Preparturient caesarean section in the bitch: justification, timing, execution and outcome evaluation

By

Kurt Guido Mireille De Cramer

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Acknowledgments

The need for this study evolved from the dilemma in my own professional life. The dilemma of the after-hour caesarean section intervention in the absence of support staff. The dilemma of the ever-present emergency caesarean section and elusive planned fixed time elective caesarean section. The knowledge that the human obstetrician had solved the problem suggested that the same luxury may potentially be afforded to the veterinary obstetrician. The driving force behind this study was a combination of stubborn and brazen intent to solve the problem, an unabating passion for my work and the desire to, albeit a very small contribution, contribute to matters of veterinary advancement.

So, started an arduous path spanning more than two decades with my supervisor, Professor Johan Nöthling. He ensured that the academic banquet was filled with clear achievable goals, publishable data and practical approaches. His ability to always see an angle that I had missed, never failed to impress. His quest for meticulous planning helped me avoid both academic and ethical potholes. Because of my natural tendency to gravitate towards the innate practical clinician in me, he had hard work to jolt the researcher in me. Despite all, he magnificently managed to harmonise academic discipline with the freedom to explore responsibly on my own. After this all, I unashamedly announce that I am proud of the work we have done and our thesis. I have no regrets that the path has been this long. The problem required a sober incremental approach with matured candidates. Johan, the biggest thanks of all belongs to you. I hereby express my sincere gratitude for all you have done. It suffices to say that I could not have done this with anyone but you. However, somehow, I think it was not our last round!

I thank Professor Martin Schulman and Daniela Steckler for their valuable moral support, help and friendship. Having walked the same doctoral path, the knowledge that they had understanding of my “suffering” was both comforting and inspiring. Thank you also to Rachel who understands the sweet and sour of my profession. I thank Professor Jeffrey de Gier for eloquently translating the summary to Dutch. This translation is the only tiny bit of proof that I have left of my heritage in this thesis and Jeffrey made it just perfect. Thank you also to Org, the human obstetrician, who’s brain I picked along the way and always answered promptly how he does it in the real world. I thank Darren, my training partner of very many years, to help keep body, mind and soul vitalised with interesting new routines and motivating me to keep on doing it. To my long-lost friend, Werner Barnard who always insisted that I balance effort with reward and cautioned of the dangers if you get it wrong, thank you for that wisdom and for caring.

My colleagues at work Freek, Garreth, Tania, Gena, Roxanne, Marelize were involved from

its inception and later Corli and Karen were all very much part of this research and great people to work with. They all helped; collect data, monitor oestrous cycles, collect blood samples and helped with many other duties this research entailed. I thank you all for the good work you did, but most of all, for sharing my enthusiasm. Although I wish to think that I brought excitement and spice, I am very much aware that I added more to an already very busy environment. Sometimes it was not easy. Thanks for putting up with me guys.

I am indebted to the co-authors of the various chapters in this manuscript. Johan always made mammoth contributions to all aspects of the articles but with special reference to statistical manipulation of the data, his contributions were immaculate and invaluable. All those in academia that know Johan's flair with statistical analyses, concede that he is undeniably just a cut above the rest. Carolynne impressed with her ability to produce independently. Kenneth more than impressed with his practical insights and very rapid response time. I feel honoured to have worked with individuals of your stature. I feel indebted to the very many researchers before me who are referenced in the text. I am truly in awe of the great work they have done.

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The owners of the bitches were just fabulous. They just knowingly went along with all I did and planned. Always trusting me, always believing in me. Thank you for that.

To my mom, Suzanne, whom through failing health will not be able to read the content of this thesis, I thank you mom for all that you have done, wished for us and tried to do, in our early days, despite very challenging circumstances. I will always remember her reminding us that the spine in our book is not nearly as important as the spine in our back. To my dad Elias, I hope he is as proud of this work as I am of the work he did for me. I am surrounded by the technical gadgets he made for me, that helped me in my work and at home. I am so fortunate to have inherited some of his good hands. Even though I want to, I cannot forget the many hours he toiled, showing us what old-school work ethics was all about. Thank you also to my niece, Inge, who shares the passions of my world and always thinks that I am the greatest, even if I am not.

Thank you to my many siblings; Chantal, Geert, Ingrid, Donald, Caroline, Gretel, Jan and Hellmut for always supporting me, and each other, no matter what. I might have earned their respect for raising the academic flag in the family? They earned my respect for proving it is not a prerequisite!

I owe gratitude to my lovely wife Zelda. I am so proud of all her stunning creations in her art studio and the Eden she created in the garden with her green fingers. It inspired me to create in my world as well. Her love for all God's little creatures is surpassed by none. She was always fully supportive of my endeavours. Because it was important to me, it was important to her. She has always been a most splendid parent to our children Kyle and Mira, whilst I was not. They were victim of a frequently absent father, albeit at work or at home, behind a computer. I see little sparks of the budding investigators in them, in the engineering careers they have chosen. I wish to think I had something to do with that. It is my sincere hope that the work ethics they witnessed will motivate them in their own lives to distinguish themselves, ask questions and seek the answers irrespective of the efforts by others and experience gratification in both the journey and goal, as I have. I thank them for the gracious response en-route.

Finally, I owe gratitude to our Creator whom has given me both the tools and opportunity and always rewards my efforts.

This poem and this thesis is dedicated to the dog as a species, perhaps the most magnificent and diverse species on earth, which has suffered greatly from our meddling. I wrote this poem in acknowledgement of our guilt. This thesis might not have been necessary if we had left it to nature. I however hope that it will lead to further research and improvement in the obstetric health of my beloved species.

Plight of the bitch to mankind

Near primitive man, did my ancestors dwell
With cousin Lupus, all was still well
Carefully in the shadows, for left-overs they looked
Forever following, when mankind cooked
So, over time, developed this -symbiosis
Now I can tell of, our grand -metamorphosis
Since that first wolf-litter, was raised by man
On natural selection, there was a ban
Until then natures fittest, had been our mate
But now it was mankind, which determined our fate
What emerged was variety, oh, so great
In breeds, we number, so many to date
Each were selected, for an eccentric trait
Long, smooth, curly or hairless, our coats could be
Leaving each country, with their own to see
From tall big and bulky, to the smallest toy
The hearts of our masters, we fill with joy
Within our ranks, stand proud the terrier
For some it would seem, the more the merrier
Amongst us some were, selected for sight
Strengthening our masters, in their hunting might
On their farms and travels, this need emerged
Tiss no surprise, herd loving breeds diverged
So please mankind, do understand our plight
As this matter is not clear, in black and white

With us normal birth, is always desired
For some C-sections, are sometimes required
So, this forms the base, of our plight
Tiss all in your hands, and not in our might
Those genes in us, you so have found
Does not make nature's, perfect hound
For breathing we should, us all be able
Without a visit, to the operating table
Whilst our folds and wrinkles, endear so many
Tiss our wish for sure, we did not have any
Short crooked legs, and a very long back
May lead to weak disc, and a surgeon with knack
Our ears are in some, not upright erect
Causing discomfort, and meds to correct
No longer do we search, and hunt for our meal
Explaining why often, our weight's not ideal
It's probable this change is, forever and ever
Going back to the wild, is a futile endeavour
So, with this I do not, proportion you blame
But making us better, should be your first aim
We don't mind the oddities, which make us so cute
But there are some genes, which you should permute
Tiss thought that variety, in genes do protect
So, from changing nature, we could resurrect
When we are too few, the breed is called rare
That's when new genes, become a desperate affair
We know that you love us, and want us to thrive
Please therefore keep in us, some wolf genes alive
Because of your choices, determining our fate
We are now having, this important debate
So please mankind, do understand my plight
Against bad genes, you all should unite

Kurt De Cramer 2015

Declaration of originality

Full names of the student: Kurt Guido Mireille De Cramer

Student number: 81267046

Declaration:

1. I understand what plagiarism is and am aware of the University's policy in this regard

2. I declare that this thesis is my own original work. Where other people's work has been used (either from a printed source, Internet or any other source), this has been properly acknowledged and referenced in accordance with departmental requirements.

3. I have not used work previously produced by another student or any other person to hand in as my own.

4. I have not allowed, and will not allow, anyone to copy my work with the intention of passing it off as his or her own work.

Signature student: 

Signature Supervisor:

February 2017

Ethics statement

I, Kurt Guido Mireille De Cramer, the author of this thesis have obtained, for the research of this work, the applicable research approval required by the Faculty of Veterinary Science of the University of Pretoria's ethics and animal use committees. The approval certificates are; v071-13, v010-14, v010-14 (Amend 1), v048-14, v048-14 (Amend 1), v021-15, v079-15 and respective copies thereof can be viewed in the addendum. For the survey study Chapter 3, I obtained permission from the Dean. I further declare that I have observed the ethical standards required in terms of the University of Pretoria's *Code of ethics for researchers* and the *Policy guidelines for responsible research*.

Signed 

March 2017

Definitions and synonyms used in text

Where synonyms apply, preferred terms are used in this manuscript. In the case of the absence to reference of species in the text, it may be assumed that the species referred to is the domestic dog. When the word canine is used, it refers to the domestic dog unless specified otherwise. Based on the preference of British Small Animal Veterinary Association, the word veterinary surgeon will be used in preference to veterinarian, to describe a person who is duly qualified and registered to practice veterinary medicine or clinician to refer to a veterinary surgeon in clinical practice. When reference is made to a veterinary obstetrician, it refers to a veterinary surgeon that is dealing with an obstetric case and is the preferred word in this thesis. The words "veterinary obstetrician" in this thesis, do not imply any form of specialisation. In order to avoid confusion, the reader should be familiarised with the terminology regarding the timing of intervention by caesarean section.

Concepts of preterm, early term, term, late term and post term when referring to pregnancy in humans

The period around the estimated date of delivery in humans has been divided into the designations: Preterm (before 37 0/7 weeks of gestation), early term (37 0/7 weeks of gestation through 38 6/7 weeks of gestation), full term used synonymously as term (39 0/7 weeks of gestation through 40 6/7 weeks of gestation), late term (41 0/7 weeks through 41 6/7 weeks of gestation) and post term (42 0/7 weeks of gestation and beyond) (Spong, 2013). This classification system was adopted by the American college of obstetricians and gynaecologists committee ([ACOG] American College of Obstetricians and Gynaecologists, 2015). The World Health Organization's definition of "term", is the age at birth that is supposed to have the lowest neonatal risk (De Luca et al., 2009).There are however other classifications systems with minor differences (Fleischman et al., 2010a; Zegers-Hochschild et al., 2009). These differences entail the number of days in early term, term (also known as full term) and late term. There is however consensus about preterm being before 37 0/7 weeks and post term being after 42 0/7 weeks (Fleischman et al., 2010a; Zegers-Hochschild et al., 2009; Spong, 2013). These classification systems were decided upon by comparing the risk of mortality in births between term births (combination of early term, full term and late term births)—seen as one group— to both the preterm and post term groups, respectively. There is significance in subcategorizing early term, full term and late

term. The clinical significance in humans is that the early term subgroup benefits from prenatal maternal corticosteroid administration (corticosteroid priming) whereas these benefits are less clear in the full term and late term subgroup (Hansen et al., 2008). From this discussion, in the human, the safe period wherein the human foetus may be delivered, has been well defined and subcategorised based on outcomes of various sub-categories of gestational age deliveries in very large studies (Fleischman et al., 2010a; Zegers-Hochschild et al., 2009; Spong, 2013; Hansen et al., 2008). The safe period of intervention in the human is thus synonymous with "term" which is sometimes used as the collective noun for early term, full term and late term. To summarise, in humans a full-term or term-birth is a live birth or stillbirth that takes place between 37 completed and 42 completed weeks of gestational age (Zegers-Hochschild et al., 2009). An important implication of the subcategories term and preterm is, that foetuses delivered preterm are considered premature and foetuses delivered from term gestations, are considered mature.

Concepts of full term, term and preterm when referring to pregnancy in dogs

In contrast to the terminology used in the human, in dogs, a full-term birth would be a birth where the bitch has completed her entire gestation and not only a portion thereof. Currently, in veterinary nomenclature, a pregnant bitch is either preterm, full term or post term. Preterm being at any time before onset of spontaneous parturition, full term being at the onset of spontaneous parturition and post term is when the duration of gestation in a bitch has extended beyond the maximum range of the predicted date of parturition in conjunction with foetal demise. The problem with this terminology is the following. If a caesarean section (CS) were to be performed in a bitch at the time of onset of cervical dilatation (thus late stage one of parturition) this intervention would be considered a full-term intervention. However, if the CS were to be performed at hypothetically six hours before the onset of cervical dilatation (generally considered a preparturient CS), in veterinary obstetrics, this intervention would be considered a "preterm" intervention. The problem with this approach is that the foetus delivered from the latter intervention would not necessarily be premature, yet—given the understanding of the term with respect to the human—the word "preterm", implies it would be premature. Therefore, before the full extent of the safe period of intervention in the dog is known, one cannot refer to preterm or term in the dog. We can however say that if a preparturient CS were to result in mature-looking puppies and these puppies have survival rates comparable to the survival rates of puppies delivered by

parturient CS, that the preparturient CS likely occurred within this unknown period of safe intervention “term”. In veterinary obstetrics relating to the bitch, we will only be able to use the word “term” correctly once the full extent of the safe period of intervention “term” is known in the dog. In this manuscript, the safe period of intervention by CS in the dog is defined as that period before the onset of spontaneous parturition wherein foetuses may be delivered by CS and have survival rates comparable to those of puppies delivered by parturient CS.

This discussion on terminology at the onset of this thesis was necessary because it deals with a new concept in veterinary obstetrics of the bitch namely “preparturient” CS. A preparturient CS is defined as a CS that is performed before the onset of cervical dilatation. The puppies delivered from a preparturient CS may be fully mature or premature depending on whether the intervention took place within the safe period of intervention by CS or not. Planned term caesarean sections are only possible in a species when both the safe period of intervention is known and a method to precisely determine that the gestational age of the foetuses have advanced to within that critical period

List of abbreviations and acronyms

bpm	Beats per minute
BPD	Biparietal diameter
CI	Confidence interval
CLIA	Chemiluminescent immunoassay
CRL	Crown rump length
CS	Caesarean section
CSs	Caesarean sections
CV	Coefficient of variation
D0	Day one of cytological dioestrus
DCD	Day of first appearance of any degree of cervical dilatation
FC	Female control
FF	Female foetus
FNF	False negative fraction
FPF	False positive fraction
FTA	Fast technology for analysis of nucleic acids
Htafter	Haematocrit after
Htbefore	Haematocrit before
ICC	Inner diameter of the chorionic cavity
ISAG	International Society for Animal Genetics
LH1	First or only day of the LH surge
MC	Male control
MF	Male foetus
nonTOLAC	Not by trial of labour after caesarean section
NPV	Negative predictive value
OR	Odds ratio
P0	Day of spontaneous parturition, due date or whelping date
PC6	First day that PC rises above 6 nmol/L
PaO2	Partial pressure of oxygen
PC	Progesterone concentration in blood (plasma or

	serum)
PC16	First day that PC exceeds 16 nmol/L
PCR	Polymerase Chain Reaction
PPV	Positive predictive value
progRIA	Concentration of progesterone in serum or plasma of bitches determined by RIA
progImm	Concentration of progesterone in serum or plasma of bitches determined by Immulite
RCS	Repeat caesarean section
RR	Relative risk
SD	Standard Deviation
SE	Standard Error or Sensitivity
SEM	Standard Error of the Mean
SP	Specificity
SPO2	Percentage saturation of oxygen bound to haemoglobin
SRY	Sex determining Region of Y-Chromosome
STR	Short tandem repeat
TCD	Time of first appearance of any degree of cervical dilatation
TOLAC	Trial of labour after caesarean section

Summary

Preparturient caesarean section in the bitch: justification, timing, execution and outcome evaluation

By

KURT GUIDO MIREILLE DE CRAMER

Supervisor: Prof J O Nöthling
Department: Production Animal Studies
Degree: PhD

It is known that the prevalence of CSs in some breeds approaches 100%. In addition, this study identified previous CSs, fewer than eight and more than 11 puppies per litter in Boerboel bitches as obstetric risk factors (resulting in emergency CSs and stillbirths). It also showed that a trial of labour after caesarean section was associated with considerable obstetric risks. In such high-risk pregnancies, using the signs of parturition to time elective CSs in the bitch is problematic, as by then there may already be foetal distress or demise. Also, the signs of parturition may present at an inconvenient time of the day, when staff shortages may impede professional service. This study aimed at solving these problems by investigating various means of predicting the day and time of onset of parturition (cervical dilatation) in the bitch. This study showed that a chemiluminescent immunoassay (Immulite® 1000 LKPW1) is a reliable replacement for a discontinued radio immune assay to measure PC in serum or plasma. It proved that the first day of cytological dioestrus (D0) is the most precise peri-oestrous predictor of the day of cervical dilatation. This study showed that the variation in the foetal biparietal diameter is too large to accurately predict readiness for CS and that the preparturient PC may be used to predict the time of cervical dilatation, thereby allowing timeous planned CSs in bitches where D0 is unknown. The use of medetomidine hydrochloride as premedicant combined with propofol as induction agent and sevoflurane as maintenance, is safe and is associated with good maternal and puppy survival rates at delivery, 2 h and 7 d after CSs. Performing elective CSs upon the first appearance of any degree of cervical dilatation proved successful. For bitches in this study

with high-risk pregnancies, we proved that it is safe to perform fixed date preparturient CSs on D57 if the cervix has not dilated by then. This study showed that bitches have haematocrits at the time of cervical dilatation that are at the lower end of the normal reference ranges for non-pregnant dogs and that the decline in haematocrit associated with CS is similar for parturient (open cervix) and preparturient (closed cervix) CSs. As incidental findings, this study discovered two puppies sharing one placenta in each of two litters. The one case of placenta sharing proved to be monozygotic twins whilst the other case proved to be a case of dizygotic monochorionic canine foetuses with blood chimaerism and suspected freemartinism. This study provides the veterinary obstetrician with a protocol that can be used to safely perform elective CSs in a large proportion of the obstetric population at a convenient time of the day but more research is required with larger numbers to establish whether this practice is routinely safe and safe in all breeds.

Opsomming

Preparturiënte keisersnit in die teef: regverdiging, tydsberekening, uitvoering en uitkomsevaluering

Deur

KURT GUIDO MIREILLE DE CRAMER

Promotor: Prof J O Nöthling

Departement: Produksiedierstudies

Graad: PhD

Die voorkoms van keisersnitte bereik 100% in sommige rasse. Hierdie studie het getoon dat 'n vorige keisersnit, kleiner werpsels as agt en groter werpsels as 11 in die Boerboel, verloskundige risikofaktore is. Dit het ook getoon dat 'n poging tot spontane kraam nadat 'n teef voorheen 'n keisersnit ondergaan het, geassosieer is met 'n aansienlike risiko van nood-keisersnitte en doodgebore hondjies. Om in sulke hoë risiko dragtighede te wag vir tekens van kraam voordat 'n elektiewe keisersnit uitgevoer word is riskant, omdat fetale nood of dood reeds kon intree. Die tekens van kraam verskyn dikwels op 'n ongeleë tyd van die dag, wanneer 'n personeeltekort 'n professionele diens belemmer. Hierdie studie was daarop gemik om hierdie probleme op te los deur verskeie metodes te ondersoek om die dag en tyd van aanvang van servikale ontsluiting in die teef te voorspel. Verskeie bevindings spruit uit die studie: 'n Chemiluminessensie immunotoets (Immulite® 1000 LKPW1) is 'n betroubare plaasvervanger vir 'n gestaakte radioimmunotoets. Die eerste dag van sitologiese diestrus (D0) is die mees presiese en praktiese peri-estrus voorspeller van die dag van servikale ontsluiting. Die bipariëtale deursnit van honde fetusse tydens laat dragtigheid varieer soveel binne rasse en binne werpsels dat dit ongeskik is vir die akkurate en betroubare voorspelling van gereedheid vir keisersneë. Die voorgeboortelike progesteroon konsentrasie in die bloedplasma of serum dien as 'n voorspeller van kraam in tewe waarvoor D0 nie bekend is nie. Die binneaarse toediening van 7 mg/kg medetomidine hidrochloried as premedikasie,

gekombineer met 1–2 mg/kg propofol as induksiemiddel en 2% sevoflurane in suurstof vir die onderhoud van narkose vir keisersnit in tewe is veilig en lewer hoë oorlewings peile in tewe en kleintjies by geboorte, 2 ure en 7 dae na keisersnit. Keisersnitte wat uitgevoer word sodra die eerste tekens van servikale ontsluiting waargeneem word, is suksesvol. Hierdie studie het getoon dat vir tewe met hoë risiko swangerskappe, dit veilig is om preparturiente keisersnitte uit te voer in tewe met n geslote serviks, sewe en vyftig dae (D57) na D0 vir tewe met werpsels > 1 as die serviks teen daardie tyd nog nie ontsluit het nie. Tewe se hematokrit aan die begin van kraam stem ooreen met die laer normale waardes van vir nie-dragtige honde en dat vermindering in hematokrit geassosieer met parturiente keisersnitte (oop serviks) soortgelyk is aan bloedverlies geassosieer met preparturiente (geslote serviks) keisersnitte. Toevallige bevindings was dat twee fetusse uit elk van twee werpsels ’n plasenta gedeel het. In een geval was die fetusse monosigotiese tweelinge en in die ander geval was hulle monochorionies en disigoties, met moontlike freemartinisme. Hierdie studie stel die veterinêre verloskundige in staat om elektiewe preparturiënte keisersnitte op ’n geleë tyd van die dag uit te voer in ’n hoë persentasie van die obstetriese populasie maar verdere navorsing met groter getalle word benodig om vas te stel of dit roetine gewys veilig is en veilig is in alle rasse.

Samenvatting

De antepartum uitgevoerde electieve keizersnede bij de hond: rechtvaardiging, optimale moment van uitvoeren, werkwijze en analyse van het resultaat.

Door

KURT GUIDO MIREILLE DE CRAMER

Promotor: Prof J O Nöthling

Departement: Studie van productiedieren

Graad: Doctor

De prevalentie van keizersneden benadert in sommige hondenrassen 100%. Uit dit onderzoek blijkt dat verschillende risicofactoren, zoals een eerder uitgevoerde keizersnede, kleine worpen (< 8 pups) en grote worpen (>11 pups) in de Boerboel ras, de kans op een spoedkeizersnede en doodgeboorte vergroten. Ook bleek uit de studie dat een natuurlijke geboorte na een eerdere keizersnede geassocieerd is met een toegenomen verloskundig risico. In dergelijke hoog risico drachten is het lastig om de voortekenen van een naderende geboorte te gebruiken om het moment van uitvoering van een electieve keizersnede vast te stellen, omdat er op dat moment al sprake kan zijn van foetale stress of pupsterfte. Tevens start de geboorte regelmatig op een tijdstip van de dag of nacht waarop onvoldoende geschoold personeel voorhanden is waardoor de kwaliteit van de professionele service in het geding kan komen. Dit onderzoek begon met het oplossen van deze problemen door methoden te evalueren waarmee de start van de geboorte (ontsluiting) kan worden voorspeld. Een chemiluminescentie immunoassay (Immulite® 1000 LKPW1) om de progesteronconcentratie (PC) te bepalen in plasma of serum bleek een betrouwbare

vervanger van de niet meer beschikbare radio immunoassay. De eerste dag van de cytologische metoestrus (D0) is de betrouwbaarste parameter in de periode rondom de oestrus om de dag van ontsluiting mee te voorspellen. In dit onderzoek werd ook aangetoond dat de variatie in biparietale diameter te groot is om betrouwbaar de gereedheid voor een keizersnede te voorspellen maar dat de PC antepartum wel gebruikt kan worden om het moment van ontsluiting te voorspellen, waardoor het tijdig plannen van keizersneden mogelijk wordt bij teven waarbij D0 onbekend is. Een anaesthesieprotocol met medetomidine als premedicatie, gevolgd door inductie met propofol en sevofluraan voor onderhoud is veilig en geassocieerd met hoge overlevingskansen voor zowel de moederdieren als de pups op 2 uur en 7 dagen na een keizersnede. Het uitvoeren van keizersneden op het eerste moment dat enige mate van ontsluiting waarneembaar was bleek succesvol. Het antepartum uitvoeren van electieve keizersneden bleek veilig bij drachtige teefjes met een verhoogd risico op D57 bij die teven waarbij op dat moment nog geen ontsluiting waarneembaar was. Wij hebben aangetoond dat de hematocriet bij teven op het moment van ontsluiting rondom de ondergrens van de referentiewaarden bij niet drachtige teven ligt en dat het vermindering van de hematocriet als gevolg van de KS niet verschilt tussen dieren waarbij de keizersnede werd uitgevoerd *in partu* (open cervix) en *antepartum* (gesloten cervix). Als toevallige bevindingen hebben wij twee voor de hond, zeldzame tweelingen, die beide één placenta deelden, ontdekt. In een geval ging het om een eeneiige tweeling en in het andere geval om een twee eiige tweeling waarbij bloedchimerisme werd aangetoond en die verdacht is van freemartinisme. De resultaten van dit onderzoek voorzien de veterinair obstetricus van een protocol waarmee keizersneden veilig uitgevoerd kunnen worden op een geschikt moment van de dag in een groot deel van de verloskundige bevolking maar meer onderzoek is nodig met grotere aantallen om vast te stellen of deze praktijk routinematig veilig is en veilig is in alle rassen.

