of zinc- and $CaCl_2$ based solutions in male and female animals and factors likely to improve responses to treatment. Animal Reproduction Science 169, 119-120.

Manuscript: Cavalieri, J (2016) Chemical sterilisation of animals: A review of the use of zinc- and $CaCl_2$ based solutions in male and female animals and factors likely to improve responses to treatment. Animal Reproduction Science (to be submitted August 2016).

Manuscript: Cavalieri, J, Hayes, L (2016) Examination of the use of intraovarian administration of $CaCl_2$ and zinc gluconate as potential chemosterilants in *Bos indicus* heifers. Australian Veterinary Journal (to be submitted August 2016).

Poster: Applied Andrology conference, Tours, France 2016.

Chemical sterilisation of animals: A review of the use of zinc- and $CaCl_2$ - based solutions in male and female animals and factors likely to improve responses to treatment

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Introduction

Sterilisation of animals: helps to control animal numbers, gene transfer, modify animal behaviour and carcass composition.

Potential advantages of chemical sterilisation:

- Single, low cost, permanent method of sterilisation
- Requires no or only light sedation
- Avoids surgery and its potential complications
- An alternative where surgical castration is not preferred

Responses in males

Chemosterilants: Solutions containing zinc salts (zinc gluconate and zinc acetate) or calcium ($CaCl_2$) have been most widely used in male animals but have seldomly been applied in female animals. Responses to treatments in male and females have been variable and are reviewed.

Zinc gluconate: Efficacy in male dogs is high. Azoospermia occurred in >80% of dogs in most studies,[1,2,3] 73% of cats,[4] but epididymal sperm were found in 87.5% (7/8) of adult bears.[5]

In *Bos indicus* calves treated with zinc acetate 57.9% (11/19) became azoospermic, total testicular mass was reduced but effects were variable (Fig. 1).[6]

Adverse reactions: In dogs these are uncommon but include transient testicular swelling, scrotal pain (6.3%, n = 270), orchitis, scrotal ulceration (1 to 6%), vomiting (4.4%), anorexia (4.0%), lethargy (2.2%) and an abnormal gait.[2,3,7,8]

Pain: Is uncommon in dogs[2,3] and cats[4] treated with zinc gluconate but was observed in some calves treated with zinc acetate.[6]

Testosterone: Decreased in calves treated with zinc acetate[6] and in some but not all studies conducted with dogs treated with zinc gluconate.[1,2,9]

$CaCl_2$: Decreased circulating concentrations of testosterone in rats,[10] goats[11] and dogs[12,13] along with a dose-dependent decrease in testicular mass.

Side effects such as transient scrotal swelling and ulceration, testicular necrosis have been observed.[12,13,15]

Reductions in testicular mass appear less when testicular size is large, for example in bulls[14] and donkeys.[15]

Literature cited:

[1]Anim Reprod Sci, 2012;132:207-212
[2]USFDA, 1993 http://www.fda.gov/downloads/AnimalVeterinary/Products/ApprovedAnimalDrugProducts/FOIADrugSummaries/ ucm116024.pdf
[3]Proceedings of the 3rd ACC&D International Symposium on Non-surgical contraceptive methods for Pet Population Control, 2006;
[4]Vet J, 2013;197:307-31
[5]Theriogenology, 2011; 75:1444-1452
[6]Anim Reprod Sci, 2015; 156:23-33
[7]Am J Vet Res, 2008; 69:140-143
[8]Vet Pathol, 2014; 51: 820-823
[9]Theriogenology, 2015; 83: 1021-1027;
[10]Contraception, 2006; 73:289-300
[11]Anim Reprod Sci, 2005; 86: 89-108
[12]Acta Veterinaria Scandinavica, 2014; 56:1-7
[13]Acta Vet Scand, 2014;56:62;
[14]Revue de médecine vétérinaire, 2006; 157:420
[15]BMC veterinary research, 2016; 12:1
[16]Arksciences, 2014b. Zeuterin™ Injectable Solution package insert, ArkSciences, Irvington, NY.

Responses in females

Bos indicus heifers were treated with intraovarian injections of either saline (n = 10), $CaCl_2$ (in 95% ethanol; n = 10), zinc gluconate, buffered in arginine (ZG; n = 10), or a combination of $CaCl_2$ and zinc gluconate in an aqueous solution (Ca + ZG; n = 10).