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Chapter 1. General introduction

1.1. The dilemma of parturition management

The management of parturition in bitches is a frequent request in veterinary hospitals and veterinary surgeons with a special interest in canine reproduction (henceforth referred to as veterinary obstetricians), feel obliged to comply. An added consideration is the ethical obligation to concede to this demand when many of these bitches conceived by assisted reproductive techniques performed by the same veterinary obstetricians. Breeders expect the best possible outcome for each case, namely a litter of viable puppies and a live, healthy bitch that can produce litters in future (Smith, 2007). More specifically, in the context of this study, the term "outcome" following CS is used, and refers to short term (2 h) and medium term (7 d) maternal and puppy survival rates following CS.

The demand by owners for a favourable outcome following parturition in the bitch is driven by both financial and emotional considerations. Owners with experience of bitches with dystocia and related puppy mortality demand timeous intervention. Some owners are a long distance away from veterinary care and fear the extended transit time may compromise the litter in case of dystocia. Travelling at night to the veterinary facilities in some parts of the world is a serious security risk resulting in both clients and veterinary staff to avoid after-hour service. In addition, after-hours veterinary facilities available to clients may not be adequately equipped to appropriately deal with dystocia cases or to perform a CS. Some bitches and their litters are considered particularly valuable (Smith, 2007). The life style factors such as working-hours, night-hours commitments, planned holidays or business trips, may make it impossible for the owner to supervise the parturient bitch. Extrapolating from human obstetrics, many owners assume that carefully timed preparturient "term" elective CS in the bitch is possible as it is in women (Bergholt et al., 2004). Finally, some owners insist on a CS for their bitch and it has been proposed that as veterinary obstetricians we should be prepared and allowed to counsel, treat and respect a client's right to elect a CS for their bitch (Smith, 2007) as is the case with women (Ben-Meir et al., 2005; Bergholt et al., 2004; Husslein and Wertaschnigg, 2002).

The concept of a reliable protocol allowing planned preparturient CS resonates with the veterinary obstetrician for numerous reasons. Planned and timed CS obviates the need for after-hour interventions when staff shortages are a serious impediment to the execution of a

professional service. Some high-risk pregnancies do not show signs of impending parturition or the signs are subtle and difficult to recognise leading to foetal compromise. Parturition management is very time consuming. Parturition management exposes veterinary obstetricians to criticism if retrospectively intervention or lack thereof is speculated to have reduced puppy survival. As is the case with medical obstetricians (Ben-Meir et al., 2005; Bergholt et al., 2004; Husslein and Wertaschnigg, 2002) parturition management would allow veterinary obstetricians to perform CSs at times convenient to them.

For various reasons, there is a tendency for veterinary hospitals to discontinue after-hours services and refer their patients to alternative after-hour facilities. In many cases these facilities are staffed by less experienced veterinary obstetricians exposing them to possible allegations of misconduct and increasing the pressure on particularly the specialist theriogenologist in private practice, to avail themselves for after-hour assistance for obstetric cases. Complaints to veterinary regulatory bodies are not uncommon following unfavourable outcomes in alleged incompetent management of parturition. This has resulted in both academic institutions and veterinary obstetricians opting to leave the responsibility of managing parturition to the breeder with obvious associated complications resulting from failure to intervene timeously.

Veterinary supervised parturition management is problematic. Most veterinary hospitals do not have the capacity for 24 h around the clock supervision. Decision making in bitches presenting with dystocia or parturition is challenging in high risk pregnancies. Singleton litters present a unique challenge because they frequently fail to enter Stage 1 of parturition, go over-term and present with vaginal discharges and a dead foetus (Johnson, 2008a). Bitches in late pregnancy of which only mating dates are known, present a serious challenge and represent most of the parturition management requests. Veterinary obstetricians do not have at their disposal accurate objective timing criteria that will allow them to perform safe obstetric intervention in these bitches. Since the duration of pregnancy in the bitch is relatively short (Concannon et al., 1983; Johnson, 2008b) and the major part of foetal growth in the dog occurs in the last third of gestation (Evans and Sack, 1973; Salazar and Yllera, 1991; Moriyoshi et al., 1996), it is critical that foetuses are fully mature prior to delivery for them to survive. Premature delivery of puppies leads to fatal outcome for the puppies and potential danger of excessive uterine haemorrhage (Smith, 2007). Knowledge of the safe period of intervention, accurate prediction of parturition date and confirmation of imminent

parturition in the immediate preparturient period, all may prove helpful in managing parturition in bitch. This knowledge is known and commonly used in clinical practice in human obstetrics and was the main driving force in the current study for seeking the veterinary obstetric solution to planned elective preparturient CSs in the bitch.

1.2. Ethical considerations

Caesarean section in the bitch has in some circles raised ethical concerns because it is claimed that not all CS may be necessary, that CS is an unnatural artificial intervention and by doing so we are artificially propagating the very same genes which prompted the assistance in the first place. A classic example of this is artificial insemination and CS in brachycephalic and some other breeds. However, until such time that this dilemma is resolved, veterinary obstetricians will continue to be confronted with the request for planned CS in these breeds. Ethical concerns are also raised about pain control before, during and after CS. The matter of pain control for CS brings the theriogenologist in conflict with the anaesthesiologist. This is because the administration of non-steroidal anti-inflammatory drugs (NSAID), opioids and other analgesic agents may either suppress cardiorespiratory function or adversely affect the newborn puppies. Also, some theriogenologists have strong objections against epidural analgesia for CS in the bitch because it extends the duration of the procedure, causes hypotension, may hinder post-operative ambulation and may delay time of discharge. In some countries, surgical intra-uterine artificial insemination is prohibited by law because of strong objections against exposing the bitch to surgical and anaesthetic risks for the sake of artificial reproduction in aid of supposedly little else but financial gain for their owners. The same objections are now emerging in some countries against CS. The objections to CS in the veterinary profession mirrors those encountered in the medical profession where concerns are expressed about unnecessary CS performed in woman and exposing mother and babies to unnecessary surgical and anaesthetic risk, again in many cases supposedly only to convenience patient and hospital staff. It is also alleged that the medical profession may have perverse financial intent in recommending CS for non-medical reasons (Ben-Meir et al., 2005). However, fear of possible complications associated with vaginal births and possible litigation, influences the recommendation made by the human obstetrician. Similarly, in the veterinary profession, it has been suggested that many veterinary obstetricians might be inclined to opt too soon for a CS. This is because they might feel more comfortable in performing a surgery to preclude puppy losses because

of an expected dystocia (Taverne and Van Der Weijden, 2008). In most breeds, most litters will always be born naturally with the occasional emergency CS. A successful and uncomplicated vaginal delivery of the conceptus forms the final step of the reproductive cycle in mammals and should always be the ultimate aim (Taverne and Van Der Weijden, 2008). In this thesis, the research aimed at preparturient CS is considered justified if performed on pregnancies where CS is considered the only safe way of delivery.

1.3. Overall aims of this thesis

The overall aim of the studies in this thesis is to increase our knowledge in order to identify high-risk pregnancies in the bitch, improve parturition management, obviate the need for full-time observation, accurately time a preparturient elective CS and safely perform it.

1.4. Problems in need of investigation in this study

1.4.1. A need to gather evidence on incidence of caesarean sections in bitches

A plethora of reports (with sufficiently large numbers of subjects) are available in humans on trial of labour, trial of labour after CS also known as vaginal birth after CS, vaginal birth and repeat CS (Curtin et al., 2000; Mahoney et al., 2010; Stone et al., 2000). This enables the medical profession to provide pregnant patients with accurate prediction on probabilities of outcomes in trial of labour, vaginal birth after CS and repeat CS. Hence, human obstetricians can advise confidently and pregnant woman can subsequently make informed decisions, all based on these very large studies. Unfortunately, this information is not available with respect to bitches.

There is a need for information in the bitch on the outcome of trial of labour after CS, the proportion of bitches requiring CS and the proportion of litters delivered by CS in different breeds, the effect of a CS to deliver the previous litter on the need for a CS to deliver the current litter, the proportion of stillborn puppies with vaginal delivery, emergency CS and elective CS on parturient bitches. This will enable the veterinary obstetrician to better predict which bitches are likely to experience dystocia and may require CS (categorise bitches in either general obstetric population or high risk pregnancy subpopulation) and allow the veterinary obstetrician and the bitch owner to make informed decisions regarding method of delivery (trial of labour after CS) based on these predictions.

1.4.2. A need to establish whether the use of low dose medetomidine premedication for caesarean section in the bitch is safe

The use of alpha2-adrenergic agonist prior to anaesthesia for CS is controversial. This is because xylazine was identified as a risk factor for increased puppy mortality (Navarro and Friedman, 1975; Moon et al., 2000) and which is associated with an increased risk of death in the dog (Clarke and Hall, 1990; Dyson et al., 1998). In more recent surveys however, premedication with medetomidine was not identified as an increased risk factor (Brodbelt et al., 2008b). Using alpha2-adrenergic agonists as premedicants significantly decreases the dosages required to induce and maintain anaesthesia (Hammond and England, 1994), reduces the risk of induction apnoea and makes anaesthesia more cost effective. The analgesia following a single dose of alpha2-adrenergic agonists is unquestionable. Medetomidine has the advantage that it can be quickly and completely reversed using its specific antagonist atipamizole ("Antisedan," Zoetis, South Africa) in both the bitch and the puppies immediately after delivery. Despite all the potential benefits of medetomidine as premedicant for CS in the bitch, its safety for the puppies and the bitch needs to be conclusively established.

1.4.3. A need to establish normal haematocrit values and ranges in bitches before and after CS

Knowledge on normal range pre- and post CS haematocrits may assist veterinary obstetricians in risk assessment. Despite the pioneering studies by Concannon and co-workers whom initially reported the anaemia of pregnancy (Concannon et al., 1977b), our knowledge of the haematocrit of periparturient bitches is incomplete. We also do not have accurate normal ranges of haematocrits of bitches that underwent CS. Without reference values of haematocrits in periparturient bitches and the change thereof during CS, the veterinary obstetrician has no clear guidelines by which to identify danger indices regarding haematocrit and undue blood loss during CS.

The establishment of baseline data is important for future comparisons. For instance, the extent of blood loss may be different when a CS is performed as opposed to natural delivery, or when the placentas are left in the uterus as opposed to being removed during CS or when an ovariohysterectomy is performed at the time of CS as opposed to when not. The latter were not studied in the current study but are mentioned as potential topics for future research.

Of greater importance to the entire PhD study was to evaluate whether preparturient CS influenced maternal haematocrit and thus whether preparturient CS poses a greater risk of post CS haemorrhage than does parturient CS. If we do not have the normal haematocrit values following parturient CS, we will not be able to compare it to preparturient CS haematocrits.

1.4.4. A need to quantify the variability of biparietal diameter of dog foetuses at the time of birth within breeds

The biparietal diameter (BPD) of human foetuses is routinely used to estimate the date of delivery. Batista et al. (2014) concluded that the attainment of a minimum BPD of dog foetuses during late gestation confirms their readiness for delivery. The conclusion of Batista et al. would pose a clinical risk if not all viable foetuses attain the proposed minimum BPD prior to birth or if some potentially viable foetuses attain it too soon to survive if delivered. Given the clinical importance of these risks, there is a need to quantify the variability of BPD of dog foetuses at the time of birth within breeds.

1.4.5. A need to compare the progesterone concentration in serum as determined with the Immulite® 1000 LKPW1 chemiluminescent immuno assay to the PC in plasma as determined with the Coat-A-Count RIA

The Coat-A-Count RIA for progesterone and the Immulite 1000 were compared by Kutzler et al. (2003) and Volkmann (2006) and both have been widely used in practice and research. The former was due to be withdrawn from the market by the end of 2014 and the latter changed in 2012, from when onwards it was known as Immulite 1000 LKPW1 (Schmicke et al., 2016). There thus was a need to compare PC measured with the Coat-A-Count RIA in plasma of bitches with those measured in serum with the Immulite 1000 LKPW1 assay before Coat-A-Count progesterone assay was removed from the market.

1.4.6. A need to determine the agreement between the days on which PC determined with Immulite LKPW1 and Coat-A-Count RIA respectively reached 6 or 16 nmol/L, and the PC determined by each on LH1

Having determined that the PC as determined by Immulite 1000 LKPW1 does not perfectly agree with that determined with the Coat-A-Count RIA, and that the former was generally lower than the latter, the need arose to compare the agreement of the days on which PC

measured with each would reach 6 or 16 nmol/L, and the PC determined with each on LH1.

1.4.7. A need to assess the precision of observations made during the peri oestrous period to predict the day of parturition

Levy et al. (2009) and Vannuchi et al. (2012) performed preparturient CSs in 37 and three bitches, respectively at known intervals after PC or LH had reached threshold concentrations during oestrus, from which they estimated when ovulation would occur. Levy et al. estimated the extent to which the CSs they performed curtailed the duration of pregnancy by subtracting interval between the estimated day of ovulation and CS from the generally accepted mean interval between ovulation and parturition. Vannuchi et al. estimated the number of days by which the CSs shortened gestation by comparing these intervals to the intervals between PC or LH having reached the same thresholds and parturition in their three control bitches. The precision with which PC and LH, as they used it, would predict the day of parturition is unknown.

Apart from Levy et al. (2009) and Vannuchi et al. (2012) who timed preparturient CSs based on events during oestrus, various studies report the precision of the intervals between peri-oestrous events such as the LH surge, the LH peak, first rise of progesterone above 19 nmol/L (Levy et al., 2009), ovulation and the onset of cytological dioestrus and parturition. The precision of these variables in predicting parturition has not adequately been compared.

In bitches with a high-risk pregnancy, where an elective CS is planned, it may be convenient to perform a preparturient CS because foetal compromise may already be present at the earliest signs of impending parturition (personal observation) and may be the motive behind a substantial proportion of pregnant brachycephalic bitches that undergo elective CS before natural parturition begins (Evans and Adams, 2010). A precise method of predicting the day of parturition will limit the time spent observing such bitches during the preparturient period in order to identify the optimal time for the CS.

1.4.8. A need to assess the precision of observations made during the preparturient period to predict the day and time of the onset of parturition

A precise method of predicting the date and time of the onset of parturition (as identified by the onset of cervical dilatation) will allow one to determine whether a bitch is ready for

preparturient CS or not and, if not, how to optimise further observation, firstly to find out when she would be ready and, secondly, to avoid cervical dilatation that started sooner than anticipated from going unnoticed.

1.4.9. The need to assess the feasibility of D57 as the day for preparturient caesarean section

In an earlier study (Chapter 8) of the thesis it was shown that the first day of cytological diestrus (D0) is the most precise and practical predictor of the onset of cervical dilatation. D0 predicted the date of cervical dilatation with a precision of ± 1 d, ± 2 d and ± 3 d in 88%, 99% and 100% in 242 oestrous cycles. The interval between D0 and the day of cervical dilatation in 242 pregnancies was normally distributed with a mean of 56.74 days (SD 0.96).

1.5. Research questions and hypotheses in the chapters

1.5.1. Research questions and hypotheses for Chapter 3

1) Survey 1

a) Question: What is the odds of bitches of the common breeds in South Africa of ever undergoing a CS during her reproductive lifespan?

Hypothesis: This was a survey and a descriptive study and no hypothesis was stated.

2) Survey 2

a) Question: What is the odds of CS in German Shepherd Dog bitches in South Africa and what is the effect of parity thereon?

Hypothesis: Determining the odds of CS was descriptive and no hypothesis pertains.

The hypothesis with respect to the effect of parity is as follows: The odds of CS increases with parity ($P < 0.05$).

b) Question: What is the effect of the method to deliver the previous litter (CS vs. natural birth) on the odds of CS in the current litter in German Shepherd Dog bitches in South Africa?

Hypothesis: The odds of CS in a German Shepherd Dog bitch in South

Africa is higher if her previous litter was born by CS compared to when it was born naturally ($P < 0.05$).

3) Survey 3

a) Question: What proportion of puppies are stillborn from Boerboel bitches that whelped naturally and never had a CS before in a large South African kennel?

Hypothesis: This survey descriptive study and no hypothesis was stated.

4) Survey 4

a) Question: What is the odds of bitches requiring a CS following trial of labour compared to the odds of bitches requiring a CS where the previous litter was born naturally?

Hypothesis: The odds of CS following trial of labour is higher than the odds of CS where the previous litter was born naturally ($P < 0.05$).

b) Question: What is the odds of stillbirth in litters born by trial of labour after the previous litter was born by CS compared to the odds of stillbirth in litters where the previous litter was born naturally?

Hypothesis: The odds of stillbirth in a litter born by trial of labour after a previous CS is higher than the odds of stillbirth after the previous litter was born naturally ($P < 0.05$).

5) Survey 5

a) Question: What is the odds of stillbirth of puppies born by emergency CS to that of puppies born by elective parturient CS?

Hypothesis: The odds of stillbirth is higher in emergency CS than in elective parturient CS ($P < 0.05$).

1.5.2. Research questions and hypotheses for Chapter 4

a) Question: What are the Apgar scores and puppy survival ratios at delivery, 2 h and 7 d, as well as the maternal survival ratio following the

use of low doses of medetomidine as premedication for CS in bitches?

Hypothesis: This was a descriptive study and no hypothesis was stated.

1.5.3. Research questions and hypotheses for Chapter 5

a) Question: What is the average and range of haematocrit in normal healthy pregnant bitches at the time of CS?

b) Question: What is the average and range of haematocrit in bitches 1–2 h after CS and standardised fluid administration?

Hypothesis: This was a descriptive study and no hypothesis was stated.

1.5.4. Research questions and hypotheses for Chapter 6

a) Question: Does a minimum BPD exist that all viable foetuses of a breed will attain when they are ready for birth but not before?

Hypothesis: No BPD exist that all viable foetuses of a breed will attain by the time that they are ready for birth but not before.

1.5.5. Research questions and hypotheses for Chapter 7

a) Question: How well does the PC in the serum of bitches as determined with the Immulite® 1000 LKPW1 chemiluminescent immunoassay agree with PCs below 25 nmol/L in plasma as determined with the Coat-A-Count radioimmunoassay?

Hypothesis: Immulite® 1000 LKPW1 and Coat-A-Count RIA yield similar PC at concentrations below 25 nmol/L.

Hypothesis: Immulite® 1000 LKPW1 and Coat-A-Count RIA yield similar precision.

b) Question: How well do the days on which PC determined with Immulite LKPW1 and Coat-A-Count RIA respectively reached 6 or 16 nmol/L, and the PC determined by each on LH1, agree?

Hypothesis: Immulite and Coat-A-Count RIA agree in their identification of the days on which PC reached 6 nmol/L and 16 nmol/L and in their

PCs on LH1.

1.5.6. Research questions and hypotheses for Chapter 8

a) Question: With what level of precision do the day of first rise in PC above 6 nmol/L, day on which the PC in blood plasma or serum reaches 16 nmol/L, the first or only day of the LH surge and D0, respectively, predict the date of parturition and which of these is the most precise predictor thereof?

Hypothesis: At least one variable, measured during the peri-oestrous period will allow prediction of the day of cervical dilatation with no more than a four-day range in at least 95% of bitches

Hypothesis: The precision of the peri-oestrous predictors differ ($P < 0.05$).

1.5.7. Research questions and hypotheses for Chapter 9

a) Question: Will the concentration of progesterone and cortisol in blood plasma during the preparturient period enable one to predict the date or time of cervical dilatation and how precise are the dates or times so predicted?

Hypothesis: In this study the data were scouted for patterns from which criteria for prediction could be derived. No hypothesis was stated.

1.5.8. Research questions and hypotheses for Chapter 10

a) Question: Would preparturient CSs, performed on the morning of D57, yield similar odds of stillbirths and neonatal death than parturient CSs and how would the ratios of stillbirth and neonatal death of puppies born by preparturient CSs compare to those after CS as reported in the literature?

Hypothesis: The odds of stillbirth and neonatal death with preparturient CS is similar to those of parturient CS ($P > 0.05$).

Hypothesis: The probability of stillbirth and neonatal death with

preparturient CS in English Bulldogs is similar to those reported by Wydhooghe et al. (2013) and those in Boerboels is similar to those reported by Moon et al. (1988).

1.6. Scope of the thesis

The scope of this thesis is wide because numerous factors that impact on the outcome of CS had to be investigated on parturient CSs parallel with those of preparturient CSs to allow for accurate comparison of the two. This led to other planned studies peripheral to the main theme as well as incidental findings that we reported. Chapter 1 is the introduction and highlights the dilemma in canine obstetrics that led to this study, explores the ethical considerations and lists the aims and needs for this study. It finally explains the limitations of the current knowledge we have and formulates the research questions and hypotheses. Chapter 2 is a review of the literature reporting on the relevant aspects related to pregnancy, parturition and CS in the bitch. Because this thesis dwells on unchartered territory, it necessitated selective review of the literature of other species and that of human obstetrics in view of potential overlap. Chapter 3 is a retrospective and prospective study that reports on CS in the management of obstetric risk in bitches. It identifies obstetric risk factors in the bitch and elucidates the existence of subpopulation of dogs that should be considered to carry high risk pregnancies where CS or preparturient CS is justified and a good option for delivery of the puppies. Chapter 4 examines puppy survival and vigour associated with the use of a low dose medetomidine premedication, propofol induction and maintenance of anaesthesia using sevoflurane gas-inhalation for CS in the bitch. Chapter 5 reports the haematocrit changes in periparturient bitches undergoing CS. The effects that CS performed on date of onset of spontaneous parturition (P0) and preparturient CS has on the maternal haematocrit was of interest in this study. A study conducted decades ago concluded that haemodilution occurs in the dog as it does in man, causing the so called “anaemia of pregnancy”. Our knowledge about haematocrits of bitches in late pregnancy is incomplete. We have no knowledge of haematocrits before and after CS. This is a stumbling block in the post-parturient risk assessment. Assessing risk prior to discharge following CS in bitches is particularly important because the discharge usually takes place as soon as 2 h after surgery. The effect of preparturient CS on maternal haematocrit has not been reported in the bitch. It is of interest that this effect be evaluated as it requires comparison with preparturient CS haematocrits in order to include or exclude increased risk associated with the latter. This

study finally also assessed whether preparturient CS is associated with increased risk of haemorrhage when compared to parturient CS. Chapter 6 investigates and reports on whether foetal biometric measurements in late gestation have potential in predicting readiness for CS in dogs. Midway of planning this study, it had been suggested that biparietal diameter (BPD) having reached a minimum in the English Bulldog, indicated readiness for CS (Batista et al., 2014). Since readiness for CS was central to this study, an additional study was immediately added to evaluate foetal biometric parameters as predictors of readiness for CS. Chapter 7 covers the first of the three experiments central to this study. It validated Immulite® 1000 LKPW1 for PC against the "gold standard" radioimmunoassay (RIA) (Coat-A-Count radioimmunoassay). This study aimed at comparing the agreement between the concentrations of progesterone in plasma or serum of bitches respectively measured with these assays. This study also evaluated the ability of Immulite® to estimate the days on which Coat-A-Count radioimmunoassay would first exceed 6 nmol/L or 16 nmol/L as well as the ability of Immulite® and Coat-A-Count radioimmunoassay to identify the day of the LH surge. Chapter 8 and Chapter 9 respectively compared the accuracy of the interval variables used to predict date of parturition in the bitch based on observations during the peri-oestrous and preparturient periods. In the peri-oestrous period the PC, luteinizing hormone in the serum (LH) and cytological dioestrus (D0) was used to predict the parturition date. In the preparturient period, both the concentrations of cortisol in the blood and PC were evaluated for usefulness in predicting imminent parturition. This study finally assisted in selecting the variable in the peri-oestrous that best predicted the day of parturition. This study also helped reveal the usefulness of the concentration of cortisol and PC during the preparturient period as predictor of the time of cervical dilatation and thus onset of imminent parturition. Chapter 10 reports on the outcome associated with performing preparturient CSs based on dates predicted as described in the preceding chapter. Chapter 11 and Chapter 12 respectively report on the first ever dizygotic monochorionic canine foetuses with blood chimaerism and suspected freemartinism and the first ever confirmed monozygotic twinning in the dog. Chapter 13 is the overarching and concluding discussion followed by Chapter 14 elaborates on future research needs and finally Chapter 15 gives the references.

Chapter 2. Literature review

General overviews are given of the relevant literature and where required an in-depth discussion is done on the value and relevance of specific information to this study.

2.1. Vaginal cytology

The vaginal epithelium and morphology of the cells of the ad luminal layers of the vaginal epithelium, change during the oestrous cycle (Schutte, 1967c). Vaginal cells may be obtained by passing a cotton tipped ear bud moistened with sterile saline into the vaginal canal. The vestibule and clitoral fossa should be avoided during the procedure as superficial cells from these areas could alter the cytological interpretation. This is because they may harbour cells not representative of the relevant cells in the vaginal mucosa (personal observation). Vaginal smears may be stained with a trichrome stain (Schutte, 1967b), Wright's Giemsa stain (Holst and Phemister, 1974), rapid modified Wright's Giemsa stain (Olson et al., 1984) or Diff Quick stain. Keratinized cells stain orange with the trichrome stains in contrast to non-keratinized cells that stain blue and the percentage of orange stained cells resembles the eosinophilic index (Schutte, 1967a). Even though all cell types described by Schutte (1967a) and Christie et al. (1972) as well as the onset of cytologic dioestrus can be identified on smears stained with Giemsa or modified Giemsa (Holst and Phemister, 1974), the modified Giemsa stain is the preferred method because of its ease of use and reliability (Olson et al., 1984). In two separate studies, Schutte (1967) used nuclear size to classify the vaginal epithelial cells as superficial, intermediate or anuclear whereas Christie et al. (1972) included nuclear pycnosis as a criterion to classify an epithelial cell as superficial. The classification by Holst and Phemister (1974) compared the incidence of anuclear and superficial cells combined, with the incidence of parabasal cells and small intermediate cells combined. Their method is the more practical one. Vaginal smears were made once daily and stained as previously described (Olson et al., 1984) to establish D0, using the criteria set out by Holst and Phemister (1974). The bitch was considered to be in cytological dioestrus when her vaginal smear showed an abrupt change in the relative numbers of vaginal epithelial cells from the previous day, when nearly 100% of epithelial cells were of the superficial type. These changes were a decrease by at least 20% in the percentage of epithelial cells that were of the superficial type, irrespective of whether they were nucleated or anuclear, and an increase of at least 10% in cells from the deeper layers

of the vaginal epithelium, such as early intermediate cells or parabasal cells (Holst and Phemister, 1974).

In the current study, vaginal cytology was employed in conjunction with visual inspection of the vagina through a speculum to determine the stage of the oestrus cycle of the experimental bitches to decide when to start blood collections and to decide on the optimal time for breeding. Vaginal cytology was used to determine the onset of D0 which was ultimately used to time preparturient CS.

2.2. Visual inspection of the vagina through a speculum

Visual inspection of the vagina through a speculum allows the evaluation of the macroscopic appearance of the vaginal mucous membrane. Lindsay (1989), described the macroscopic changes that occur in the vaginal mucous membrane of the bitch throughout the oestrous cycle. Using a 4.7 mm diameter, rigid, paediatric telescope she examined the luminal surface of the paracervix. Anoestrus was characterised by low, simple, rounded vaginal folds with a pink or red colour. Pro-oestrus was initially characterised by increasing oedema, and later in oestrus by decreasing oedema of the folds resulting in a concertina-like appearance of the folds. During pro-oestrus all profiles of folds remained rounded. During oestrus, the vaginal folds became pale and dry with profiles that became increasingly more angular. During early dioestrus, there was a rapid lowering and rounding of the profiles of all vaginal folds. The vagina also becomes pinker during early dioestrus. Lindsay et al. (1988) and Jeffcoate and Lindsay (1989) showed that the first sign of angularity of the vaginal folds occurred 2–4 d after the LH peak, thus coinciding with the time of ovulation or thereafter, during the period of post-ovulatory maturation of oocytes. Visual inspection of the vagina through a speculum in the current study was useful to determine the optimum time of artificial insemination as well as to help estimate when to start collecting blood to ensure that our blood sample contain within them the day that the first rise of PC above 6 nmol/L in the peri-oestrous period occurred.

2.3. Initiation of parturition in the bitch

Uterine quiescence during pregnancy is maintained by mainly progesterone and relaxin (Heap and Flint, 1984; van der Weyden et al., 1989). It is not clear what triggers the onset of whelping in the dog but it is generally accepted that it involves initiation by the foetus or

foeto-placental unit as is the case in other mammals (Liggins et al., 1973; Flint et al., 1978; Mitchell, 1994; Whittle et al., 2000) and that cortisol and prostaglandin likely plays a role in the bitch similar to that of the goat (Flint et al., 1978). The initiation of normal parturition in mammals and the bitch is orchestrated by a neuroendocrine cascade of events during which there is both the abolition of the inhibitory effects on the uterus and the recruitment of factors promoting uterine activity. Prostaglandin $F_{2\alpha}$ and oxytocin stimulate uterine activity (Mitchell, 1994; Norwitz et al., 1999). In the bitch, concentrations of cortisol in maternal circulation (and probably also concentrations of cortisol in foetal circulation) and concentrations of PGF2α (Concannon et al., 2001; Kowalewski et al., 2010) or PGFM (Baan et al., 2008) increase before parturition. In several species, the concentration of oestradiol in serum increases before parturition and stimulates PGF2α secretion (Challis, 1971). In the bitch, no increase in preparturient oestradiol has been reported (Edqvist et al., 1975; Austad et al., 1976; Concannon et al., 2001; Luz et al., 2006). Although the concentration of oestradiol in the serum had not increased, there would have been an increase in oestradiol production if one considers the increase in plasma volume in late pregnancy (Concannon, 2009). At the time of normal parturition a clear rise in circulating oxytocin is demonstrated (Hoffmann et al., 1999; Klarenbeek et al., 2007; Hollinshead et al., 2010) and lower levels of oxytocin have been demonstrated in bitches with uterine inertia (Bergström et al., 2006a). Other mechanisms or hormonal changes than oestradiol is thought to increase the sensitivity of oxytocin receptors in the bitch. Prostaglandin $F_{2\alpha}$ causes luteolysis (Concannon et al., 1988; Challis et al., 1997; Veronesi et al., 2002) and subsequent decrease in the serum PC in the bitch to <3.18 nmol/L) 24 h before parturition (Veronesi et al., 2002). PGF2α also stimulates uterine contractions (Challis and Lye, 1986; Challis et al., 1997). Relaxin is produced by the placenta and declines precipitously at parturition (Steinetz et al., 1987; Klonisch et al., 1999).

Prolactin concentrations in the serum of bitches increase prior to parturition following the decline in PC (Concannon et al., 1978). The concentration of cortisol in the maternal circulation increases with approaching parturition in the bitch (Concannon et al., 1978) but there is no data proving whether the origin of the cortisol is maternal, foetal or both. It is suspected that the cortisol in the maternal circulation causing the preparturient increase in concentration originate from firstly foetal cortisol and secondary to maternal stress (Taverne and Van Der Weijden, 2008). It has further been speculated that the additive effect of this foetal cortisol (Taverne and Van Der Weijden, 2008) as well as maternal stress associated

with a large litter (Taverne and Van Der Weijden, 2008) may contribute to the inverse relationship between litter size and gestation length (Eilts et al., 2005). Glucocorticoids are effective abortifacients in some species (Anderson et al., 1975) but in the bitch lengthy treatments with dexamethasone were required and worked better if given twice a day (Zone et al., 1995). The endocrine events discussed above focus on the maternal effects and subsequent tonicity or quiescence of the uterus. Synchronous to these events in women are the effects endocrine events have on foetal maturation and in particular lung surfactant production (Sun et al., 2006). Research in pregnant sheep (Liggins et al., 1973) and nonhuman primates, shows clearly that the foetus determines the duration of gestation (Taylor et al., 1983) but the mother determines the time of day at which parturition begins (Honnebier et al., 1989; Honnebier et al., 1991). This is probably an evolutionary adaptation to ensure maximal survival rate of offspring through ensuring that parturition occurs mostly at times when there is least chance of predation. The above circadian nuances at parturition may affect hormone concentrations, body temperatures, behaviour and other parameters as well as response to pharmacologic agents. Also, circadian nuances at parturition may influence hormonal assay results depending on what time of day they were collected. From this discussion, it may be deduced that there is likely a beneficial role of increased maternal preparturient cortisol concentrations in the bitch that may play a vital role in parturition, placental release and puppy maturation and ultimately post-delivery puppy survival.

2.4. The origin of PGF2α and its transfer to the corpus luteum

In several species PGF2α or its metabolite PGFM has been implicated in initiation of parturition via its uterotonic and or luteolytic effects (Thornburn and Challis, 1979; Thorburn et al., 1977; Lohse and First, 1981). In these species (porcine, ovine, caprine) the placenta or uterus appears to be the source of the luteolysin (Thornburn and Challis, 1979). Dogs are polytocous species that are dependent on luteal progesterone throughout pregnancy. In the pregnant dog, luteal progesterone production is dependent on pituitary gonadotrophins. There is no evidence of a placental luteotrophin in bitches but preparturient rises in the concentrations of cortisol and prolactin in the circulation have been demonstrated (Concannon, 1986) as well as preparturient rise in plasma PGF2α from the gravid uterus (Concannon et al., 1988). In the non-pregnant bitch the uterus does not make any contribution to luteolysis (Okkens et al., 1985). A preparturient increase in oestrogen has not been reported in the bitch (Concannon, 1986). Simple local transfer of PGF2α from the

utero-ovarian vein to the ovarian artery has not been demonstrated in the bitch but the association between these blood vessels could allow for such transfer (Ginther et al., 1973). It has been demonstrated that there is venous anastomoses between the vasculature of the two uterine horns in the region of the cervix in the bitch (Del Campo and Ginther, 1974), the extent and significance thereof is unknown. Any PGF2α in the blood crossing these anastomoses (from one horn to the other) would be diluted by the blood from the contralateral uterine horn. The dilution factor in the systemic circulation would obviously be far greater. Prostaglandin $F_{2\alpha}$ is suspected not to reach the arterial circulation in the bitch (Gerber et al., 1979).

Thus considering that the gravid uterus is the origin of PGF2α (Concannon et al., 1988) and the non-gravid uterus not (Okkens et al., 1985) and if it is true that no PGF2α reaches the systemic arterial circulation in the bitch, the sidedness of foetuses in canine pregnancies may be important. It can therefore be speculated that, given the anatomy of the vasculature in the bitch (Del Campo and Ginther, 1974), it is possible that PGF2α has to be secreted in both uterine horns in order to ensure that bilateral luteolysis occurs (Irons et al., 1997) and that failure of which may lead to incomplete luteolysis of corpora lutea on ovaries of non-gravid horns in pregnant bitches, leading to prolonged gestation and foetal demise. The relevance of this information will become evident in the discussion that follows.

2.5. Incomplete luteolysis and singleton pregnancy

It has been established that in pregnant bitches, a rapid, preparturient decrease in PC is consistently found within a variable period (around 48 h) before whelping (Concannon P. et al., 1977; Veronesi et al., 2002; Williams et al., 1999; Concannon et al., 1989; Luz et al., 2006; Kowalewski et al., 2010; Chakraborty, 1987; Austad et al., 1976; Hoffmann et al., 1994; Edqvist et al., 1975; England and Verstegen, 1996b; Leroyer et al., 2002; Meyer, 1994; Verstegen-Onclin and Verstegen, 2008; van der Weyden et al., 1989). The only exception is speculated to be the singleton pregnancy (Johnson, 2008a). The exact mechanism by which parturition fails to occur in some singleton pregnancies and not in others, is unknown. Singletons have been identified as high risk pregnancies by some authors (King, 1978; Lopate, 2008; Smith, 2007; Beccaglia et al., 2008; Johnson, 2008b; Wykes and Olson, 2003; Munnich and Kuchenmeister, 2009) and is a serious challenge to the veterinary obstetrician. In singleton pregnancies in large breeds, failure of parturitions to occur are more likely when compared to singleton pregnancies in small breeds (personal

observation). The foetuses in these singleton pregnancies that do not progress normally are not necessarily oversized in either small breeds or large breeds. Increased gestation length has been reported in bitches carrying singletons (Holst and Phemister, 1974; Okkens et al., 1993) and a significantly higher incidence of total primary inertia in bitches with litters of one or two foetuses has been observed (Darvelid and Linde-Forsberg, 1994). It is suspected that incomplete luteolysis might have a role in the singleton enigma (Lopate, 2008; Johnson, 2008b; Irons et al., 1997). The puppies in some but not all singleton litters may be disproportionally large, leading to obstructive dystocia. In most however, the bitch appears to go over-term and the puppy dies without the bitch ever having shown any signs of parturition. In some cases, the owners might not have known that the bitch was pregnant and only suspect something when they see vaginal discharges. Even if the veterinary obstetrician is presented with a known singleton litter, their options are limited. The veterinary obstetrician may have no means of knowing whether this bitch will manifest signs of active parturition or not. Around the clock 24 h observation also has its limitations as the veterinary obstetrician may only know that immediate intervention is required when there is a green vaginal discharge by which time it is usually too late. Veterinary obstetricians thus have difficulty in deciding when to perform obstetrical intervention. Routine foetal monitoring also has its limitations. This is because the foetal heartbeat may fall from within the normal range to as low as 120 bpm (Zone and Wanke, 2001) in less than half an hour (personal communication, Nöthling) and (personal observation), indicating emergency CS. In many of these cases, the puppy dies before it is rescued by CS. It is not known why some singletons frequently go over-term and others not. We do not know whether foeto-placental release of PGF2α occurs in singletons or why some singleton cases appear to have incomplete luteolysis. Knowledge on the endocrine changes occurring around parturition may reveal information useful in managing it.

Incomplete luteolysis has not been clearly defined in the literature. Incomplete preparturient luteolysis has been reported in a few cases of bitches with normal sized litters. The reason for incomplete preparturient luteolysis was not established in these cases (Irons et al., 1997; Hoffmann et al., 1999). In these two reports of prolonged gestation, PC was above 12 nmol/L, suggesting that luteolysis was incomplete. Knowing the distribution and variability of PC at the time of parturition may provide the defining value of PC above which an “parturient” bitch may be regarded as suffering from incomplete luteolysis. The mean concentration of progesterone in blood plasma during spontaneous parturition was

3.2 ± 1.2 nmol/L in seven bitches (Veronesi et al., 2002), 5.9 ± 1.2 nmol/L in six bitches (Baan et al., 2005; Baan et al., 2008), 2.23 ± 0.64 nmol/L, varying from 0.64 to 5.41 nmol/L (Concannon et al., 1988), below 3.12 nmol/L (Edqvist et al., 1975) and 3.18 nmol/L (Leroyer et al., 2002). Plasma PC declined after the start of metergoline administration in all pregnancies but levels below 4.8 nmol/L were required for successful abortions (Nöthling et al., 2003). These data suggest that a parturient bitch will have a PC of below 6.4 nmol/L, above which the bitch may be considered to suffer from incomplete luteolysis. Owing to the paucity of data available on incomplete preparturient luteolysis (only two reports on one bitch each), it is fair to assume that it is probably rare.

Large breeds have a higher ovulation rate and are thus more likely to have larger numbers of corpora lutea (CL) (Marinelli et al., 2009). This may offer an explanation why large breed bitches carrying small litters (singletons or two puppies) are more likely to suffer from incomplete luteolysis and failure of parturition to progress normally than in the same bitches had they carried large litters. This is because in the large litters there may be more PGF2α available to exert its luteolytic effect and induce spontaneous parturition than in the same bitches with a singleton (or two puppy litter), the foeto-placental unit produces insufficient PGF2α to lead to the demise of a high number of CL, leaving the bitch to go over-term. Also, the side of the uterine body on which the CL (or the low number of CL) lies relative to the side on which the only conceptus lies may be important. If, for example, the only conceptus lies in the left uterine horn and the corpora lutea are in the left ovary, PGF2α from the foeto-placental unit on the left may more effectively reach the corpora lutea and cause their demise than would be the case if the CL were in the right ovary. This is because the close association between the utero-ovarian vein to the ovarian artery could allow for the local transfer of PGF2α between those vessels (Del Campo and Ginther, 1974). Irons et al. (1997) suggested that PGF2α must be secreted in both uterine horns to ensure bilateral luteolysis in the bitch. It may hypothetically be speculated that in bitches with only one ovary and the ipsilateral uterine horn, normal delivery may occur if sufficient PGF2α escapes metabolism and reaches the contralateral ovary via the systemic circulation. Again, in such hypothetical bitch, the size of the litter and thus amount of PGF2α available for systemic transport to the contralateral CL, may influence whether luteolysis will occur or not. It is therefore important to take note of the sidedness of foetuses and CL in studies designed to investigate the singleton enigma. The dog's CL seems to have an internally programmed life span (Meyer, 1994). The implication is that if the foetus remains in utero

and dies because of placental release, the PC may decline because the CL have reached their end of their functional life span and not necessarily because of some luteolytic factor released or inflammatory process.

The phenomena of incomplete luteolysis in normal sized litters and the singleton enigma requires further research.

2.6. Hypoluteodism

Although hypoluteodism is often suspected (Krachudel et al., 2013; Günzel-Apel et al., 2006; Verstegen-Onclin and Verstegen, 2008) and treated based on history or suspicion (Tibold and Thuroczy, 2009; Purswell, 1991; Gorlinger et al., 2005), there are no conclusive case reports on luteal insufficiency during gestation in the bitch. Hypoluteodism is characterized by insufficient secretion of progesterone by the corpora lutea during pregnancy which may lead to loss of pregnancy (foetal resorption or abortion depending on whether it occurs in first half or second half of gestation) (Gobello and Corrada, 2002). A PC of 8.3 nmol/L is considered just above the threshold necessary to maintain a pregnancy in the bitch (Gorlinger et al., 2005) with 6.4 nmol/L being the threshold (Johnson, 2008a).

The “condition” of hypoluteodism is important to this study as PC forms integral part of study throughout.

2.7. Timing of ovulation using progesterone concentration

In cases of frozen semen where sperm lifespan may be in the order of hours (England and Ponzio, 1996; Linde-Forsberg et al., 1999), it is critical that one or more inseminations occur after the time of oocyte maturation but before oocyte degradation. This critical time has been estimated to be at 5–6 days after the LH peak and the associated rise in the concentration of progesterone in the blood (Concannon et al., 1977a). The timing of ovulation has been correlated to PC by direct means. Concannon et al. (1977), removed ovaries and dissected them to confirm ovulation and found that approximately 48 h after the LH peak, when plasma progesterone was 17.3±3.0 nmol/L (SEM, n = 6), the ovulated: non-ovulated mature follicle ratios were 100%. When ultrasound was used to determine the day of ovulation, it was determined that the PC on ovulation day was 7.4 nmol/L (range 5.7–8.8 nmol/L, n = 6). Hase et al. (2000) concluded that a PC of 6.4 nmol/L confirmed day of ovulation. However, in another study where ultrasound and direct ovarian dissection was used in a group of

37 bitches, the mean PC at ovulation was 20.41 ± 4.86 nmol/L (SEM, range values: 14.08–31.19 nmol/L) and when a bitch is within one day of ovulation, 55 of 59 bitches had PC of 15.9 nmol/L and a mean concentration at the end of ovulation of 23.2 nmol/L (SEM 6.1 nmol/L) (Fontbonne, 2008). When recoveries of oocytes were used to confirm that ovulation had taken place, the highest numbers of oocytes were recovered from bitches with PCs of 19 nmol/L–25 nmol/L (Hossein et al., 2008).

Absolute PC at key times in the peri-oestrus period are important in this study as it has relevance to predicting the date of parturition.

2.8. Factors that may affect the concentration of progesterone in blood plasma or serum

2.8.1. Diurnal pattern in progesterone concentration

A diurnal pattern of PC occurs in the bitch. In mid-gestation the PC was approximately twice as high at 8:00 am as at 3:00 pm, although, during the first and last week of gestation there was not a significant effect of time of day the at which blood was collected for progesterone assay (Steinetz et al., 1990). Although all blood collection in the current study is expected to take place before, during the first week and during the last six days of gestation, the effect of a diurnal pattern on PC will be avoided by collecting samples at the same time of day.

2.8.2. Haemodilution

Pregnancy increases blood volume and may have an effect on complete cell counts and serum biochemistry profiles in dogs (Kimberely et al., 2006). In the current study variables in blood, such as haematocrit and the concentrations of hormones will be studied during advanced pregnancy, without comparing them to non-pregnant bitches. It will be assumed that the extent of haemodilution is similar among bitches in late gestation. The effects of haemodilution among bitches in late gestation will thus be ignored in this study.

2.8.3. Blood collection volume and frequency

The effect of frequency and volume of blood collections presumably has little influence on haematocrit. This is because the maximum blood collection volume is 20 ml once daily for a maximum of 7 d in dogs that weigh 20 kg or more in all the experiments which are reported on in this manuscript.

2.8.4. Hyperlipaemia

Hyperlipaemia is a common sequel to ingestion of a meal in dogs. The normal concentration of triglycerides in the blood plasma or serum of dogs varies from 0.34 to 3.6 mmol/L (Vaden et al., 2011). Hyperlipaemia has an effect on certain biochemistry profiles (Walker and Crook, 2013). The Coat-A-Count RIA Progesterone assay brochure shows that triglyceride concentrations as low as 500 mg/dL (5.65 mmol/L) causes a 5%–14% decrease in PC. In contrast the manufacturer's pamphlet detail on Immulite® 1000 confirms that lipaemia does not influence results significantly. This suggests that high normal post-prandial triglyceride concentrations may cause some difference in the values obtained with Immulite® 1000 and RIA. Therefore, this study will ensure that the plasma and serum samples are not lipaemic by assuring that they are from healthy dogs. Most bitches in the preparturient period will become anorexic and may have starved in the 12 h prior to sampling. Direct visual inspection of plasma or serum samples can identify gross hyperlipaemia. In general there was a good correlation between the degree of turbidity and the lipid content of the serum (Klatskin and Gordon, 1952). When the serum was lipaemic, its milky appearance was detectable, almost as soon as the blood clotted, as a translucent greyish veil (Klatskin and Gordon, 1952).