- Total ovarian mass was significantly less in heifers treated with $CaCl_2$ than heifers in the other treatment groups
- Complete atrophy of one ovary was observed in 40% (4/10) of heifers treated with $CaCl_2$ alone
- No significant differences in the total oocyte count or concentrations of anti-Müllerian hormone were found between treatments
- Pain responses were observed in ZG and ZG + $CaCl_2$ treated heifers but not observed in saline or $CaCl_2$ treated heifers

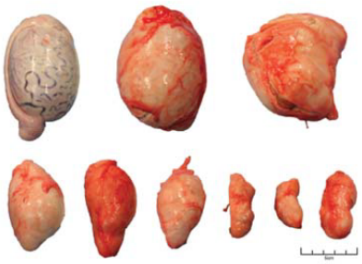

Fig. 1. Representative images of testes of *Bos indicus* bull calves treated with saline (upper left) or zinc acetate 860 days before slaughter. The images illustrate the variation and range in sizes of testes recovered from calves treated with zinc acetate.[6]

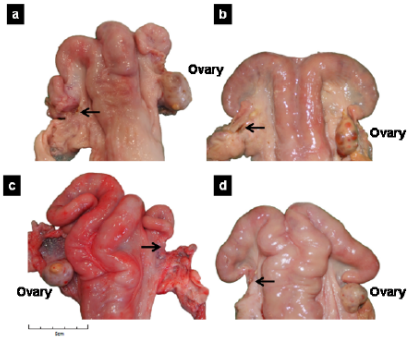

Fig. 2. Reproductive tracts of heifers treated with an intraovarian injection of $CaCl_2$ illustrating complete atrophy of one ovary (→; left: a, b, d; right: c) and failure of treatment in other ovaries.

Factors affecting responses

- A new, small diameter needle should be used on each testis
- Adequate restraint to avoid movement during administration. If needed light sedation should be used
- Pressure on the testes at the time of injection should be avoided by holding the skin below the testis rather than the testis themselves.
- Injection into the scrotal sac or skin must be avoided
- The chemosterilant should be administered slowly
- Administration should cease if resistance to injection is encountered
- The volume administered should be adjusted to the size of the testis; pain, scrotal necrosis are more likely with excessive doses
- On completion of injection, a 3 second pause should be observed and the needle removed rapidly[16]
- Responses may be better in younger animals or animals with small testes. Avoid animals with large testes
- With $CaCl_2$, an alcohol base maybe more effective than an aqueous base
- In females: further research is needed but guidelines in males or a modified approach may be applicable

Conclusion

- Chemical sterilisation can play a role in the sterilisation of animals but careful attention to dose, volume, chemical composition, administration and technique are needed to avoid adverse side effects such as pain and scrotal necrosis.
- Use in large animals is likely to be hampered by the need for careful administration, failure to suppress testosterone synthesis in every animal and the slower speed by which the technique can be carried out compared to surgical castration.
- More variable responses in animals with larger testes may mean that the technique is less satisfactory in animals with larger sized testes.
- Chemosterilants such as $CaCl_2$ have potential for application in female animals as a means of sterilisation but further research is needed to determine appropriate dose rates and optimum administration techniques.
- While zinc gluconate can be successfully used in males it appears to be ineffective as a potential chemosterilant in females and can cause pain.

Acknowledgement:

This project was funded in part by Meat and Livestock Australia Ltd Project #B.AWW.0219.

10 Acknowledgements

This project was funded by Meat and Livestock Australia Ltd Project #B.AWW.0219. The help of Martin Holzwart in managing cattle and Tony Parker and Andrew Krockenberger in providing access to animals is very much appreciated. Expert technical assistance was provided by Scott Blyth, Nigel Breen, Rob Jack, Tennille Otremba, Jeff Palpratt, Jo Penny, Laurie Reilly, Virginia Simpson, and Sally Watts. The efforts of Greg Walsh and Ian Johnson in provided assistance in video capture of behaviour and Lynda Hayes with histopathological examination of ovaries is greatly appreciated. We thank Paul Hemsworth for advice on aversion testing.