2.8.5. Progesterone assay method

Depending on the purpose for the progesterone assay, its ability to qualitatively assess or quantify PC may be of importance. Qualitative assays may be adequate to determine when to mate or artificially inseminate bitches with fresh semen. Quantitative assays are required to determine when to inseminate bitches with frozen-thawed semen or to assess the preparturient decline in PC. Although practical and of value for some applications (Hospes et al., 2004), semi-quantitative rapid progesterone assays are not suitable in the current study.

a) Radioimmunoassay of progesterone

The RIA (Coat-A-Count radioimmunoassay) has been established as the "gold standard" in terms of precision and accuracy against which other progesterone assays may be compared to (Moxon et al., 2010). This is confirmed by the observation that almost all results concerning PC are validated against this standard (Reimers et al., 1991) or results are compared with other assays based on previous validation of progesterone against RIA (Kutzler et al., 2003a).

a) Chemiluminescent immunoassay (Immulite®)

Progesterone may be accurately measured by chemiluminescent immunoassay. Progesterone CLIA is available through human clinical and some veterinary diagnostic laboratories and it is comparable in cost with the RIA. In addition, CLIA has the advantage of continuous processing because of the rapid turnaround time without the disadvantages associated with isotopic assay (e.g. radioactivity, sample batching). The CLIA method was validated for use in determining PC in canine serum and its specificity, accuracy, and precision of the CLIA were comparable to those obtained with RIA (Kutzler et al., 2003a). However, another showed that RIA measured significantly higher serum progesterone concentration than CLIA (Volkmann, 2006). Because crucial decisions are based on PC during peri-oestrous and preparturient periods, it is vital that we validate the agreement between and precision of Immulite® compared to RIA for PC quantification at the low concentrations expected during early oestrus and in the last 3 d before parturition.

2.9. Need to measure progesterone concentration precisely and accurately

This study concerned itself mainly to correctly identify day on which the concentration of progesterone first exceeds 6 nmol/L, 16 nmol/L in peri-oestrous bitches and to correctly identify time and day on which potentially low (< 6 nmol/L) concentration in the preparturient bitch occurs. There are however many other reasons why the PC may be of importance to the researcher and veterinary obstetrician. These include, staging of the normal oestrus cycle (Concannon et al., 1977a), investigating, abnormal oestrous cycles (Meyers-Wallen, 2007), hypoluteodism (Gobello and Corrada, 2002), conception problems in bitches (England and Russo, 2006) and incomplete preparturient luteolysis (Irons et al., 1997; Hoffmann et al., 1999). Whichever the reasons for PC assay, CLIA would be a great addition to the veterinary obstetrician's arsenal if proven both precise and accurate in all mentioned cases.

2.10. Synchrony of ovulation

In a polytocous species such as the dog, the question may arise over what timeframe ovulation occurs. If this time frame is very short, then ovulation may be considered synchronous. Synchrony of ovulation in the dog needs be discussed and convince the reader that differences in puppy size or apparent maturity and or gestational stage has little to do with ova presumably having been released at so vastly different times to account for different

sizes and apparent maturity or lack thereof.

Holst and Phemister, (1971) were the first to examine synchrony of ovulation and concluded that all follicles in a bitch ovulated synchronously 1–2 d after the onset of behavioural oestrus. Concannon et al. (1977) removed the ovaries from eight beagles at various times after the LH peak and inspected them for ovulation. No bitches had started to ovulate by 38 h after the LH peak. One bitch was in the process of ovulating at 44 h after the LH peak. Ovulation was complete in two bitches at 50 h and 96 h after the LH peak, accounting for an approximate period of 48 h for ovulation in these bitches. In another study, Wildt et al. (1978) found that 77.2% of 98 follicles ovulated during the period 24–72 h after the LH peak (Wildt et al., 1978).

Only 6.5% of ovulations occurred more than 96 h after the LH peak. Tsutsui, (1989) performed laparotomies on 132 beagles at different times after the onset of standing oestrus. Each beagle was operated on only once during oestrus. He observed that most ovulations had occurred between 48 h and 60 h after the onset of behavioural oestrus (Tsutsui, 1989). Concerning the duration of the ovulation process, Boyd et al. (1993)suggested that the ovulation process seemed to begin in the right ovary and that the whole process may take as long as 36 h to be completed. In one study, the embryonic development was synchronous in relation to the LH peak (Bysted et al., 2001). Ultrasonography of ovaries showed that ovulation was completed in both ovaries for around 50% of the bitches in less than 12 h and appeared synchronous between the two ovaries (Fontbonne, 2008). In summary, it may be concluded that ovulation in the bitch occurs over a period of not more than 36 h and probably less.

The duration of fertility of dog ova is also important as prolonged fertility may allow for the asynchronous fertilization of ova leading to different gestational ages. In one study, it was found that canine oocytes are fertile for as long as 4–5 d after maturation (Tsutsui et al., 2009) but that this fertilization was only possible under artificial conditions using intra uterine inseminations. This is so because sperm entering the canine vagina can only enter the uterus up to on average 5 d after ovulation because cervical closure appears to occur at that time (Tsutsui et al., 2009; Verstegen et al., 2001). It thus follows that the prolonged duration of fertility of canine ova allows for the potential asynchronous fertilization of ova only under artificial conditions (Tsutsui et al., 2009). It is also important to note that the

fertilization of aged ova results in high resorption rate (Tsutsui et al., 2009). From this it follows that unless a bitch has been inseminated over many days by means of intra uterine inseminations or has been mated or vaginally inseminated before closure of the cervix and inseminated into the uterus after the closure of the cervix, and then only if the conceptus does not resorb, may the bitch carry foetuses in the same litter that differ in gestational age by more than 48 h.

From the above neither asynchrony of ovulation nor duration of fertility of dog ova under natural conditions explain the vast differences in puppy sizes as well as apparent differences in maturity of puppies in the same litter.

The differences in size of puppies in a litter may most likely be explained in most cases by genetic variation and (or) conditions in the uterus that influence foetal growth. Some observational studies show a direct association between small size in early life (for example, low birth weight) and current, adult health outcomes. However, in others this relation has emerged only after body size at some later period (notably current weight or body mass index) has been adjusted for (Lucas et al., 1999).

In conclusion, the puppies in a litter will, under normal conditions, not vary in age by more than 24–48 h.

2.11. Superfoetation

Superfoetation is thought to occur when a pregnant female, already carrying one or more live foetuses, is bred again and a second conception occurs. Although it has been reported in ruminants and pigs (Smith, 1927) as well as man (Baijal et al., 2007), it has never been reported in the bitch and is probably not possible in this species. Superfoetation may be excluded as a cause of foetuses of discordant sizes in the same litter and pregnancy.

2.12. Parturition induction in the bitch

The hormonal events at parturition induction are discussed where this may have relevance to puppy survival in planned CS as well as the timing of this intervention relative to hormone concentrations in the various situations. Furthermore, it may be possible that from the results of this study, a safe parturition induction timing protocol may evolve, providing additional tools to manage parturition.

The ideal parturition induction protocol should:

- Induce whelping with a high efficiency
- Have a consistent predictable time frame between administration and induction
- Have acceptable expulsion times, inter-pup intervals and total whelping time span
- Have no side effects
- Be safe for bitch and her puppies
- Not affect lactation
- Not affect colostral transfer to milk and uptake by puppies
- Not affect placental separation and expulsion
- Not affect uterine involution
- Not affect future fertility
- Be effective and suitable in all breeds

Breed may be important with respect to the induction of parturition. Apart from the study by Fontbonne et al. (2009), all other studies were performed in beagles. No study has evaluated the efficacy of parturition induction in high risk breeds such as brachycephalic breeds. Considering that the mean interval between two successive expulsions was significantly longer in the induced group than in the control group (115.6 ± 82.8 min (range 34–265) versus 68.8 ± 24.5 min (Fontbonne et al., 2009), and that brachycephalic breeds are prone to dystocia (Darvelid and Linde-Forsberg, 1994), caution is warranted in applying parturition induction protocols to brachycephalic and dystocia prone breeds. Dopamine agonists are not useful for the induction of parturition due to the extended interval and unpredictability of treatment to onset of parturition. Prolactin secretion inhibitors would also decrease or halt lactation. Studies with the progesterone-receptor blocker mifepristone have shown variable results. In one study an incomplete parturition, which did not proceed beyond the stage of dilatation of the cervix was induced (Nohr et al., 1993) whilst another reported a normal course of parturition using mifepristone (van der Weyden et al., 1989). Another progesterone receptor blocker, aglepristone, has also been employed for the induction of parturition (Baan et al., 2005; Fieni and Gogny, 2009). Both these progesterone receptor blockers could however also only induce an incomplete parturition in some instances (Nohr et al., 1993; Hoffmann et al., 1999; Baan et al., 2005). The work of Kowaleski et al. (2010) may provide an explanation for the observation that aglepristone-induced parturition in the dog may or may not require ecbolic support

(Kowalewski et al., 2010). Cyclooxygenase 2 is the essential enzyme allowing for the formation of the common precursor of PGF2α and PGE_2 and the hormones leading to uterine contractility and cervical dilatation respectively. Provision of adequate amounts of these precursors seems to be restricted to the immediate phase prior to parturition. It thus follows that if aglepristone is administered prior to the precursor formation, ecbolic support will be required and conversely, if aglepristone was administered closer to normal expected parturition date, none is required (Kowalewski et al., 2010). In another study, it was confirmed that plasma PGFM concentrations were significantly lower in aglepristone induced bitches when compared to untreated bitches prior to normal parturition (Baan et al., 2008). When ecbolic support was required in aglepristone induced parturition, it was successfully achieved using either oxytocin almost hourly (Fieni and Gogny, 2009; Fontbonne et al., 2009) or prostaglandins once or twice (Riesenbeck et al., 1999; Fieni et al., 2001).

The temporal events following parturition induction are important. In one study, expulsion of the first puppy occurred between 32 and 56 h after the first treatment with aglepristone which represents a 24 h variation. The variation in expulsion phase was 12.8 h, estimated gestation length shortening was 2.7 d and puppy survival at 7 d was 83% (Baan et al., 2005). In the study by Fieni and Gogny, (2009), gestation length was shortened by approximately 24–48 h, variation in response after induction was 18.9 h and puppy survival 86%. From these time frames, it follows that bitches induced require observation for a minimum of 36 h following induction. This approach thus necessitates around the clock observation and does not meet the requirements of this study as the aim is to be able to leave the bitch unattended for night-hours.

The dose of aglepristone (Alizine, Virbac Laboratories, Carros, France) used in two studies (Fontbonne et al., 2009; Baan et al., 2005) was 15 mg/kg given either once or twice with 9 h interval between administrations. This dose is higher than the 10 mg/kg used to terminate pregnancy (Pettersson and Tidholm, 2009; Galac et al., 2000). It is not clear why a second dose 9 h (Baan et al., 2005) later would be required if therapeutic levels are maintained for 24 h following a single administration of aglepristone.

Gestation timing in the induction of parturition is very important. In one study (Fontbonne et al., 2009) all four of one litter of Yorkshire Terrier puppies in the induced group were

premature at the time of birth and died between 19 h and 29 h after delivery. When comparing parturition induction protocols, it is important that conclusive evidence is provided whether the gestation period has indeed been shortened. This can only be achieved if accurate and precise methods are used to time gestation and when the experimental number is large enough to yield statistically significant results.

In the study where premature puppies were delivered following parturition induction (Fontbonne et al., 2009), the aglepristone administration before parturition was neither able to prevent prematurity nor to induce the release of lung surfactant (induce maturation), as indicated by the post-mortem examination.

Birth weight of the puppies was not significantly different in aglepristone induced group and control bitches (Fontbonne et al., 2009). This was an unexpected finding because Evans and Sack (1973), Salazar and Yllera (1991) and Moriyoshi et al. (1996), reported a very pronounced growth rate of canine foetuses in late pregnancy. This suggests either that aglepristone treatment had no effect on the weight gain of the puppies or that the aglepristone was administered very close to due date, or lastly, that the growth rate in the last few days is indeed not that pronounced as is suspected?

The number of interventions following induction of parturition until the birth of the last puppy is of interest. In the study by Baan et al. (2005) and Fontbonne et al. (2009) ecbolic support had to be given numerous times and examined every half hour to monitor response to treatment. It is concluded that the induction of parturition using aglepristone may have some value in facilitating placental separation in preparation for a planned CS and thus preventing possible post CS haemorrhage.

2.13. Aglepristone

Aglepristone, marketed under the name Alizine®, is a synthetic steroid with an anti-progesterone action by competing with endogenous progesterone at the level of the progesterone receptors. It is a potent progestin antagonist with no agonistic effect, even at high doses (Hoffmann and Schuler, 2000). Aglepristone has a very strong binding affinity for the uterine progesterone receptors in the bitch, cat and rabbit and competitively binds to the uterine progesterone receptor with three times the binding affinity of progesterone, but without the physiological effect, resulting in displacement and blocking of the progesterone

hormone (Hoffmann and Schuler, 2000). Due to the specific, targeted, local effect, Alizine is able to work effectively, and without the side effects related to other pharmacological means of abortion. In early pregnancy, aglepristone inhibits endometrial changes and modifies uterine secretions, thus preventing embryo implantation (Fieni et al., 2001). Later administration prevents progesterone from maintaining gestation and therefore induces a true abortion with expulsion of foetuses (Fieni et al., 2001). The luteal demise following aglepristone administration is not well understood. The luteal demise occurs in pregnant bitches where termination of pregnancy is desired (Galac et al., 2000) and in non-pregnant bitches treated for pyometra (Fieni, 2006).

After two injections of 10 mg/kg/day at 24 h-intervals, the peak plasma concentration of aglepristone in the plasma is around 280 ng/ml. This peak is reached within 2.5 d with the mean time wherein aglepristone can still be detected (residence time) is around 6 d. Excretion is very slow with only 6% of the administered dose being excreted during the first 10 d. The injection of an oily substance by intramuscular (im) or subcutaneous (sc) routes causes lesions of varying intensity. The sc route is better tolerated. Massaging the area can lower local reaction at the injection site. If more than 5 ml is required, more than one injection site should be used.

Aglepristone was reviewed extensively to establish whether it may theoretically play a role in facilitating placental release and or foetal maturation when used prior to preparturient elective CSs.

2.14. Prediction of parturition dates based on events during the peri-oestrous period

The prediction of parturition date is discussed under various headings with specific reference to accuracy, influence of numerous variables and most important, significance and value for use in this study. Gestation length in the bitch can vary depending on which event in the cycle is determined as the starting point or chronological landmark. Historically, since the pioneering studies of Concannon et al. (1977) and Wildt et al. (1978), the chronological landmarks have been the LH surge, LH peak and estimated day of ovulation based on the known temporal relationship between LH and ovulation. Due to practical difficulties in determining LH, first rise in PC above 6.4 nmol/L was later adopted as chronological landmark.

2.14.1. Parturition date relative to the appearance of the vagina during oestrus

Visual inspection of the vagina through a speculum is valuable to examine the vagina of the bitch (Borkowska et al., 2003; Lulich, 2006). The macroscopic changes in the vaginal mucous membrane of the bitch throughout the oestrous cycle were first described by Lindsay et al. (1988). Frequent visual inspection of the vagina through a speculum during oestrus allows one to predict the optimal day of mating (Lindsay et al., 1988) but is not reliable to predict the day of parturition (Fay et al., 2003; Seefeldt et al., 2007).

2.14.2. Parturition date relative to vaginal cytology

Vaginal cytology was first described by Schutte (1967b). Vaginal cytology has been employed to determine time of ovulation (Jeffcoate and Lindsay, 1989; Bouchard et al., 1991) and other physiological events. It was concluded that cytology is not useful in breeding management to determine the optimal mating period (Hiemstra et al., 2001). It can be used, however, to determine the stage of the cycle in general (Bell and Christie, 1971; Holst and Phemister, 1975) and to determine the onset of cytological dioestrus which has retrospective value in indicating the time of the fertilization period. Vaginal cytology is influenced by staining methods and examiner (Ehlers, 2004). Vaginal cytology was found unreliable to predict day of parturition in the bitch (Fay et al., 2003).

2.14.3. Parturition date relative to first day of cytological dioestrus

The first day of cytological dioestrus is not a new concept but since accurate counting of days from one event to another in this manuscript is crucial, no confusion may exist with regards to potential confusing denotations. Generally, cytological dioestrus starts 6 d after the estimated time of ovulation (Bouchard et al., 1991). Since Holst and Phemister (1974) defined the onset of cytologic dioestrus, they and others thereafter, denoted this date as day 1 denoted as (D1) (Holst and Phemister, 1974; Olson et al., 1984). From the above some may start counting from 0, 1, 2, whilst others might start from 1, 2 and so forth. This constitutes a difference of 24 hours which may be critical when planning a CS. Therefore, in this manuscript, the first day of cytological dioestrus will be denoted as D0. Eilts et al. (2005) reported mean intervals of 56 d with a SD of 2.8 d in 152 pregnancies which represents a larger spread than the mean intervals of 56.88 d with a SD of 1.61 d in 93 pregnancies as found by Holst and Phemister (1974). Although knowledge of D0 is very helpful in

approximating parturition dates, it is not known if these dates can be used for safe intervention by CS or can be safely used to time preparturient CS.

2.14.4. Parturition date relative to breeding dates

The disparity between apparent and actual gestation length in the dog is due to the long period over which dog spermatozoa remain fertile in the bitch (Doak et al., 1967; Concannon et al., 1989) together with the long period of oestrus (Concannon et al., 1989) that causes the apparent variation in gestation length. Another reason is the wide variation in apparent gestation length is the interval from onset of behavioural oestrus to ovulation (Tsutsui et al., 2009). Because there is also a variable time between ovulation and fertilization it is more correct to state that the variation in interval between the onset of oestrus and the time of fertilisation of oocytes is the greatest contributor to the apparent variation in gestation length.

The apparent gestation length can vary by as much as 14 d (57–72 d) when timed from the first of multiple matings or by as much as 11 d (58–69 d) when timed from a single mating (Holst and Phemister, 1974). This range of 14 d is of no help in the management of parturition. Mating dates in absence of time of ovulation is useless (Tsutsui et al., 2006; Shimatsu et al., 2007). Use of breeding dates alone does not provide due dates with adequate accuracy (Lopate, 2008). The variation in interval between a fertile mating and the time of fertilisation may bring about the variation in the apparent duration of gestation in the bitch. Breeding dates are useless in predicting the date of parturition.

2.14.5. Parturition date relative to LH surge

The actual gestation length in the bitch is 65 ± 1 d when timed from the preovulatory LH surge in peripheral blood (Concannon et al., 1983). The day of the LH surge is a reliable physiologic event in canine reproduction, from which ovulation, oocyte maturation, implantation, foetal development, and parturition date can be determined (Concannon et al., 1975). It is important to emphasize that high LH concentrations, (concentrations of not less than 10 ng/ml), may last 6–18 h around the LH peak and this duration varies considerably among individuals (Hase et al., 2000). The estimated peak value of plasma LH is 24.8 ± 3.1 ng/ml on average (range 13.6–42.4 ng/ml) (Hase et al., 2000). The mean plasma LH levels are 7.0 ± 2.1 and 14.7 ± 4.4 ng/ml at 12 h and 6 h before the peak, respectively, and 14.2 ± 4.1 and 5.8 ± 2.4 ng/ml at 6–12 h after the peak (Hase et al., 2000). From this it

is clear that to obtain reliable results, several determinations per day are necessary rendering this method impractical and expensive. Hase et al. (2000) used the first day that LH exceeded 10 ng/ml in the plasma as the day of the LH surge whereas another worker used the first day of elevated concentrations of LH as the day of the LH surge (Onclin et al., 2002).

There may be value determining the first day on which the LH surge started and the day on which it ended or determining merely one or more days on which the concentration of LH in the blood plasma (serum) is elevated. One study used an in-house Witness® LH test (Zoetis) on saved serum samples from the date when PC rose to above 4.8 nmol/L (Cohen et al., 2009). In this study, Day 0 was defined as the day serum tested positive for LH (LH surge) on the in-house LH test. The predicted parturition date, 65 d following the day of the LH surge was compared to actual parturition date (the day the first puppy was delivered). Cohen et al. (2009) predicted the day of parturition to within ± 1 d and ± 2 d in 82% and 100% of 98 pregnancies in bitches. The precision of prediction was not affected by breed or litter size. These results compare favourably with those obtained in another study using prebreeding PC alone (Kutzler et al., 2003a).

The LH peak will remain an accurate pivotal or key event in the canine oestrus cycle in experimental studies which may be used as chronological landmark but it remains unavailable in clinical practice (Goodman, 2001). Since the time of the LH surge detected by the canine LH assay kit was very similar to the LH surge detected by RIA (Nishiyama et al., 1999), there may be value in using these kits in controlled studies. It is crucial that in any trial involved in establishing time of ovulation or prediction of parturition, the time of the LH peak be established by either direct means by measuring the LH using RIA or commercial LH kits or it may be estimated by indirect means by determining the first rise in progesterone above 6.4 nmol/L. This discussion was necessary as the current study wished to evaluate the interval between the first day of the LH surge determined using the witness LH assay kit (Zoetis) and the onset of parturition

2.14.6. Parturition date relative to absolute progesterone values at around the time of ovulation

In cases of frozen semen where sperm lifespan may be on the order of hours, it is critical that one or more inseminations occurs after the time of oocyte maturation, at 5–6 d after the LH surge and 3–4 d after ovulation (Concannon, 2011). The first day of the oestrous cycle

on which the concentration of progesterone exceeds 6 nmol/L is important as that day allows one to predict the days on which the highest fertility should be expected with the use of frozen-thawed spermatozoa (Steckler et al., 2013). The mean duration of pregnancy from the estimated day of ovulation (PC exceeding 19 nmol/L) was 63.1 d (SD 2.1 d, ranging from 60 d to 68 d) (Mir et al., 2011).

Previous studies of canine reproductive physiology indicate that PC can vary widely after the LH surge. Ovulation was estimated to occur when PC was 15.58 d (SD 3.18 d, n = 15) (Bouchard et al., 1991). Reports using quantitative results from RIA to estimate ovulation 2 d after LH surge with PC ranging from 15.9 to 19.1 nmol/L or the time of insemination 4 d after LH surge with PC ranging from 19.08 to 25.44 nmol/L have predicted gestation lengths ranging from 58 d to 70 d (Badinand et al., 1993; Jeffcoate and Lindsay, 1989; Linde-Forsberg and Forsberg, 1993; Linde-Forsberg et al., 1999; Okkens et al., 1993). In the study by Okkens et al, (1993), parturition in the dog took place after a pregnancy with an average length of 61.4 d, measured from the single day of mating which was determined based on the plasma progesterone pattern but the variation in the duration of gestation ranged from 4 d to 7 d.

Seefeldt et al. (2007) critically evaluated the value of progesterone at the time of ovulation to predict the day of parturition. The day of ovulation was estimated by semi-quantitative or quantitative measurement of PC ranging from 15.9 to 25.4 nmol/L. Though 66.1% of the bitches whelped 62–64 d after the estimated day of ovulation, the remainder did not and the variation in gestation length in them was 59–70 d. It was concluded that this variation was essentially caused by methodical inaccuracies in determination of the time of ovulation and an individual variability of the PC at ovulation. Other workers found that assaying progesterone around the time of ovulation instead of ovarian ultrasonography to detect ovulation may lead to variability in estimating the time of ovulation by up to 24 h in up to 20% of the cases (Seefeldt et al., 2007; Marseloo et al., 2004). Further research is however required to establish the accuracy of gestation length in the bitch based on counting from an absolute progesterone value.

2.14.7. Parturition date relative to the time (or day) at (on) which progesterone reached a concentration above the preovulatory threshold (6 nmol/L)

The time (or day) at (on) which progesterone reached a concentration above the preovulatory

threshold has been used in a variety of ways to time a managed breeding or insemination. Parturition occurs 65.1 ± 0.1 d (SEM, n = 54) after the LH surge in the bitch (Concannon et al., 1983). The LH surge can be measured directly, or the time of its occurrence estimated by the concomitant initial preovulatory rise in plasma PC that occurs during oestrus (de Gier et al., 2006). Progesterone concentrations in the plasma or serum around the time of the LH surge have been reported (de Gier et al., 2006; Badinand et al., 1993; Wright PJ, 1990; Kutzler et al., 2003a; Concannon et al., 1977a; Wildt et al., 1979). One study evaluated the effect of body mass on peri-oestrous progesterone profiles and defined the day of the preovulatory rise in serum progesterone as the day that the progesterone rose to above 4.77 nmol/L followed by a continued rise to 9.54 nmol/L the following day (Kutzler et al., 2003a). Kutzler et al. (2003a) found that the mean PC at day of the initial rise in preovulatory progesterone was 6.42 ± 0.57 nmol/L for all body mass groups. This study showed that the rate of the rise in PC between day of initial rise and 4 d later did not vary significantly in bitches of the same body mass, but did differ significantly between bitches of varying body mass (Kutzler et al., 2003a). This is in contrast to previously reported PC concentrations on the day of ovulation (2 d after LH surge) as reported by Concannon et al. (1983), which do not reflect the significant variation as found by Kutzler et al. (2003a). Predicting the date of parturition using PC after the LH surge was inaccurate but using the initial rise in PC allowed prediction thereof as falling in the interval of 65 ± 3 d in all bitches, irrespective of body mass or litter size (Kutzler et al., 2003a). In this study, ovulation was considered to have occurred when PC exceeded 6.4 nmol/L. Kutzler et al. (2003a) concluded that pregnancy length from the day of the preovulatory rise in progesterone, defined as the day when PC rose to 5.7 nmol/L was 65 ± 1 d, 65 ± 2 d or 65 ± 3 d interval with a 67%, 90% and 100% of accuracy, respectively.

In another study, the interval from the estimated day of ovulation based on progesterone exceeding 6 nmol/L to parturition ranged from 61 to 66 d with the mean 63.9 ±0.2 d (SEM, n = 36) (Tsutsui et al., 2006).

The mean (mean ± SD), estimated pregnancy length based on estimated day of ovulation (PC reaching 19 nmol/L) was 63.1 ± 2.1 d (range 60–68) (Mir et al., 2011). It is important to note that the accuracy approached 100% only when the interval approached 65 ± 3 d. This leaves the veterinary obstetrician with essentially eight potential parturition dates to observe.

2.14.8. Parturition date relative to ultrasonographic timing of ovulation

Earlier, workers found that the ovaries of the bitch are difficult, and sometimes impossible, to observe by means of conventional ultrasound because they are small, difficult to resolve from surrounding tissues (particularly if lying deep in large dogs) and are often obscured by intra-intestinal gas (Yeager and Concannon, 1990; Boyd et al., 1993; Hayer et al., 1993). In a study using ovarian ultrasonography for determination of the time of ovulation in bitches, implementing PC determination concurrently with ultrasound examination may help in improving the reliability of the ovulation time detection (Wallace et al., 1992; Marseloo et al., 2004). The process of ovulation could be assessed with reasonable accuracy with improved quality of equipment, although the exact fate of the follicle could not be imaged (Boyd et al., 1993).

More recently, workers found that accurate identification of the ovaries depended on the presence of significant follicles or corpora lutea (Davidson and Baker, 2009). Real-time ultrasound imaging was used in a clinical study to estimate the number of follicles of different sizes, ovulation and conception rates, and to study follicle dynamics following the induction of oestrus (England et al., 2009). A direct detection of ovulation can be done by ultrasonographic examination (Reynaud et al., 2006; Reynaud et al., 2009). It must be considered that there has been considerable technological progress made in the ultrasound equipment, which has improved the ability to both locate the ovary and identify structures on it. With tissue harmonic imaging good images are often obtained with less effort (Hohl et al., 2004). Repeated visualisation (two times a day) is required to determine the time of ovulation (Hase et al., 2000).

Determining the date of ovulation by means of ultrasound appears possible given high resolution apparatus and an experienced operator. However, the most advanced technology cannot overcome the impediment of gas-filled structures overlying the ovary in some bitches. Lack of operator skill is likely to reduce ultrasonography as means of ovulation timing to third choice after determination of the preovulatory rise in progesterone and determination of the LH peak. Studies to evaluate this direct measurement of ovulation as predictor of the onset of parturition have not been completed to date. In a study by Fontbonne (2008), it was shown that ovulation could be precisely and accurately determined using once daily ovarian ultrasonography in the bitch. In only 15.3% of bitches was the day of ovulation detected more precisely with ultrasound than was the case with PC However, Fontbonne

(2008), conceded that even with a high-quality machine, features of ovulation may be difficult to visualise in large breeds.

Although ultrasound has potential, access to high resolution sonogragphs and operator skill remains a problem and was not used in this study.

2.15. Prediction of parturition dates based on events during gestation

2.15.1. Prediction of parturition date based on ultrasonography of gravid uterus

Ultrasonography is an excellent tool to diagnose pregnancy, monitoring high risk pregnancy (Zone and Wanke, 2001; Di Salvo P. et al., 2006; Miranda and Domingues, 2010) and even determining foetal sex (Garand et al., 2009). The efficacy of ultrasonography to determine the parturition day (when used on a breed per breed basis e.g. Yorkshire Terrier) renders accurate results (Jabin et al., 2007).

When no information on the bitch is available and the bitch is first examined for pregnancy diagnosis, the day of parturition can be predicted by the ultrasonographic evaluation of foetal size and the prediction is expressed as days from parturition instead of days of pregnancy (Luvoni and Beccaglia, 2006).

The ultrasonographic measurement of certain extra-foetal structures is an accurate method to evaluate gestational age and to predict the day of parturition when the bitch is examined for pregnancy diagnosis during early gestation. During early gestation, the most suitable parameter is the inner diameter of the chorionic cavity (ICC) (England et al., 1990; Luvoni and Grioni, 2000; Kim and Son, 2008; Socha et al., 2008). It can be imaged for the first time 45 d before onset of spontaneous parturition (P0) as a spherical anechoic structure and remains spherical up to 25 d before P0, where after it becomes elongate (Luvoni and Grioni, 2000). Whilst spherical, the measurement of the ICC is determined by taking the mean of two ICC diameters made at 90º angles from one side of the trophoblastic decidual reaction to the other. After it has become elongate, one needs to measure the interpolar diameter and the equatorial diameter, as well as the two perpendicular diameters in the equatorial plane. The ICC is significantly related to gestational age (England et al., 1990) and to predict the parturition day. Different equations, derived from growth curves, can be applied in small (Luvoni and Grioni, 2000; Son et al., 2001) and medium size bitches (Yeager et al., 1992; Luvoni and Grioni, 2000). Correction for giant size is needed (Kutzler et al., 2003b). The

accuracy using ICC when predicting the delivery day (± 1 d) was approximately 77% in small and medium size bitches, while within ± 2 d was 88% and 85% respectively and prediction was not affected by litter size or sex ratio. The ultrasonographic measurement of the outer uterine diameter at the implantation sites; the placental thickness and the length of zonary placenta are also significantly and linearly related to gestational age (England et al., 1990; Yeager et al., 1992; Luvoni and Grioni, 2000; Son et al., 2001). Predictions obtained by these measurements are less accurate than those obtained by ICC, because the uterine wall and foetal membranes are characterized by less defined margins than the chorionic cavity (Luvoni and Grioni, 2000).

In late gestation, less than 25 d to P0, foetal parameters are more easily imaged. Crown-rump length (CRL) is the distance between the most rostral point of the crown to the caudal edge of the perineum (England et al., 1990; Yeager et al., 1992; Son et al., 2001). Although reliable at 25 d before P0, the CRL becomes difficult to measure closer to P0 because of foetal flexion, foetuses overlying each other and foetal length that exceeds the size of the sector image. CRL may therefore be difficult to measure in large breeds. The diameter of foetal heart may also be measured by measuring the major and minor axes of the maximum cross section of the heart (Moriyoshi et al., 1996). However, the reliability of this method is impeded by the error made due to cardiac cycle contractions. Body diameter is measured taking the two diameters made at 90º angles in the transverse plane at the level of foetal liver and stomach (England et al., 1990; Yeager et al., 1992; Moriyoshi et al., 1996). This measurement, when combined with BPD to predict the same end-point, increases the accuracy of the prediction.

The BPD is the most suitable measurement to predict the day of parturition during late pregnancy and it is taken on the same longitudinal section of CRL (England et al., 1990; Luvoni and Grioni, 2000). In late pregnancy, the foetal head is easily identified but it may be more difficult to image it on a longitudinal plane. For the measurement to be accurate, the parietal bones of the skull must be parallel to measure the correct distance between them. When done correctly this measurement is highly correlated with gestational age. Applying the BPD equations, the accuracy of prediction of P0 ± 1 d was 75% and 63% in small and medium size bitches, while within P0 ± 2 d, it was 88% and 81% respectively and not affected by litter size and sex ratio (Beccaglia and Luvoni, 2006). The foetal head diameter has been proposed as a predictor of delivery date (Yeager et al., 1992; Moriyoshi

et al., 1996). The foetal head diameter is measured as the largest cross-sectional diameter of the head when this structure was well identified in longitudinal section. The symmetry of the head and the central location of the echoic *falx cerebri* have to be taken into account to obtain reliable measurements (Son et al., 2001). The ultrasonographic study of the growth of the deep portion of telencephalic vesicle promises encouraging results. This structure is represented by thalamus and basal nuclei primordia and can be visualized between 30 d before P0 and 8 d before P0 (Beccaglia and Luvoni, 2004). This measurement is feasible in dogs of different sizes and it could be usefully combined with BPD measurements in order to increase examination reliability (Luvoni and Beccaglia, 2006).

Several authors derived different equations from the growth curve of the cited structures, which can be applied to different sizes of bitches. Body mass did not affect the accuracy of parturition day prediction in medium and large bitches, but a correction factor for small and giant size is necessary (Kutzler et al., 2003b). Several workers have employed ultrasonographic findings to establish a prediction table of parturition day. In one study in small breeds, extra foetal and foetal structures were measured from all conceptuses. The parameters that exhibited the best correlation to parturition were the inner chorionic cavity diameter from 18 d to 37 d after the estimated day of ovulation (when PC first increased above 15.2 nmol/L) and the foetal head diameter from 38 d after the estimated day of ovulation to parturition (Son et al., 2001). Using this method, 64.3% of bitches delivered exactly on the date predicted and 32.1% delivered within 1 d of the date predicted (Son et al., 2001). In another retrospective study on foetal measurements obtained by transabdominal ultrasonographic examination, gestational age of 32 breeds of different sizes was estimated using two published tables correlating either embryonic vesicle diameter, crown-rump length, body diameter and BPD to the LH surge in mid-gestational beagles or body diameter and foetal head diameter to parturition in late-gestation. Parturition date was predicted by obtaining the difference between the gestational age estimate and 65 d. The accuracy of the prediction was not affected by litter size but was affected by maternal body mass for small and giant body mass groups only. When adjusted for body mass, the accuracy of prediction within ± 1 d and ± 2 d intervals was 75% and 87%, respectively (Kutzler et al., 2003b). The most accurate prediction of parturition date was obtained when foetuses were measured at 30 d after the LH surge, regardless of body mass or litter size. It is important to note that parturition date predictions made after 39 d of gestation using only biparietal and body diameter foetal measurements were less than 50% accurate within ± 2 d (Kutzler et al.,

2003b; Lenard et al., 2007) whereas foetal head diameter was the most accurate for estimation of gestational age after Day 38 to parturition (Kim and Son, 2008).

The main shortcoming of ultrasound in staging gestation is that its accuracy increases by the number of conceptuses measured as well as with the number of parameters measured. Furthermore, depending on method, correction factors for body mass need to be employed.

The predictors discussed above are quantitative. Therefore, the researchers that did the work mostly derived regression formulae from which to calculate the most likely date of parturition. Possibly, the requirement for adjustments for bitch body mass are required due to differences in litter size and individual size of the conceptuses. Qualitative changes, may well not be influenced by size of bitch, litter size or the genotype of the foetus (which is the main determinant of its size relative to the expected size in the breed of the dam) and may provide useful predictors of the onset of parturition. The foetal heartbeat, calcification of the foetal skeleton, the line of contrast between the hypo-echoic liver and the hyperechoic lungs, the foetal stomach, the foetal gall bladder, the foetal urachus and foetal intestinal peristalsis are qualitative parameters that have been evaluated. Other potential qualitative variables may include "the time at which the allantoic cavity or the allantoic membrane is first seen", "the time at which the amniotic cavity or the amniotic membrane is first seen" and "the diameter of the yolk sac as a proportion of the transverse diameter of the conceptus". Such parameters have not been investigated and are potential subjects for future research.

2.15.2. Parturition date relative to foetal heart rate monitoring

The heart rate of dog and cat foetuses in utero studied using real-time B and M mode ultrasonography were recorded at 238 ± 16.1 beats per min (SEM, n = 15) at Day 40 following the LH peak. A reduction was noted near parturition (Verstegen et al., 1993). Another study in humans concluded that late decelerations of the foetal heart rate are an early sign of foetal hypoxia (Monheit et al., 1988). In a case where foetal heart rate was monitored every half hour with intent to perform a CS on a singleton bitch, the foetal heart rate declined rapidly and the puppy died before emergency CS could be performed (Personal communication, Nöthling). In one study in the bitch, foetuses were considered to be normal when heart rate, determined by a Doppler flow meter was > 220 bpm; suffering from slight foetal distress when heart rate was between 180 and 220 bpm, suffering from severe foetal distress when heart rate was < 180 bpm (Zone and Wanke, 2001). There appear differences

in the literature regarding the heart rates that signal foetal distress. In contrast to the above study, in another study the foremost indication for CS was considered a reduction in foetal heart rate, indicating foetal stress. A foetal heart rate < 150 bpm is considered an emergency and CS should be performed as soon as possible (Traas, 2008b). A rate of 150–170 bpm indicates moderate to severe foetal stress, whereas a rate of > 180 bpm should be considered normal (Traas, 2008b). It is noteworthy that a brief reduction in heart rate (usually determined with ultrasonography) may occur due to passage of a uterine contraction over a foetus. Therefore, any foetus with a low heart rate should be monitored for 30–60 s, or reassessed 1–2 min later, to ensure that the low heart rate is caused by foetal distress, rather than a uterine contraction (Traas, 2008a). Foetal heart rate is an excellent tool to establish foetal health but has no practical predictive value of when to expect the onset of spontaneous parturition.

2.15.3. Parturition date relative to abdominal palpation

Canine pregnancy diagnoses performed by abdominal palpation performed between days 25 and 35 of pregnancy is about 90% accurate in confirming pregnancy (Taverne et al., 1985) but false negatives are common in bitches carrying small litters (Shille and Gontarek, 1985). Palpation has no value in predicting parturition dates (Socha et al., 2008).

2.15.4. Parturition date relative to radiography of gravid uterus

Late-term radiography is the best modality for accurate assessment of litter size. Radiography may also reveal an emphysematous foetus or foetal skeletal or skull collapse (Spalding sign), all of which are consistent with foetal death (Johnston et al., 1983). Radiography can be used to approximate foetal maturity. Radiographs can detect mineralized foetal skeletons 43–54 d after breeding (Rendano Jr et al., 1984). The foetal pelvis, ribs, radius, and ulna appear on radiographs 11 d before parturition, and teeth appear 4 d before parturition (Rendano Jr et al., 1984). Radiography is a diagnostic modality whereof the results depend on factors relating to the apparatus, operator and finally the subject being radiographed. Under ideal circumstances, good quality radiographs may result but this is not always the case in clinical practice. The positioning of the foetuses in relation to gut loops, faecal balls and gas has a major influence on demonstration of fine details as described above. To date late-term radiography has not been employed to time CS and appears to have poor potential in precise prediction of the date of onset of parturition.

2.15.5. Parturition date relative to preparturient decline in progesterone

It has been established that PC decreases prior to parturition (Concannon et al., 1975; England and Verstegen, 1996a; Onclin and Verstegen, 1997). The temporal relationship between the steep decline in PC and onset of parturition is important. Identifying a threshold of PC below which all bitches are within a consistent and definable interval prior from the onset of spontaneous parturition would be very helpful.

Most studies on the decline in preparturient PC were not designed to evaluate usefulness for predicting parturition for our purposes (Edqvist et al., 1975; Hoffmann et al., 1999; Leroyer et al., 2002). The experimental numbers were too few, seldom exceeding six and the frequency of blood collections was inadequate. There are many other shortcomings in these studies for our purposes. The stage of onset of parturition from which counting of hours commenced retrospectively was not standardised. For instance in the literature counting may have started from first signs (also poorly defined) of Stage 1 of parturition (Nohr et al., 1993), time from expulsion of first puppy (Baan et al., 2005), or when bitch is parturient (Veronesi et al., 2002). Owing to these shortcomings but mainly to the long interval between blood collections, it is not possible to accurately compare the data of any of the workers that examined preparturient decline in PC with those of Concannon et al. (1988), which is further discussed below.

It is clear from the above requirements that a study that would provide data with potential high precision, would require very frequent blood collections and assays in the last days of pregnancy. The study by Concannon et al. (1988) meets all the requirements for the current study and provided data central to the design of the current study. Key to this study was the short interval of 3 h in between blood collection starting from 4 d prior to parturition, enabling establishment of a PC curve of high resolution.

This frequent blood collection by Concannon et al. (1988) allowed them to align the data from onset of parturition retrospectively with a small loss of precision due to the short sample collection interval of only 3 h. A summary of their results is given: Concentrations of progesterone were measured in plasma collected from 6 bitches every 3 h starting 2.8–4.6 d before the birth of first puppy and continuing until 0.4–0.8 d after delivery of the last puppy. The mean (SEM) concentrations of progesterone in six bitches at 48, 24, 12 and 3 h before the delivery of the first puppy were 8.90 (0.95) nmol/L, 7.00 (1.28) nmol/L,

3.82 (0.64) nmol/L and 2.23 (0.64) nmol/L.

Various studies report a PC of 6.4 nmol/L below which most of the bitches studied were within 48 h of P0 (Concannon et al., 1975; England and Verstegen, 1996a; Onclin and Verstegen, 1997; Austad et al., 1976). These studies do not reveal whether exceptions and outliers, which are important to the current study, occurred. An example of an outlier is demonstrated in a study where a fall in PC to undetectable levels was reached in one bitch as much as 10 d before whelping (Austad et al., 1976). Another exception was in at least one bitch in their study, they reported that PC were less than 6 nmol/L for 6 d before parturition (Onclin and Verstegen, 1997). In contrast to the steep decline 24–36 h prior to parturition reported by Concannon et al. (1989), Luz et al. (2006) reported a steady but variable decline in PC towards parturition in late gestation.

The preparturient decline in PC requires investigation for usefulness as predictor of the time of onset of parturition. In this study, we will retrospectively assess the pattern of decline in PC and the absolute value of PC during the few days before parturition to determine whether there is a pattern of decline during a period of 1 d or shorter, or whether there exists some absolute value of PC which will enable one to predict when parturition will occur.

2.15.6. Parturition date relative to the preparturient fluctuations in serum oestradiol

Plasma 17 ß-Oestradiol concentrations were monitored daily from 15 d before the LH peak to reach maximum values (79.1 ± 12.2 pg/ml) 24–48 h before the LH peak and 44.4 ± 9.2 pg/ml at the time of the LH peak (Onclin et al., 2002). Nine days following the LH peak, oestradiol decreased to basal values where after it increased again and remained significantly elevated throughout the luteal phase both in pregnant and non-pregnant animals (Onclin et al., 2002). Similar results were confirmed in another study (Hoffmann et al., 1994). In humans (Fuchs and Fuchs, 1984) and sheep (Challis, 1971), oestrogens play a key role in sensitising oxytocin receptors prior to parturition. The dog seem to be different from most other domestic animals in this respect (Hoffmann et al., 1994). The role of oestrogens at the onset of parturition in the bitch remains undefined (Verstegen-Onclin and Verstegen, 2008). It is unknown what sensitises oxytocin receptors in the preparturient bitch.

These studies suggest that oestradiol will not be useful in the current study.

2.15.7. Parturition date relative to the preparturient fluctuations of concentrations of cortisol

The cortisol in the maternal blood may originate from the dam, the foetus or both. Measurement of hormonal concentrations in the foetus is theoretically possible but impractical. In the current study, the concentration of cortisol will be determined in maternal blood plasma, without considering its origin.

Studies on the periparturient cortisol profile varied. Whilst all studies concurred that the concentration of cortisol remains elevated during parturition (Concannon et al., 1978; Concannon, 1986; Hoffmann et al., 1994; Veronesi et al., 2002; Olsson et al., 2003; Baan et al., 2008; Bergström et al., 2010), only three report the cortisol concentrations during the 24–30 h before the onset of expulsion (Concannon et al., 1978; Baan et al., 2008; Bergström et al., 2010) and two of these confirmed elevated cortisol concentration prior to parturition. The concentrations of cortisol in serum samples collected at 8–16 h intervals 2–4 d before parturition ranged from 30 to 119 nmol/L (11–43 ng/ml) and averaged 63.2±3.3 nmol/L (22.9 ± 1.2 ng/ml) (n = 57) (± SEM). A distinct increase in cortisol concentration occurred prior to parturition, with peak concentrations of 172.5 ± 19.8 nmol/L (range 116–240) (62.5 ± 7.2 ng/ml (range 42–87) occurring 12.7 ± 2.6 h preparturient (range 8–24 h) (Concannon et al., 1978). Cortisol concentrations were subsequently reduced 53.2±9.9 nmol/L (19.3 ± 3.6 ng/ml) at 8–12 h after parturition. Mean concentrations of cortisol during lactation and after weaning was between 60.1±3.3 and 73.7±14.9 nmol/L (21.8 ± 1.2 and 26.7 ± 5.4 ng/ml). Based on these values, it may be postulated that all dogs within 8 h of parturition will have a cortisol concentration of 116 nmol/L (42 ng/ml) or more (Concannon et al., 1978).

Cortisol concentrations are difficult to interpret as stress may influence them. Nevertheless, cortisol concentrations should be considered a worthy candidate for investigation. Prof. Henk Bertschinger (personal communication) referred to an earlier, unpublished study that he did in which he found that concentration of cortisol in the serum or plasma of beagles was highest during the early morning, then declined before rising again to reach a second-high level during the late afternoon following which levels decline to a nadir during the night. These observations demonstrate the importance of considering the time of day on which cortisol concentrations are measured, and of comparing levels not only across time of

day in the same bitch but also at the same time over different days in the same bitch. In this study, we will retrospectively assess the pattern of change of concentrations of cortisol at the same time of day over different days and across times in the same day during the few days before parturition. The aim would be to determine whether there is a pattern of change in the concentration of cortisol in the blood during a period spanning 1 d (following measurement at the same time of day on two consecutive days) or shorter, or some absolute concentration, that will enable one to predict when parturition will occur.

2.15.8. Parturition date relative to the preparturient fluctuations of relaxin

The syncytiotrophoblast produces relaxin in the canine placenta (Klonisch et al., 1999). Since then relaxin has been used in rapid enzyme linked immunoassay (Bencharif et al., 2001) and to indicate pregnancy in dogs and wild canids and felids (Bergfelt et al., 2010; Bunck et al., 2002; Carlson and Gese, 2007; Fontbonne et al., 2000; Einspanier et al., 2002). The temporal relationship of relaxin concentrations before parturition has not been examined and its usefulness is therefore not known. It may play a permissive role in parturition without relaxin concentrations increasing. It may be of value to assess relaxin concentrations in the 48 h before parturition in future studies.

2.15.9. Parturition date relative to preparturient fluctuations of oxytocin

The oxytocin concentrations taken at intervals throughout whelping showed large variation (Klarenbeek et al., 2007). The concentrations were obtained from normal pregnant bitches and bitches suffering from uterine inertia (Klarenbeek et al., 2007). This study found that there was no correlation between oxytocin peaks and expulsion of puppies during whelping. Thus determination of clinically significant levels of oxytocin at any specific time point during whelping or determining a ‘‘normal’’ reference range may be very difficult (Hollinshead et al., 2010). It was, however, established that low plasma oxytocin levels is a cause of primary inertia in bitches with normal serum calcium concentrations and aggravates the condition in bitches with low calcium levels (Bergström et al., 2006a) and that progesterone and PGFM was higher and the oxytocin and vasopressin concentrations lower in the dystocia dogs than in the control dogs. The findings indicate that these hormones are involved in the pathophysiology of total primary uterine inertia in bitches (Bergström et al., 2010). Although there appears little doubt that oxytocin increases during parturition (Olson, 2003) the pulsatile erratic nature towards late pregnancy would complicate its interpretation

and make it an unlikely variable for use in this study.

2.15.10. Parturition date relative to preparturient fluctuations of prolactin

Prolactin appears to be the main pituitary hormone sustaining steroidogenesis by the corpus luteum (Okkens et al., 1990). Prolactin concentrations rise in mid-gestation and remain elevated throughout gestation and lactation. Due to individual, diurnal, and or stress-related variations, serum prolactin concentrations are unreliable for pregnancy diagnosis (Onclin and Verstegen, 1997).

Serum prolactin concentration in samples collected at 8–16 h intervals from the fifth to the third day before parturition ranged from 14 to 97 ng/ml and averaged 40.1 ± 7.4 ng/ml. Peak prolactin levels occurred 8–32 h, (21.3 ± 3.4 h) before parturition (Concannon et al., 1978).

It is important to note that prolactin is elevated during the nonpregant diestrus where it functions the same as during pregnancy for luteal support and that increased prolactin concentrations in the serum are also observed in cases of overt false pregnancy.

From this it can be concluded that at least prolactin increases towards parturition and that the temporal relationship between prolactin and parturition is worth investigating in future studies.

2.15.11. Parturition date relative to preparturient decline in rectal temperature

Progesterone appears to be thermogenic in the dog. Decreased PC are followed by decreased rectal temperature. Decreased rectal temperatures in bitches are noted whether the progesterone decreases naturally following normal whelping (Tsutsui and Murata, 1982) or in induced luteolysis (Concannon and Hansel, 1977; Zonturlu et al., 2008; Williams et al., 1999; Meier and Wright, 2000) or when progesterone receptor blocking agents are used (Baan et al., 2005; Corrada et al., 2005; Veronesi et al., 2002).

For body temperature response to be useful in predicting onset of parturition it must be repeatable and reliable. Body temperature does not seem to be a suitable variable to clinically monitor abortion following aglepristone treatment (Veronesi et al., 2002). A decline in rectal temperature was of some value but was found to be too variable between individuals to use as the sole criterion in predicting onset of parturition (Long et al., 1978; Tsutsui and Murata, 1982; Veronesi et al., 2002). Some bitches do not demonstrate a

detectable decline in rectal temperature prior to parturition even when monitored three times daily (Johnston et al., 2001b). Impending parturition and the progress of parturition and delivery can be monitored by assessing rectal temperature but final decision is not based on temperature alone (Johnson, 2008b). Some veterinary obstetricians recommend owners to monitor rectal temperature 2–3 times daily during the last 2 week of gestation to establish a baseline. Temperature decreases below baseline by 1.1–1.7^{0}C which usually occurs 6–18 h before parturition. In small breeds it may decline as low as 35.8^{0}C, in medium size breeds as low as 36.8^{0}C and in large breeds to 37.8^{0}C (Linde-Forsberg and Eneroth, 2000). In some bitches, the temperature fluctuates. In a study of 100 canine pregnancies where rectal temperature was taken approximately every 12 h, the preparturient decline in rectal temperature was not detected in 19 animals before the delivery of the first puppy (Copley, 2002). Some veterinary obstetricians planned CS based on temperature decline as sole criterion (Smith, 2007). In fact the same veterinary obstetrician concedes to CS in bitches where the client requested CS based on temperature decline (Smith, 2007) whilst in another case report by the same author there had been no temperature decline prior to cervical dilatation and some foetuses going into distress (Smith, 2007). The rectal temperature, which was around 38.5^{0}C during 144 h (6 d) before parturition decreased to 37.4^{0}C at the onset of whelping (Zonturlu et al., 2008). However, following the onset of parturition, it is found that there is a statistically significant substantial increase in body temperature beginning 12 h after the onset of parturition (Williams et al., 1999). Furthermore, it was suggested that if any significant increase in body temperature is recorded at the end of pregnancy without the beginning of the expulsion of foetuses, it could indicate problems at parturition (Veronesi et al., 2002).

It is important to note that the temperature of the dam may fluctuate during the day (Honnebier et al., 1991) therefore temperatures must be taken at the same time daily (usually mornings). One worker found that, despite bitches showing circadian rhythm in rectal temperature profiles, the temperature changes caused by parturition were more marked than the circadian effect.

From the literature study, it may be concluded that the preparturient temperature decline can give important clues but that it is not accurate or reliable enough to use as sole predictor of time the of parturition or of indicating foetal maturity and safety for intervention. For these reasons, in this study preparturient temperature will not be used.

2.15.12. Parturition date relative to visual inspection of the vagina through a speculum in preparturient period

Digital and unaided visual examination of the bitch's reproductive tract is complicated by its anatomic features and dimensions. Without visual inspection of the vagina, through an endoscope or speculum, cervical examination is impossible in bitches (Schweizer and Meyers-Wallen, 2000). Visual inspection of the vagina through a vaginoscope is a valuable tool in examinations of the vagina and cervix (Borkowska et al., 2003; Lulich, 2006) but can also be achieved using a speculum.

Vaginal speculum examinations in woman may be considered the equivalent of visual inspection of the vagina through a speculum examinations in the bitch. The cervix can be easily visualised in woman but in the bitch the cervix is only clearly visible following relaxation of the paracervix through a speculum unless a thin scope is entered into the paracervix. Cervical dilatation is used as a parameter to monitor progress of parturition in women (Mahoney et al., 2010; Zhang et al., 2010). In the bitch, cervical dilation is an objective parameter that can be monitored during parturition observation to indicate that the bitch is parturient. It has been reported that the cervix remained closed despite the preparturient decline of both PC and rectal temperature in some bitches (Smith, 2007). It is not known if cervical opening and the decline in preparturient PC are synchronised. Furthermore, it has been the experience of the author that it may be difficult to observe opening of the cervix in some bitches because of ventroflexion of the vagina in heavily pregnant bitches. To overcome this problem correct handling of the bitch is required and the ventroflexion can be corrected by gently having a handler lift the abdomen. This inability to sometimes identify cervical dilatation may result in foetal compromise before a CS is done. Another exception is premature cervical dilatation which is commonly reported in man (Eggers and Doyle, 1979).Premature cervical dilatation and partial abortion has rarely been reported in the dog (Linde, 1983).

Visual inspection of the vagina through a speculum is very valuable in establishing and confirming that a bitch is parturient but has limited predictive value because when the cervix is found to be open, the bitch is already in Stage 1 parturition and if it is not open yet, there is nothing to tell you when it will open besides re-examinations. Visual inspection of the vagina through a speculum during management of parturition should be performed every 6 h but more often when other signs of Stage 1 parturition are exhibited. Cervical dilatation

is one of the later signs of Stage 1 parturition and usually follows nesting, restlessness and hyperventilation. In this study, cervical dilatation and or exhibiting of active contractions of parturition will be used to indicate readiness for CS. In eutocia it will be assumed that the cervix opens within a few hours from Stage 1 of parturition. This assumption must be made because many of the bitches in this study will undergo a CS based on showing dilatation of the cervix. If we cannot make this assumption it might be argued that the gestation length in CS bitches was indeed shortened by more than a few hours because a CS was performed within 6 h after the onset of cervical dilatation.

2.15.13. Parturition date relative to behavioural signs of impending parturition (Stage 1 of parturition)

The signs of impending parturition were first described by Bleicher (1962). With regards to the current study, the establishment of a standardised modus operandi for all bitches in the trial is important and that normal progress of parturition is defined.

The three stages of normal parturition or eutocia are described as reported by Linde-Forsberg and Eneroth (2000). In Stage 1, the bitches appear restless, may nest and pant but differs from Stage 2 in that no uterine contractions are detected but uterine tone increases and progressive cervical dilatation occurs. This stage may last 6–12 h or possibly 24 h. Primiparous bitches may have a Stage 1 parturition in the longer part of the normal range. Stage 2 is characterised by strong abdominal contractions, a vaginal discharge and later expulsion of puppies. The first foetus is normally delivered within 30 min of the onset of abdominal contractions but this may vary. The progress of Stage 2 parturition is very important for puppy survival. The true inter-puppy interval is of importance but even short intervals in some breeds do not guarantee favourable outcome in survival of each individual in the litter. Stage 2 should be completed in 12–24 h, with a foetus delivered every 0.5–4 h. Excessively long inter-puppy intervals of 34 h (Romagnoli et al., 2004) and longer (Smith, 2007) with favourable outcome have been reported but these should be seen as exceptions. Most extended Stage 2 of parturitions will end in increased puppy mortality and possible complications for dams as well. Stage 3 is the phase where the placentas are expelled (Linde-Forsberg, 2005; Wykes and Olson, 2003). Multiple placentas may be passed after several puppies are delivered close together. Bitches commonly bite the amniotic and allantoic membranes, sever the umbilical cord, and ingest the placenta after parturition (Wykes and

Olson, 2003; Schweizer and Meyers-Wallen, 2000).

It is speculated that a bitch may exhibit voluntary inhibition of parturition because of excessive stress and excitement (Wykes and Olson, 2003). This is important in cases where bitches are hospitalised for management of parturition. It has been suggested that opioids and benzodiazepines may be used as anxiolytics in these cases (Gendler et al., 2007), but this practice should be questioned as it is not known if these drugs interfere with normal parturition or affect the survival of the litter particularly in bitches prone to uterine inertia. It has been suggested that intuition (pattern recognition) may play a part in knowing when to wait and when to intervene (Von Heimendahl and Cariou, 2009), but this practice is neither scientific nor helpful for the inexperienced veterinary obstetrician.

The main problem with predicting parturition from behavioural signs is that it requires near constant observation and failing to do so may prove disastrous. Behavioural signs of impending parturition are inconsistently displayed in bitches and parturition supervisors may fail to observe them. The time interval between first displays of behavioural signs of parturition during Stage 1 of parturition to Stage 2 of parturition is also very unreliable. Nesting behaviour has been observed up to 7 d prior to P0 (Johnston et al., 2001b). Even with constant observation it will become clear (from section on dystocia), that waiting until intervention is required based on behavioural signs of parturition, is also not without risk (section 2.21).

2.15.14. Parturition date relative to measurements obtained by commercial external monitoring devices to record uterine activity

Continuous electronic foetal monitoring was developed to assist in the diagnosis of foetal hypoxia during parturition and attempt to reduce mortality in humans (Bailey, 2009). In humans, foetal electrocardiography serves as a reliable method for electronic foetal monitoring during high-risk labours (Rzepka et al., 2010). In a recent literature review it was concluded that widespread use of cardiotocography with CS for foetal distress has led to significant declines in stillbirth rates in humans (Haws et al., 2009). Foetal monitoring has also become commonplace at home for human pregnancies and consideration should be given to uterine and foetal monitoring at home for bitches (Davidson, 2008) with high risk pregnancies and those in which Stage 1 of parturition goes unrecognized (Johnson, 2008a). Intrapartum monitoring of the foetus and uterine activity allows the veterinary obstetrician

to better medically manage parturition and identify the need for early surgical intervention. Current monitoring tools available to the veterinary obstetrician include B-mode ultrasonography, Doppler (foetal heart rate) ultrasonography (Blanco et al., 2008), and tocodynamometry (Groppetti et al., 2010), which can detect changes in intrauterine pressure (Davidson, 2001). Tocodynamometry can be used as a non-invasive method for monitoring parturition patterns, delivery and dystocia in the bitch to reduce neonatal mortality (Schroeder et al., 2006). During late pregnancy (greater than 50 d following estimated day of ovulation) the pattern of uterine electrical activity is characterized by episodes of activity lasting 3–10 min and recurring at a low frequency (maximum 2.5/h). During the last 7 d before delivery there is a progressive qualitative change in activity which is correlated with the decrease in PC. Concomitant with the sharp fall in PC there are significant quantitative changes in uterine activity which occur during the last 24 h before parturition (van der Weyden et al., 1989). Although several commercial external monitoring devices claim to record uterine activity and foetal heart rates during whelping (Johnston et al., 2001b), these devices have not been evaluated with physiological studies.

Uterine and foetal monitoring has the advantage of detecting foetal distress that may otherwise have been missed by veterinary obstetrician in bitches that show no signs of parturition. Despite this proven and potential value in detecting foetal distress it has no predictive value and does not negate the overnight monitoring to which this study is aimed at avoiding.

2.15.15. Parturition date relative to onset of lactation and progestogen and ionic concentrations of mammary secretions prior to parturition

Onset of secretion of milk by bitches varies from 2 weeks before until several days after parturition (Roberts S.J., 1986) and is not an accurate predictor of parturition. Not all bitches have milk prior to parturition and this limits the usefulness of using milk as predictor of impending parturition. The initial mean progestogen concentrations in the mammary secretions were high and started to decline from five days before parturition, in a similar way to changes in plasma progesterone concentration (England and Verstegen, 1997). However, there were marked individual and day variations which prevented this method from reliably indicating the time of impending parturition (England and Verstegen, 1997). Calcium, Magnesium and Sodium concentrations in the milk were found not to be useful for predicting impending parturition (England and Verstegen, 1997).

2.16. Determining number of foetuses by ultrasound

It is unlikely that ultrasonography will allow one to measure any parameter in all the foetuses in all litters. This assumption is made because the limitations of ultrasonography were born out in a study attempting to accurately determine litter size (Lenard et al., 2007; England et al., 1990). If it is impossible to accurately count the number of foetuses during mid-pregnancy, it may be more difficult to make foetal biometric measurements in late pregnancy where other foetuses and the gastro-intestinal tract of the bitch may obscure foetuses in the ultrasonographic field of interest. Therefore it has to be questioned whether obtainment of BPD in all the individual puppies in all litters (Batista et al., 2014), is truly achievable.

2.17. Determining number of foetuses that are dead or alive by ultrasound

It is of importance to establish prior to a treatment or procedure whether all the foetuses are alive and well prior to intervention. This is so because otherwise an erroneous deduction (false overestimation) of dead foetuses may be made as having been caused by the intervention. It is likely that error will be reduced but not eliminated because again not all the foetuses (dead or alive) would be counted correctetly in all bitches.

2.18. Foetal biometric measurements as means of estimation of gestational age and establishing readiness for caesarean section

2.18.1. Foetal biometric measurements in humans

Before the advent of ultrasonography, pregnancy length was calculated from the last menstrual period (Kramer et al., 1988). Since then pregnancy dating curves and reference curves for foetal growth were derived based on foetal biometric measurements taken at various stages of pregnancy. Biparietal diameter, head circumference, abdominal circumference and femur length have all been detected by the ultrasound estimation of gestational age (Davis et al., 1993) demonstrate a similar pattern of increase with gestation and no large inconsistencies with other frequently used curves (Verburg et al., 2008). The crown-rump length is considered by some as the highly reliable gold standard for gestational age estimation (Benson and Doubilet, 1991) but crown rump length and BPD had similar predictive value in estimating gestational age (Bovicelli et al., 1981).

Generally, irrespective of parameter measured, the earlier in pregnancy ultrasound

measurements were performed to establish gestational age, the more accurate the prediction of the date of delivery (Campbell and Newman, 1971; Bovicelli et al., 1981; Benson and Doubilet, 1991) with an optimum at 10–12 weeks (Campbell and Newman, 1971).

In conclusion, ultrasound measurement of foetal dimensions is routinely used for estimating gestational age and planning CSs in man (Henrihsen et al., 1995).

2.18.2. Using foetal biometric measurements in dogs to establish readiness for caesarean section

A study involving English Bulldogs concluded that CSs can be scheduled safely when the foetal BPD shows a minimum value of 29.5 mm (Batista et al., 2014). Although several studies involve gestational age estimation in dogs by ultrasonographic assessment of foetal biometric measurements, none explores the safety of using such estimated dates to time CS.

In woman with normal twin pregnancies, a separate twin BPD prediction table is required to permit a more accurate assessment of twin gestational age and foetal growth (Leveno et al., 1979).Given this it seems likely that the large variations generally encountered in bitches regarding litter size, body mass of dams, birth mass of puppies and breed may even further complicate establishment of accurate prediction tables in the dog. Foetal biometric measurements require further investigation to critically evaluate usefulness in predicting readiness for CS.

2.18.3. Accuracy of biparietal diameter measurements in dogs

To draw reliable conclusions on foetal biometric measurements in a polytocous species one must ensure that the measurements obtained are representative of those of all the foetuses in the litter. The accuracy of ultrasonographically obtained foetal biometric measurements depends on the spatial arrangements of the foetuses, ultrasonographic planes and may further be influenced by intra- and inter-observer variability (Dudley, 2005). Variability may be reduced because of erroneous re-assessment of the same foetuses and result in artificial homogenization of the results. It is of interest to establish whether the variability in BPD and bodyweight in a large number of puppies of two breeds would allow the veterinary obstetrician to potentially plan CSs based on these measurements having reached a minimum value as suggested by Batista et al. (2014).

The current study aims at reaching a high level of accuracy in procuring individual BPD by

making direct measurements at birth using digital calipers and critically evaluating BPD as predictor of readiness for CS.

2.19. Foetal growth curve

In the current study, it is critical that foetuses are sufficiently mature at the time of delivery to survive. In the dog the duration of pregnancy is relatively short (Concannon et al., 1983; Johnson, 2008b) with consensus that the major part of foetal growth in the dog occurs in the last third of gestation (Evans and Sack, 1973; Salazar and Yllera, 1991; Moriyoshi et al., 1996; Miglino et al., 2006) but exact foetal growth curves in the last third of gestation are lacking. One study suggested that the growth curve of canine foetus is logarithmic with a steady plateau towards the end (Evans and Sack, 1973) whereas another suggested a linear growth curve throughout second half of pregnancy (Moriyoshi et al., 1996). Knowledge of foetal growth curve in the puppy is important because it may reveal whether foetal size or estimated body mass could potentially be of value in determining gestational age.

2.20. Factors that may influence gestational length

Numerous studies used data collected retrospectively to test the hypothesis that age, breed, size or body mass, parity and litter size affect duration of gestation in the bitch.

Various studies showed that parity did not influence gestation length in the bitch (Okkens et al., 1993; Eilts et al., 2005; Seki et al., 2010).

Age had no effect on gestation duration (Eilts et al., 2005). This is in agreement with studies that found no effect of age on duration of gestation within any single breed (Okkens et al., 2001) or between primiparous bitches and multiparous bitches (Okkens et al., 1993).

Body mass or size did not affect duration of gestation in the bitch (Kutzler et al., 2003a).

Whilst other workers (Linde-Forsberg et al., 1999; Tsutsui et al., 2006; Seki et al., 2010) concluded that litter size and body mass did not affect duration of gestation in their studies, other workers found that bitches whelping small litters were significantly more likely to have a longer gestation duration than those whelping large litters (Okkens et al., 1993; Eilts et al., 2005; Gavrilovic et al., 2008). Two studies quantified the effect of litter size on duration of gestation. The one study reported that in bitches whelping four or fewer puppies (in bitches that usually have large litters) prolongation of the duration of gestation averaged

1 d (Eilts et al., 2005) whilst the other study in the Drever breed, for each foetus above or below the breed average of 6.8 puppies/litter (n = 224 whelpings), gestation length was shortened or lengthened by 0.25 d, respectively (Gavrilovic et al., 2008).

Consensus was not reached whether breed affects duration of gestation as some workers showed that breed did not affect duration of gestation (Linde-Forsberg et al., 1999; Kutzler et al., 2003a) whereas others found that it did (Okkens et al., 1993; Eilts et al., 2005). The intra-breed variation in length of gestation in the five breeds represented by five or more bitches each was 3–6 d. The mean gestation of Alsatians (60.1 ± 0.5, n = 9) was shorter ($P < 0.005$) than that of the other breeds combined (62.3 ± 0.3, n = 68) (Okkens et al., 1993). Compared to Labrador Retrievers, the German Shepherd Dogs, Golden Retrievers and hounds were more likely to have a longer gestations, respectively three, four and nearly eight times as likely (Eilts et al., 2005).

From the above the findings regarding influence of litter size and breed vary between studies. These differences might reflect true effects of litter size or breed or it is also likely that the differences in methods of timing gestation have led to different conclusions regarding variation in gestation length. The sample size in some studies may have also affected results. It seems likely the difference in gestation length between breeds is either non-existent or negligible and that litter size probably has a small influence with large litters having slightly shorter gestation. The singleton pregnancy is considered an exception and will be dealt with separately. This study will consider influence of litter size and breed with regards to duration of gestation.

2.21. Safety of drugs during pregnancy

Pregnant bitches may need to be medicated with a variety of drugs for various reasons. Only drugs that are known to be safe will be used in this study. Drug safety aspects in theriogenology of man and animal are a difficult topic to research or review in the literature. A substance or procedure may have “effects” on many different levels. The “effect” may be directly on the dam and its foetus, which is the simplest form of interference. In the reproductively active female, it may also effect:

- Endocrinological events and thus follicular development
- Oocyte maturation and survival

- Ovulation
- Development of corpora lutea and progesterone production
- Fertilization
- Ovum transport and survival
- Embryonal development
- Implantation
- Foetal development, growth and survival
- Maintenance of pregnancy
- Parturition
- Uterine involution
- Effect on lactation
- Infant survival
- Effect on further development, health and longevity of offspring

The effect of a drug during pregnancy in one species is not a reliable indicator of what the effect may be in another species. Antivivisection regulations are getting stricter by the day. Liability is also a growing concern for researchers and pharmaceutical companies alike. Given all these difficulties, pharmaceutical companies will continue to report "Safety during pregnancy has not been established". The net result is that our knowledge on the safety of drugs and procedures on reproductively active animals will remain scant. It is recommended to avoid any drug administration to pregnant pets unless absolutely necessary (Pichler, 2007), especially during days 13–30 of gestation. In those cases, the first choice should be of drugs of which the safety during pregnancy is known and that are registered for use in pregnancy. Much of our knowledge originates from in vitro or in vivo mouse or rat studies as well as extrapolation of data from human medicine. The most common drugs prescribed in pregnant and lactating animals include antibiotics, antivirals, anthelminthics, antifungals, anaesthetics and vaccinations. Most antibiotics are in Category B of the food and drug administration, meaning, that they are probably safe. Amoxicillin, ampicillin, amoxicillin with clavulanic acid, cephalexin, cefadroxil, clindamycin, erythromycin, and azithromycin are considered safe for the foetus. Metronidazole should not be used in early pregnancy, but can be used near term. Antibiotics in Category C (unknown or potential concern) include chloramphenicol, clarithromycin, enrofloxacin, gentamycin, rifampin, and trimethoprim/sulfamethoxazole. Category D medications (contraindicated unless benefits

outweigh risks) include amikacin, tetracycline, doxycycline, and trimethoprim. Permanent tooth discoloration and skeletal defects (tetracyclines), impaired cartilage development (fluoroquinolones) and bone marrow suppression or autoimmune disorders (sulphonamides) are possible adverse effects in medicating pregnant bitches. Bitches may require treatment during their clinical management from conception until CS and thereafter until weaning of the puppies. Knowledge about the safety of drugs is therefore important.

2.22. Prudent use of antibiotics in elective surgery

The use of pre-, intra- and post-operative antibiotics in routine surgery is a controversial topic which requires discussion. This primary investigator routinely uses cefazolin 500 mg to 1000 mg total dose once only and enrofloxacin at 5 mg/kg once only, preoperatively. Post operatively, amoxicillin at 20 mg/kg b.i.d will be used for 5 d. It is conceded that the antibiotics used are preventative in nature and will be administered in the absence of an existing infection. The discussion that follows motivates its routine use.

There are two interest groups lobbying in the argument whether to use antibiotics routinely in surgery such as CS. The one group (epidemiologists and others) are those who attempt to protect the interests of human and animal populations and are strongly opposed to routine use of antimicrobials for routine surgery. The second group (veterinary obstetricians) consist of those whose prime aim is to protect their patients. The former want to minimize potential for antibiotic resistance formation caused by random use of antibiotics whilst the latter wish to minimize risk of bacterial infection by routine antibiotic use in patients who do not have an existing bacterial infection. Veterinary surgeons fear consequences if infection resulting from routine procedure occurs. Both groups make compelling arguments that are briefly discussed. Many regulatory bodies agree with the general description of the antibacterial resistance problem and want to participate in measures to counteract antibacterial resistance. This is because bacteria are sure to develop resistance against new and existing products (Bergström, 2011; Cisneros et al., 2010) and that resistance has already occurred against antimicrobials in species where the antimicrobial in question has been extensively used (Lei et al., 2010). This has prompted some bodies to lobby for recommendation of prudent use and by supporting the guidance for antimicrobial use which mainly includes culture and sensitivity guided antibacterial therapy (Llor, 2010; Escher et al., 2011). Total prevention of resistance formation is unlikely but prudent use of antibiotics may delay its development (Cisneros et al., 2010). In short the "Responsible use of antibiotics today will protect us

tomorrow" (Campos et al., 2010). The argument for prudent use of antibiotics is complicated by accusations of perverse financial intent in prescribing unnecessary antibiotics and quest for steps to control this (Bergström, 2011) and legal action against veterinary obstetricians when infections do occur (Pallasch, 2003). This is certain to leave many veterinary obstetricians nervous and will influence their risk benefit assessment of individual cases.

There is no doubt that antibiotics are indispensable in surgeries where there is high risk or pre-existing infections as is the case in open bone fractures (Neubauer et al., 2006) and surgeries that involve implants (Trampuz and Widmer, 2006). In closed elective surgery the incidence of infection is around 2% and may exceed 10% performing surgery of traumatised tissues (Miclau et al., 2010). The fact that surgical site infections occur (Anderson, 2011) and that infection is the most common complication after surgery (Weiss and Lauf, 2004), raised the question whether use of antibiotic prophylaxis is effective in reducing postoperative wound infection rates in elective surgeries with no evidence of pre-existing infection. Prophylactic antibiotic treatment at the time of surgery should never replace good antiseptic technique in patient preparation and during surgery but has been demonstrated to be of greater benefit than risk in some procedures with higher infection rates (Weed, 2003). Some view an uncomplicated CS as a clean-contaminated procedure and therefore routine perioperative prophylaxis is not indicated. Perioperative intravenous antibiotic therapy is indicated if foetal death has occurred, uterine infection is suspected, there has been a break in asepsis, or gross evidence of infection is present but otherwise the routine use of postoperative antibiotics after uncomplicated CS is not justified (Ryan and Wagner, 2006b; Olson and Mather, 1978). However, in contrast, in a very large study, it was concluded that the reduction of endometritis by two thirds to three quarters and a decrease in wound infections justifies a policy of recommending routine prophylactic antibiotics to women undergoing elective or non-elective CS (Hofmeyr and Smaill, 2002), hence it is generally accepted to advise prophylactic antibiotics in CS (Clifford and Daley, 2012). It is unknown whether the same is true in bitches but the possibility that it is, cannot be discounted. The use of penicillins, cephalosporins, ß-lactamase inhibitors (Landsbergen et al., 2001) and enrofloxacin at 5 mg/kg (Wanke et al., 2006) has been reviewed and are considered safe for use at the time of CS in the bitch. For the purposes of this study only these will be used preoperatively, intra-operatively and post-operatively.

2.23. Physiologic changes during pregnancy in humans and dogs

Most of the added volume of blood in pregnant women is accounted for by an increased capacity of the uterine, breast, renal, striated muscle and cutaneous vascular systems, with no evidence of circulatory overload in the healthy pregnant woman (Ciliberto and Marx, 1998). The increase in plasma volume (40%–50%) in pregnant women is relatively greater than that of red cell mass (20%–30%) resulting in haemodilution and a decrease in haemoglobin concentration (Ciliberto and Marx, 1998). The increased blood volume serves two purposes. First, it facilitates maternal and foetal exchanges of respiratory gases, nutrients and metabolites. Second, it reduces the impact of maternal blood loss at delivery. The blood loss associated with vaginal births and CS in humans are compensated for by the so-called 'autotransfusion' of blood from the contracting uterus (Ciliberto and Marx, 1998). It is not known whether these changes are present in the bitch as well. Increased cardiac output proportional to increased blood volume occurs during pregnancy in the bitch as a result of increased heart rate and stroke volume (Seymour, 1999). Peripheral vascular resistance decreases during pregnancy in the bitch, resulting from the increased capacity of blood vessels in the uterus, mammary glands, kidneys, striated muscle and cutaneous tissue, so that the mean arterial blood pressure is maintained and circulatory overload does not accompany the increased cardiac output (Hall et al., 2001). Compensatory cardiovascular baroreceptor mechanisms in response to haemorrhage or hypotension may be attenuated during pregnancy (Brooks and Keil, 1994). Cardiac work is increased and cardiac reserve is decreased during pregnancy (Seymour, 1999). Animals with cardiac disease that were previously stable or well controlled on medications can become decompensated and develop heart failure during pregnancy and parturition (Thurmon et al., 1996). During pregnancy, there is decreased functional residual capacity (the remaining lung volume measured at the end of a normal expiration), decreased total lung volume, and increased minute ventilation and oxygen consumption (Pascoe and Moon, 2001). Decreased functional residual capacity and total lung volume are due to cranial displacement of the diaphragm by the expanding gravid uterus (Greene, 1995). Oxygen consumption increases because of the metabolic demands of the foetus, uterus and mammary glands (Hall et al., 2001). The combined effect of decreased functional residual capacity and increased oxygen consumption makes bitches in late gestation very susceptible to hypoxemia (Seymour, 1999). Any period of apnoea causes rapid maternal arterial haemoglobin desaturation, decreased oxygen delivery to the foetus and foetal hypoxia. The most critical time for apnoea is at anaesthetic induction so

preoxygenation with 3–5 L/min of 100% oxygen by face mask before and during induction of anaesthesia is highly advisable to decrease the risk of hypoxemia (Seymour, 1999).

2.23.1. Anaemia during periparturient period in humans

Anaemia may be expressed as a haemoglobin concentration or haematocrit. Pregnant women are considered anaemic when their blood haemoglobin concentration is below 110 g/L (Goonewardene et al., 2012) or if they have a haematocrit below 33% (World Health Organization, 2001). The threshold blood haemoglobin content for a blood transfusion (transfusion trigger) for humans is often considered to be < 60 g/L in general clinical practice for non-pregnant patients and < 70 g/L in pregnancy (Rohilla et al., 2011). In human intensive care unit patients a transfusion trigger of < 70 g/L is used (Wilson, 2004).

Postparturient haemorrhage can be due to intraoperative- or postoperative blood lost through the vagina or concealed intraperitoneal haemorrhage. Excessive haemorrhage associated with CS in humans is commonly defined as blood loss more than 1000 ml and is documented as occurring in more than 5%–10% of CSs in humans (Fawcus and Moodley, 2013). Another study suggested that a change in haematocrit of more than 10% may indicate a need for blood transfusion in humans (Stafford et al., 2010).

Estimation of total blood loss during and after CS is problematic owing to difficulties in accurate collection of lost blood. This blood may be collected in suction bottles or estimated from soaked swabs and theatre linen. Visual estimation of blood loss during and after CS or vaginal delivery is inaccurate, tending to overestimate at lower blood loss and underestimate at higher blood loss (Knight et al., 2009).

Lack of surgical skills may mean that less experienced personnel carry out CSs, with minimal support and this factor is associated with higher incidence of post-parturient haemorrhage in humans (Fawcus and Moodley, 2013). The same may occur in veterinary practice. Other than establishing normal values of haematocrit for late pregnant bitches, no other work for anaemia in the periparturient bitch has been reported. For this reason, in the design of this study, knowledge on human periparturient blood loss was extrapolated to the bitch.

2.23.2. Physiologic changes during pregnancy that affect the haematocrit of bitches

There are several physiological changes that take place during pregnancy that affects oxygen

delivery. Oxygen delivery is dependent on oxygen carrying capacity (haemoglobin), cardiovascular function and respiratory function. Poor oxygen delivery can have a significant negative effect on bitches and the puppies following CS (Ryan and Wagner, 2006b).

In dogs, pregnancy is associated with an increase in body mass which is followed by an increase in plasma volume (+40%), relative anaemia or reduction in haematocrit of pregnancy (Concannon et al., 1977b; Ryan and Wagner, 2006b) and relative hypoproteinaemia (Kimberely et al., 2006). The normocytic, normochromic anaemia develops between days 25 and 30 of pregnancy in bitches and is most severe at term (Concannon et al., 1977b). The erythrocyte mass and circulating volume return to normal 8–12 weeks after parturition (Ryan and Wagner, 2006b).

Reference intervals for normal healthy non pregnant dogs' haematocrits are reported as average 52%, range (42%–62%) (Moritz et al., 2004) and by another worker, average 50%, range (37%–55%) (Latimer, 2012) and reference intervals for normal healthy non pregnant dogs' haematocrits at a laboratory at similar altitude (1500 m above sea-level) than the experimental animals were 37%–55% (personal communication, Scheepers 2014). An earlier study reported haematocrits in pregnant bitches around 40% in the last third of gestation, around 35% to a nadir of 30.6% ± 0.8 (SD n = 24), 60–62 d following estimated LH peak (Concannon et al., 1977b). This study has been a keystone study that has been widely cited in the literature (cited by 152, google scholar November 2016).

Non pregnant bitches' haematocrits were reported as 45.4 ± 3.6 (SD, n = 16) and haematocrits of pregnant dogs in the last third of gestation 41± 4.9 (SD, n = 16) (Dimço et al., 2013). A Japanese study of 15 beagles reported a steady decrease in haematocrit which became statistically significant on day 50 of pregnancy (Hayashi, 1974). The nadir haematocrit of 33.7 ± 1.8% was reached on day 50 and the haematocrit then increased to 38.7 ± 1.8% on day 60 of pregnancy (Hayashi, 1974). In the study by Hayashi, (1974), day 0 of pregnancy was not defined. The postpartum haematocrit was 37% (Hayashi, 1974). A study in 23 beagle dogs, showed that the haematocrit decreased from before pregnancy 52%–40% to 42%–28% at term and that the litter size had an influence on haematocrit (Hayashi, 1974; Kaneko et al., 1993). Due to varying results, litter sizes and the small sample sizes of these studies no definitive conclusions about haematocrits during pregnancy can be made.

The effects of litter size on the extent of anaemia of pregnancy are unknown (Verstegen-Onclin and Verstegen, 2008). Pregnancy associated increased body mass is followed by an increase in plasma volume, resulting in a relative anaemia of pregnancy (Concannon et al., 1977b) and a relative hypoproteinemia (Kimberely et al., 2006). This decrease in haematocrit is speculated not only to be due to haemodilution, but also due to shortening of erythrocyte lifespan (Chaudhan and Mshelia, 2006). By evaluating the results of Kimberely et al. (2006) and Dimço et al. (2013), it may be concluded that the anaemia of pregnancy may be a lot less severe as initially reported by Concannon et al. (1977). In his initial study, Concannon et al. (1977b) reported mean haematocrits in pregnant bitches 60–62 d following estimated LH peak, as 30.6% ± 0.8 (SEM, n = 12). A low haematocrit before CS has serious clinical implications. It is unknown whether haemodilution and the concept of "autotransfusion" also act as compensatory mechanism to protect against effects of maternal blood loss during natural whelp and CS in dogs as has been reported in woman (Ciliberto and Marx, 1998). There is no absolute decrease in erythrocyte mass, and the haematocrit returns to normal within 8–12 weeks after parturition as the plasma volume returns to normal (Concannon, 2002).

2.23.3. Suitability of haematocrit as an indicator of blood loss

Caesarean sections are associated with additional blood loss from surgery. There is limited knowledge about haematocrits of bitches in late pregnancy before and after CSs. This is a stumbling block in the periparturient risk assessment of bitches that delivered by CS. Assessing risk prior to discharge following CS in bitches is particularly important because this may take place as soon as two to three hours after surgery. Puppies are generally not kept in veterinary hospitals for long due to the risk of disease exposure and better nursing environment at home. Veterinary obstetricians have no clear guidelines of what the normal expected haematocrit ranges are during pregnancy and therefore what haematocrit may be problematic with the blood loss following CS. For these reasons this study examined haematocrits before and after CS.

The majority of the haematocrit tubes when used with normal blood samples, produced packed cell volume results with biases of less than half a packed cell volume unit and are deemed acceptable by comparison to reference methods (Bull and Hay, 2001). Haematocrit and haemoglobin content of blood may both be used as an indicator of oxygen carrying capacity and of blood lost. Because the mean cell haemoglobin content may differ due to

various conditions, the haemoglobin content in blood is theoretically a more accurate measurement of oxygen carrying capacity of the blood than haematocrit, which depends on mean cell haemoglobin content. Haematocrit is, however, a more practical method in clinical practice and is well correlated to haemoglobin content provided there is no other concurrent disease process influencing the mean cell haemoglobin content (World Health Organization, 2001). A haematocrit of 30% corresponds to a haemoglobin concentration of about 100 g/L (Zander, 1999). Haematocrit is an easy, quick and cheap parameter to evaluate in a clinical setting and therefore some workers focussed on using haematocrit as an objective indicator of blood loss in woman undergoing CS (Hidar et al., 2004; Stafford et al., 2010; Anorlu et al., 2008). One such study used a formula that calculates blood loss based on haematocrit changes before and after CS and concluded it to be a reliable indicator of excess bleeding (Stafford et al., 2010). However, the limitations of haematocrit in blood loss estimation have to be acknowledged (Nicol et al., 1997). If there was not enough time allowed for fluid redistribution (plasma volume equilibration) following blood loss, haematocrit is likely to be inaccurate and give false low estimates of blood lost (Ebert et al., 1941; Faxelius et al., 1977). This effect was mitigated in the current study by intravenous fluid therapy. Despite the shortcomings of haematocrit, the American College of Obstetricians and Gynaecologists has used haematocrit in their official definition of postpartum haemorrhage as a decline of > 10% in haematocrit during delivery ([ACOG] American College of Obstetricians and Gynaecologists, 1998).

The effects of altitude on haematocrit appears minimal. This assumption is based on the finding that although exposure to hypoxia in dogs occurring at moderate to marked high altitude results in measurable cardiovascular changes, including increased heart rate, increased systemic and pulmonary artery pressure and changes in systolic and diastolic cardiac function, it does not results in increase in haematocrit (Glaus et al., 2003).

2.24. Dystocia and its management

Besides a general overview of dystocia and its management, this literature review will concern itself with mainly the decision making between surgical interventions versus medical interventions and the outcomes thereof.

Dystocia, from the Greek ‘‘dys’’ meaning ‘‘difficult, painful, disordered, or abnormal’’ and ‘‘tokos’’ meaning birth, occurs when the parturition process ceases to progress normally

(Pretzer, 2008). In many of the domestic species the perinatal losses are rather high because of the direct or indirect consequences of asphyxiation during birth (Ólafsson T.H., 2007). Dystocia occurs in approximately 5% of all parturitions in dogs (Linde-Forsberg and Eneroth, 2000).

2.24.1. Causes of dystocia

Dystocia is the inability of the dam to deliver all or some of the foetuses in a litter via the birth canal without the need for assistance and is a common emergency in bitches (Gendler et al., 2007). Maternal dystocia is encountered more frequently 60% (Gaudet, 1985) to 75.3% (Darvelid and Linde-Forsberg, 1994) and 86% (Stolla et al., 1999), than foetal dystocia (Bennett, 1974; Bennett, 1980). Uterine inertia represents 40% (Gaudet and Kitchell, 1985) to 72% (Darvelid and Linde-Forsberg, 1994) to 99% (Stolla et al., 1999) of all maternal dystocias. Uterine inertia (uterine fatigue) is the failure to expel a foetus from the uterus when no obstruction exists and has been reported as accounting for 3.2% to 12.6% (Gaudet and Kitchell, 1985) of dystocias. Uterine inertia can be classified as primary or secondary and complete or incomplete. Complete primary uterine inertia occurs when parturition fails to start and no puppies are delivered. Partial primary uterine inertia is defined as initiation of normal parturition but failure to deliver all puppies. Primary uterine inertia can result because of lack of initiation of parturition as is suspected to be the case in singletons (Johnson, 2008a; Munnich and Kuchenmeister, 2009), or because of overstretching of uterine wall in very large litters (Bennett, 1980) as is speculated to be the case with the Boerboel breed (personal observation). More than 50% of studied bitches with complete primary inertia had three or fewer puppies in their litter (Darvelid and Linde-Forsberg, 1994). Obstructions can include maternal changes to pelvic or genital tract. Foetal anatomic and orientation changes accounted for most reviewed cases of foetal dystocia 24.7% (Gaudet, 1985) to 40% (Darvelid and Linde-Forsberg, 1994) of dystocias. Oversized foetuses cause 6.6% to 13.7% of foetal dystocias (Darvelid and Linde-Forsberg, 1994; Stolla et al., 1999). Primiparous bitches older than six years have a significantly higher risk to have special obstetric conditions and stillbirths compared with young primiparous bitches (Munnich and Kuchenmeister, 2009; Johnson, 2008a) whereas dystocia was not related to the age of the bitch or the number of previous litters in another study (Stolla et al., 1999).

Several dog breeds are associated with an increased risk for dystocia. Scottish terriers and Boston terriers have inherited characteristics that predispose them to obstructive dystocia

(Eneroth et al., 1999). The Chihuahua, Dachshund, Pekingese, Yorkshire Terrier, Pomeranian, and miniature poodle were the breeds most commonly represented in a retrospective study on dystocia (Gaudet, 1985). Two other studies also reported high incidences of dystocia in miniature and small breeds (Munnich and Kuchenmeister, 2009; Stolla et al., 1999). The incidence reported by Stolla et al. (1999) was as high as 59.4%. Brachycephaly was identified as a risk factor (Trautmann and Nolte, 2003; Johnson, 2008a) as well as cephalopelvic disproportion (huge head relative to maternal pelvic size) in the same breeds (Johnson, 2008b). In the Boston Terrier, English Bulldog, and French Bulldog, more than 80% of births are resolved by CS (Munnich and Kuchenmeister, 2009). Scottish terriers (Bergström et al., 2006b) and Boxers (Forsberg and Persson, 2007) have a high risk of dystocia.

2.24.2. Deciding when to institute medical management

The most challenging questions to answer during parturition observation is when to intervene and whether this intervention should be medical, surgical or both (Gendler et al., 2007).

Indications that intervention is required are:

- Stage 2 parturition lasts more than 12 h without progress
- The dam shows signs of illness or distress
- More than 30 min of tenesmus without delivery of a foetus
- Foetal heart rates indicating distress

Foetal heart rates of 160 bpm and above are considered normal, 140–160 bpm suggest poor viability of puppies not delivered within two to three hours, and foetal heart rates of less than 140 bpm indicate that immediate veterinary intervention may be required (Davidson, 2001). Medical management is contraindicated when obstructive dystocia is present or when there are still several foetuses in utero at the time of diagnosis and they are alive and important to the owner.

2.24.3. Medical management

Approaches to the medical management of canine dystocia have been elegantly described (Pretzer, 2008). Oxytocin and calcium gluconate, fluid therapy and glucose remain the

cornerstones of medical therapy in dystocia (Bergström et al., 2006a).

a) Oxytocin and its role in eutocia and dystocia

Oxytocin is a nonapeptide hormone produced by the neurohypophysis responsible for (Lee et al., 2009) stimulation of myometrium during delivery and mammary myoepithelial cells during lactation (Grigor'eva and Golubeva, 2010). Oxytocin-induced contraction of the myometrium in rats is largely dependent on the influx of extracellular calcium (Batra, 1986). Oxytocin is also produced by the large luteal cells of the corpus luteum and is involved in luteolysis (Senger P.L., 2003). Unlike some other species, oestrogen concentrations in the bitch seem to decrease instead of increase towards parturition (Edqvist et al., 1975; Hoffmann et al., 1994; Onclin et al., 2002). Apparently, preparation of the genital tract for parturition in the bitch is not oestrogen dependant and relaxin may act in a respective manner (Hoffmann et al., 1999) or unknown other mechanisms are involved in sensitising the myometrium to oxytocin. Determination of oxytocin in peripheral maternal plasma clearly indicates that, in the dog, as in other species, release occurs late in parturition and that there is an increased sensitivity of the myometrium to oxytocin in late pregnancy and parturition (Klarenbeek et al., 2007). It was established that oxytocin at a physiological concentration stimulated the immediate release of free arachidonic acid from dispersed human decidual cells in a perfusion system. This indicates that oxytocin activates phospholipase, thus enhancing prostaglandin synthesis. These new findings are consistent with a role for endogenous oxytocin in stimulating prostaglandin synthesis at the onset of parturition in humans (Wilson et al., 1988) and in cows (Fuchs et al., 1996). It is not known whether oxytocin would do the same in the bitch but it is reasonable to speculate that oxytocin could either augment parturition directly through its ecbolic effects or indirectly through enhancing prostaglandin synthesis. If the latter were to play a significant role it would likely be a minor one as parturition induction protocols using aglepristone with PGF2α to provide ecbolic support was a lot more effective than oxytocin.

Release of oxytocin is dependent on the Ferguson-reflex, which is initiated with transport of the foetus into the cervical canal (Hoffmann et al., 1999). Digital stimulation of the dorsal vaginal wall may also stimulate uterine contractions (Biddle and Macintire, 2000). Absence of oxytocin in parturition induction regimens may lead to failure to expel puppies and puppy deaths (Hoffmann et al., 1999).

Oxytocin is probably the most common ecbolic used in veterinary practice. It has been found to be the most common drug used (abused) by breeders to "aid" parturient bitches without veterinary supervision (Trautmann and Nolte, 2003). Oxytocin led to higher puppy losses compared with other medications used in dystocia therapies (Munnich and Kuchenmeister, 2009). Without veterinary supervision oxytocin has no role to play in dystocia and has no role to play in eutocia. Oxytocin may however be an effective drug to augment parturition. Initial doses of 0.1 iu/kg are recommended and the dose can be increased incrementally to a maximum of 2 iu/kg (never to exceed 20 iu/dog in any breed) administered im at 30–40 min intervals (Johnston et al., 2001b; Copley, 2002; Davidson, 2001) and between 2 and 4 iu in the cat (Jutkowitz, 2005). Excessive oxytocin may cause uteroplacental blood flow interruptions and ineffective, tetanic uterine contractions, placental separation or even uterine rupture (van der Weijden and Taverne, 1994). This adverse effect probably explains the observation that in many cases of failed medical therapy of uterine inertia, the puppy mortality increases.

Oxytocin may also be administered via a dilute iv solution at a slow drip rate in dogs (Feldman and Nelson, 1996) as is routine in humans (Seitchik et al., 1984). It was suggested that careful foetal monitoring using either Doppler ultrasonography and tocodynamometry would allow veterinary obstetricians to accurately identify the current stage of parturition, adjust oxytocin and calcium therapy with acumen, and avoid the adverse effects of excessive oxytocin (uterine tetany, foetal hypoxia, and uterine rupture) (Davidson, 2001). Intravenous administration of oxytocin is likely to increase in popularity as the equipment to perform constant rate infusion becomes more accessible in veterinary science. Since all the main objections against higher doses of oxytocin administration are related to either possible counter-productivity in progress of parturition or foetal perfusion and oxygenation (Devedeux et al., 1993; Copley, 2002; Bakker et al., 2007), there should be none in the postparturient bitch. It is custom for many veterinary obstetricians to administer higher range doses of oxytocin intra operatively during CS after the foetuses have been delivered and in the recovery phase mainly to control postsurgical haemorrhage as is the case in humans. As soon as the last puppy is delivered, there is no danger of foetal demise by tetanic contractions. In these cases, tetanic contractions associated with a high dose intravenous oxytocin bolus may indeed be beneficial. It has however been reported that the latter may lead to maternal vasodilation and hypotension (Johnston et al., 2001b) but the significance of this intra operatively is not known. Anecdotal evidence and personal observation suggests

that intraoperative oxytocin in bitches is safe and efficacious as it is in humans. One author administered oxytocin intramurally to promote uterine involution and reduce haemorrhage but it is not known whether this route of administration holds any advantages over other routes (Smith, 2007).

In this study a bolus of oxytocin (10–20 iu) was administered iv, intra-operatively immediately following delivery of the last puppy in bitches not sterilised.

b) Calcium gluconate

Contraction of all muscle cells requires adenosine triphosphate and the presence of calcium ions (Cunningham, 1992). It is therefore not surprising that in the medical management of dystocia it was found that far better results were achieved when calcium was administered together with oxytocin (Batra, 1986; Bergström et al., 2006b). This synergism was confirmed by Hollingsworth (1977) and Gaudet (1985). Since the diagnosis of hypocalcaemia may not always be practical in clinical practice (Kraus and Schwab, 1990), and since slow iv administration of therapeutic doses of calcium gluconate (0.2 ml/kg iv) or 1–5 ml per dog sc is safe (Johnston et al., 2001b), it may be given in conjunction with oxytocin in most if not all cases of uterine inertia provided it is done under veterinary supervision. Care should however be taken in intravenous administration of calcium since cardiac arrhythmias are a potential side effect. A bolus of aqueous calcium, magnesium and phosphorus (Calci 50 p.i., Virbac, SA. Halfway House) will be administered to all dogs via the sc route once only (1 ml/10 kg) at the time of CS in this study. Many dog breeders in South Africa believe that the administration of calcium makes them better mothers and settles them down and insisted on its use following CS. Although no evidence could be found in the literature to support such notion, evidence of harm could also not be found at the given dose and route, therefore we conceded to this owner request.

c) Glucose

Most bitches have normal blood glucose levels whilst some may indeed be hyperglycaemic (Bergström et al., 2006a); possibly in response to high cortisol concentrations, which have been measured during normal parturition in dogs (Olsson et al., 2003). Normally, during pregnancy, progesterone acts as a potent insulin antagonist and results in hyperglycaemia; therefore, it is very rare but not impossible for a bitch to become hypoglycaemic (Selk

Ghaffari and Najafiyan, 2009). Especially toy breeds may become hypoglycaemic (Linde-Forsberg and Eneroth, 2000). In one study, all bitches undergoing CS, irrespective of the reason therefore, had blood glucose concentrations within the normal range throughout the study, although pre-partum concentrations were statistically lower than intra-partum and post-partum concentrations (Lucio et al., 2009). Even in bitches with uterine inertia hypoglycaemia is rarely encountered (Bergström et al., 2006a). In contrast, some authors have proposed hypoglycaemia as a cause of primary inertia, especially in toy breeds of dogs (Linde-Forsberg and Eneroth, 2000).

Possibly, in neglected cases of dystocia in small breeds (mainly toy breeds) in poor condition (that have a very low fat percentage and muscle mass), the protracted dystocia bitch that may have depleted all reserves of glucose, results in hypoglycaemia.

This variability in blood glucose indicates the importance of routine blood glucose measurement in pregnant bitches presented for parturition management.

2.24.4. Arguments in favour of surgical resolution of dystocia

Since not all cases of dystocia can be managed medically, educated and careful decision making is required prior to instituting this approach because untimeous intervention can result in compromise and even death of the dam and foetuses (Pretzer, 2008). It has been suggested that many veterinary obstetricians might be inclined to opt too soon and easy for a CS. This is because they might feel more comfortable in performing a surgery to preclude puppy losses because of an expected dystocia (Taverne and Van Der Weijden, 2008). This is a valid observation and deserves in depth discussion as it has relevance to this study.

Uterine inertia that is unresponsive to medical management and foetal distress are considered the main causes for CS (Gendler et al., 2007; Traas, 2008b). Considering that more than 60% of dystocias end up in CS anyway (Bergström et al., 2006b; Polster et al., 2005) and that delays due to failed medical management may lead to increased foetal losses. A strong argument can be made for CS at first indication of dystocia (Moon et al., 2000) in cases where the survival of the foetuses is paramount. This viewpoint is supported by the finding that the duration of expulsion stage had the highest influence on puppy survival ($p < 0.001$, $n = 530$) in one study (Munnich and Kuchenmeister, 2009) and the finding that puppy mortality increases with time during second stage of parturition (Stolla et al., 1999). It was found that bitches in stage 2 parturition for longer than five to six hours, the puppy

mortality rates increased (Gaudet, 1985; Darvelid and Linde-Forsberg, 1994; Lennoz-Roland, 1998). Conservative obstetric treatment does not always succeed in delivering all the foetuses, causes delay and stillbirths and may still require CS (Michel and Reichler, 2008a).

It is the author's experience that some clients wish not to risk potential foetal demise following medical intervention for uterine inertia and insist on CS for their bitch. The influence of all therapeutic aspects on survival of the puppies is what counts most to the owner and veterinary obstetrician for o breeding bitches with potentially valuable offspring. It is the opinion of many breeders that they would rather pay for a CS with live puppies than two bills, one for medical management and another for a CS, with several dead puppies in tow. A distinct subset of bitches is presented to veterinary surgeons for parturition management. This subset includes bitches with high risk pregnancies and those whereof the owners demand a CS. In this specific subpopulation of bitches, a carefully planned elective CS may be considered as only choice of delivery.

In the case of obstructive dystocia, there is no consensus that a CS is always indicated and in the case of uterine inertia the decision is even more complex. The conservative approach favours minimalistic intervention and would dictate medical intervention first. The questions that remain unanswered regarding dystocia caused by uterine inertia are:

- Can medical intervention result in a more favourable outcome than CS and under what circumstances?
- Do we have accurate indicators as to when to resort to CS without attempting medical management first?
- What percentage of foetal losses is associated with medical management?
- Do these losses outweigh potential losses and risk associated with CS?

In one study the general condition of the dam, foetal number and vitality of the foetuses were considered the cornerstones of the decision as to whether medical management or surgical therapy is indicated (Michel and Reichler, 2008b). In retrospective studies, 75% (Stolla et al., 1999) and 62% (Darvelid and Linde-Forsberg, 1994) of the cases with dystocia, a CS was necessary. It is of special importance to note that only 19.9% (Stolla et al., 1999) and 30% (Gaudet, 1985; Darvelid and Linde-Forsberg, 1994) of the bitches suffering from uterine inertia that were treated with ecbolic drugs whelped without further therapy. The

proportion of foetuses born dead during dystocia was 31.9% (Stolla et al., 1999). If medical therapy fails, surgical intervention is mandatory (Michel and Reichler, 2008b). A prompt decision for CS will considerably improve the prognosis for survival of bitch and puppies (Stolla et al., 1999; Michel and Reichler, 2008a,b).

A special case in favour of CS can be made in high-risk pregnancies. These are pregnancies in which the prevalence of complications is likely to be higher than that of the general obstetrical population (Johnson, 2008a). Considering that it has been adequately cited that puppy mortalities are a frequent sequel to medical therapy and late intervention by CS, the anticipation of these sequels in high risk pregnancies may prompt the veterinary obstetrician to choose CS without even considering medical intervention. It may be argued that it is a questionable practice to proceed with CS only after medical therapy has been attempted and failed, as this may lead to further delays and puppy mortalities. The English Bulldog is such a breed in which the risk of dystocia that could be catastrophic to both maternal and perinatal health is extremely high, but properly planned CS is a safe and effective treatment (Linde-Forsberg and Eneroth, 2000; Davidson, 2008).

The author concludes that experience provides the strongest evidence of effects of an intervention or lack thereof in clinical practice, and suggests that with advances in the veterinary field it may no longer be acceptable to wait for potential foetal demise to occur following medical intervention for uterine inertia and only then proceed with CS. It is of utmost importance however to take note that this conclusion only relates high risk pregnancies where an elective CS considered the only safe way of delivery.

2.24.5. Value of selection against dystocia

Optimized timing methods for elective CS and preparturient CS protocols are not the long-term solutions to the problem of dystocia-prone breeds and high-risk pregnancies in other breeds. More effort should be made to persuade the custodians of affected breeds to ensure that responsible breed standards are set which promote selection for traits that have a lower correlation to occurrence of defects than is currently the case. With regards to dystocia in the bitch, veterinary obstetricians have a role to play. Research is required to ensure that selection criteria against dystocia do indeed achieve their intended goal. For instance, in the order to reduce the CS rates in many breeds, some of kennel clubs have imposed restrictions of registrations of puppies delivered after a second CS (The Kennel

Club UK, 2012). Some breeders have similar self-imposed selection protocols by not breeding a bitch again if she has required one prior CS. It is questionable whether such selection criteria would be effective. This is because selection against the occurrence of a CS may not be as effective as selection against the underlying anatomic or physiologic traits within the breed that underlies the true cause of the CS in the first place. It is suggested that such anatomic trait is the cephalic index of a breed (Johnson, 1986; Evans and Adams, 2010; Linde-Forsberg and Eneroth, 2000; Wydooghe et al., 2013; Farrell et al., 2015). Selecting against CS without selecting against brachycephaly and or other anatomical traits might be futile. The cephalic index of a vertebrate is the ratio between the transverse width and rostro-caudal length of its cranium (Stockard and James, 1941). This ratio does not concern the muzzle or face, and thus is distinct from the craniofacial ratio, which compares the size of the cranium to the length of the muzzle. The two measures are often confused in descriptions of dog breeds. Stockard and James (1941) used the cephalic index is used to classify animals into three groups. Brachycephalic (literally 'short-headed') occurs when the length of the cranium is shorter than the width, giving the top and sides of the cranium a round shape, often referred to as 'apple-head'. Mesocephalic or mesaticephalic (‘middle-headed’) occurs when the length and width are equal, giving a square shape. Dolichocephalic ('long-headed) occurs when the length is greater than the width (Stockard and James, 1941). The English Bulldog is often singled out as being the number one candidate for CS due to their large and flat faced head (“brachycephaly”) and broad chest (Linde-Forsberg and Eneroth, 2000; Wydooghe et al., 2013). Veterinary obstetricians are instrumental in the survival of many of the brachycephalic and toy breeds by performing routine artificial inseminations and CSs on these breeds. The current situation is that the incidence of elective CSs in some breeds is approaching 100%. By doing CSs they are propagating the very genes that we wish to select against. This status quo is likely to persist until the entire breeding fraternity is convinced to move away from brachycephaly and other anatomical features contributing to dystocia. This would drastically change many of these breeds or even causing them to become extinct in their current form. Voluntary change seems unlikely. Hence some are lobbying for legislation in this regard, as has happened in the UK (The Kennel Club UK, 2012).

In addition to anatomical features resulting in high incidence of CSs, owner request for CSs results in a high incidence. This demand owners base on fear of loss of puppies following potential dystocia in their breed (albeit sometimes low in their breed). Veterinary surgeons will often concede to this request in fear of consequences, should puppy losses occur

following a trial of labour suggested by the attending veterinary surgeon.

There is also the potential danger that preparturient CS may aid in propagating non-desired genes associated with incidence of CS. It is therefore strongly advised that selection against dystocia becomes a prioritised and that elective and preparturient CSs should only be planned in those high-risk breeds and those high-risk pregnancies of any breed where CS is considered the only safe way of delivery. The ultimate goal is that all breed standards promote selection for healthy traits including selection for eutocia thereby negating the necessity for CSs in our dog breeds.

Excessive fluid build-up in the amniotic and allantoic sacs is respectively referred to as hydramnios and hydrallantois. Hydrallantois is much more common than hydramnios, although the latter is frequently seen in association with specific foetal abnormalities in the cow (Pereira et al., 2011). Hydrallantois leads to severe respiratory distress in bitches, presumably due to acute dilatation of the abdomen and compression of the diaphragm and splenic vessels (Feliciano et al., 2013; Smith, 1972). The significance of these conditions is that it may place canine pregnancies at peril and necessitate preterm or preparturient CS.

2.25. Premedication

The prime objectives for using premedication for any patient prior to surgery is normally to reduce induction dose of anaesthesia (Pascoe and Moon, 2001), reduce the minimum alveolar concentration of inhalation anaesthetics (Ryan and Wagner, 2006a), counteract possible side effects of anaesthetic agents, achieve synergism, decrease maternal stress and anxiety (Ryan and Wagner, 2006a) and providing chemical restraint to allow preoperative preparation in cases where this is required.

2.25.1. Anti-emetics

Pregnant patients are more likely to vomit and aspirate because they have increased intra-abdominal pressure, decrease in lower oesophageal sphincter tone and decrease in gastric motility (Paddleford, 1992). Preoperative administration of opioids can also significantly slow gastric emptying. Metoclopramide (0.1–0.2 mg/kg) is indicated in most animals to increase gastric motility and for its anti-emetic effects (Paddleford, 1992). Placement of endotracheal tubes with cuffs of the appropriate size significantly reduce the risk of aspiration and negates the use of anti-emetics prior to CS in the dog. In this study, no

anti-emetics were used.

2.25.2. Anticholinergics

Premedication with anticholinergics is controversial because of their potential for tachyarrhythmia, production of gastric stasis and promoting reflux of gastric contents. Anticholinergics however have the advantage of reducing salivation, reducing respiratory tract secretions, causes dilatation of bronchioles, causes blockade of impulses in vagal nerve tracts and blockade of effects of drugs that stimulate the parasympathetic nervous system (Brock, 1996). The inhalation anaesthetics methoxyflurane, halothane, enflurane and isoflurane are associated with sinus bradycardia that is preventable or treatable with atropine. Brachycephalic dogs have strong vagal tone and therefore use of anticholinergic are considered essential by some (Greene, 1995). Concurrent administration of an anticholinergic is indicated to increase the heart rate if an opioid-induced bradycardia of 30% or more below the resting baseline occurs. In those cases, atropine (0.01–0.02 mg/kg iv or 0.02–0.04 mg/kg sc or im) is preferred over glycopyrrolate for reversing opioid-induced bradycardia in patients undergoing CS because it crosses the placental barrier, counteracts foetal bradycardia, and has a shorter onset and duration of action than does glycopyrrolate (Ryan and Wagner, 2006a). The physiological disadvantage to the patient with the use of atropine is that sustained rapid heart rate decreases diastolic filling time, coronary blood flow and stroke volume, increases myocardial oxygen consumption and decreases overall efficiency of heart muscle. Some anaesthetists however regard the masking of clinical indicators of anaesthetic depth (heart rate and arrhythmias) a major disadvantage of routine use of atropine. There are however few absolute contraindications for using atropine (Brock, 1996). Most small animal veterinary practices do not have dedicated anaesthetist available to monitor and manage each anaesthetised patient. Even if they do, they might not be available for after-hours procedures. The best form of cardiac monitor available in this setting may be an audible rate monitor (pulse oximeter) and the advantages associated with routine use of atropine probably outweigh its disadvantages (Ryan and Wagner, 2006a). No atropine will be used in the current study as premedication in any bitch in this study.

2.25.3. Benzodiazepines

It has been suggested that opioids and benzodiazepines may be used as anxiolytics in

nervous bitches during normal parturition (Gendler et al., 2007), but this practice should be questioned as it is not known if these drugs interfere with normal parturition or affect the survival of the litter particularly in bitches prone to uterine inertia. This concern is supported by the finding that diazepam significantly decreased uterine activity by its direct effect on contraction frequency and to its tranquilizing and muscle relaxant action (Toaff et al., 1977). Midazolam and diazepam may smooth induction and recovery from anaesthesia, but may also cause profound sedation in neonates (Luna et al., 2004). Benzodiazepines will not be used in this study.

2.25.4. Phenothiazines

Phenothiazines are contraindicated in pregnancy because of significant hypotension and reduced blood flow (Dodman, 1979). Severe foetal central nervous system depression is also seen after premedication with acepromazine and there is no specific antagonist for reversal (Ryan and Wagner, 2006a). If sedation is required for fractious bitches in need of CS, the use of a reversible narcotic is advised. During the times that barbiturates were the main induction agents, phenothiazines were commonly used as premedication. There is consensus that use of phenothiazine agents should be avoided in CS (Ryan and Wagner, 2006a) and will not be used in this study.

2.26. Analgesia and anaesthesia for caesarean sections in the bitch

Analgesia in pregnant animals for CS is problematic and some veterinary obstetricians have historically avoided them. This practice has become unacceptable to the greater part of the profession and its legislative bodies. Ethical committees regard analgesia as high priority when evaluating research protocols and papers. This trend is expected to continue with increasing accent being placed on ethical considerations in the approval of intended animal experiments. The advantages and disadvantages in choice of different painkillers in bitches before, during and following obstetrical intervention are discussed.

2.26.1. The Glasgow Composite Measure Pain Scale

The Glasgow Composite Measure Pain Scale was developed to measure acute pain in dogs in a hospital setting and for clinical trials. However, for routine clinical use, where the emphasis is on speed, ease of use, and guidance for analgesia provision, a short form of the Composite Measure Pain Scale was developed. The Composite Measure Pain Scale Short

Form comprises six behavioural categories with associated descriptive expressions (items): vocalisation (4), attention to wound (5), mobility (5), response to touch (6), demeanour (5) and posture/activity (5) (Reid et al., 2007). Items are placed in increasing order of pain intensity and numbered accordingly. The observer chooses that item within each category which best describes the dog's behaviour and ranked scores are summed; the maximum pain score is 24, or 20 if mobility is impossible to assess (Murrell et al., 2008). A copy of the Composite Measure Pain Scale Short Form can be viewed online (http://www.ingentaconnect.com/content/ufaw/aw/2007/00000016/A00102s1/art00014).

2.26.2. Non-steroidal anti-inflammatory drugs

Non-steroidal anti-inflammatory agents are increasingly used peri-operatively, alone or associated with opioids or local anaesthetics because of their analgesic and opioid sparing properties in all types of surgery. No NSAID has been approved for use in pregnant bitches. Non-steroidal anti-inflammatory agents have been reported to inhibit PGF2α (Williams et al., 1999) and preparturient luteolysis (Hoffmann et al., 1999) and delay whelping (Landsbergen et al., 2001).

Non-steroidal anti-inflammatory agents must be used with caution in patients with a pre-existing haemostatic defect or undergoing surgical procedures causing substantial blood loss (Derrier and Mercatello, 1997; Mullins et al., 2012). Non-selective NSAID such as aspirin have long been contributing risk factors for perioperative haemorrhage in both humans and dogs due to their effects on platelet function (Luna et al., 2007) and asprin is also teratogenic in the dog (Robertson et al., 1979). Preoperative use of some other NSAID may increase risk of haemorrhage by complications from antiplatelet activity (Poveda Roda et al., 2007; Risser et al., 2009). This may be a serious concern during CS. Ketoprofen (Gael et al., 2007) is one of those drugs implicated whereas carprofen (Gael et al., 2007) and meloxicam (Fresno et al., 2005) are less likely to interfere with clotting mechanisms and can be safely administered as a preoperative analgesic in dogs. During caesarean delivery, iv tenoxicam causes a slight increase in bleeding time with no significant changes in platelet marker levels (Elhakim et al., 2000). Another study suggested that firocoxib, given at the recommended therapeutic dose, did not impair primary haemostasis in healthy dogs (Steagall et al., 2007). Although they might be safe for non-pregnant dogs, their safety in pregnant bitches has not been established.

In early pregnancy, some NSAID are embryotoxic (Burdan et al., 2007) and teratogenic as is diclofenac in animals, but the mechanism of its embryotoxicity is not fully understood (Chan et al., 2002).

Use of NSAID in late pregnancy is associated with a significant increase in the risk of premature closure of the ductus arteriosus in unborn human foetuses (Koren et al., 2006; Poveda Roda et al., 2007; Burdan et al., 2007).

An important consideration in the quest to administer pre-surgical NSAID is to prevent the wind up of nociceptors and thus reducing amount and frequency of analgesics required post operatively. This concept is known as pre-emptive or preventive analgesia in acute and persistent postsurgical pain (Dahl and Kehlet, 2011). Administration of NSAID before surgery has the beneficial effects of reducing inflammation at the surgical site, minimizing peripheral and central amplification of nociceptive input, and decreasing pain after surgery (Dahl and Kehlet, 2011).

It is known that PGFM remains elevated for some time following the birth of the puppies in the bitch (Concannon et al., 1988). This may play a role in to expulsion of foetal membranes, blood and lochia in the bitch following natural birth or CS, but its exact role is unknown. Use of NSAID during the immediate post-parturient period may be of concern as it may blunt the uterine tone and ecbolic action of natural occurring prostaglandins. This may potentially adversely affect the uterine tone, may increase risk of retained placentas, cause retention of lochia with increased risk of endometritis. It has however been suggested that inadequate analgesia in nursing bitches may cause aggressive behaviour toward the young (Mathews, 2008) or negatively influence appetite and lactation.

Effects of NSAID on the breast-feeding infant have raised concerns about their use in the post-caesarean delivery setting in humans (American Academy of Pediatrics Committee on Drugs, 1994). It has been proven that the NSAID, celecoxib is transferred to human breast milk (Carvalho et al., 2006). Although no specific studies have been conducted in the dog, it may be assumed that until proven otherwise, this may also possible in that species. It is thought to be very unlikely that the average clinical dose of celecoxib transferred via the milk would cause untoward effects in breastfed infants (Carvalho et al., 2006). In children and young infants, NSAID-associated nephropathy is well recognised (Ulinski et al., 2012) but usually follows NSAID abuse. Studies suggest that NSAID may cause arrest of

nephrogenesis in the foetus (Harris, 2000). Also, renal function and maturity in the canine neonate may be less developed than that of the human infant during particularly the first week after birth (Buckley, 1986). From this, some speculate that the canine neonate may be more susceptible to the adverse effects of NSAID intake in canine breast milk and therefore avoid its use after CS in the dog (Mathews, 2008).

Having considered all the possible adverse effects of NSAID in the late pregnant bitch, its use prior to delivery of the puppies is contra-indicated. Following the delivery of the puppies, many theriogenologists administer opioids or NSAID or both to the bitch to achieve post-surgical analgesia. Amongst those that use NSAID post CS, there is consensus that administration be restricted to a single dose after CS. The Ontario Veterinary College has been administering meloxicam intravenously at a dose of 0.1 mg/kg after CS for several years, without any abnormalities noted (Mathews, 2008). Using the protocol proposed by Mathews, (2008), appropriate analgesia would be achieved, ethical concerns are addressed and the puppies are not subjected to the adverse effects of NSAID.

2.26.3. Opioids

Morphine (0.1–0.2 mg/kg) and other opioids provide analgesia but cross the placenta and can cause significant central nervous system and respiratory depression in neonates (Goodger and Levy, 1973). Elimination of opioids can take up to 2–6 d in neonates (Mathews, 2008). Avoiding the use of tranquilizers, sedatives, and analgesics until the neonates are delivered is therefore ideal. Fentanyl, meperidine, oxymorphine, and hydromorphone in order of increasing duration of activity can be used in the bitch after delivery of the last puppy, depending on the duration of desired action. Buprenorphine is not recommended because of difficulty in reversing this agent. Butorphanol can be administered during surgery to achieve mild to moderate levels of sedation and post-surgery analgesia. If opioids are used prior to delivery, reversal of their effects on the neonate can be achieved using naloxone (0.04 mg/kg sc) (Wykes and Olson, 2003; Raffe and Carpenter, 2007). Reversal of its effects in the bitch is not sensible as it then reverses the analgesic effect that was required in the first place.

Opioids delay time of discharge because it prolongs time to ambulation. It is a requirement of the local veterinary regulatory body, South African Veterinary Council that all animals following surgery, must be ambulatory prior to discharge. Some breeders also have a very

fierce objection to the use of opioids in the bitch as they claim it makes them clumsy, bad mothers and decreases maternal care and puppy survival. It was shown in mice that pup retrieval is specifically inhibited by morphine (Haney and Miczek, 1989).

In this study, no opioids will be used either pre-or post CS.

2.26.4. Alpha2-adrenergic agonists

Alpha2-adrenergic agonists are potent sedatives, may induce narcosis at high doses, act as analgesic, vastly reduce induction doses of anaesthetic and reduces concentration of anaesthetic gases required to maintain anaesthesia.

The use of alpha2-adrenergic agonist prior to anaesthesia for CS is controversial. In one study, xylazine is not recommended in patients undergoing CS because it was identified as a risk factor for increased puppy mortality (Navarro and Friedman, 1975; Moon et al., 2000), caused severe maternal and neonatal cardiovascular depression (Traas, 2008b), was associated with increased uterine tone (Jedruch et al., 1989; Wheaton et al., 1989) and possibly premature placental separation at higher doses (Navarro and Friedman, 1975). Older literature refers to xylazine as an agent which is associated with an increased risk of death in the dog (Clarke and Hall, 1990; Dyson et al., 1998). Also, xylazine displays some adverse effects on the cardiovascular system based on its lower alpha 1 : alpha 2 specificity. Medetomidine and dexmedetomidine do not have this lower specificity. In more recent surveys, premedication with medetomidine for general anaesthetic procedures was not identified as an increased risk factor (Brodbelt et al., 2008b).

The cardiopulmonary effects of the alpha2-adrenergic agonists are well known and include transient hypertension followed by hypotension, bradycardia, increased systemic vascular resistance, reduced cardiac output, and minimal respiratory depression (Pypendop and Verstegen, 1998). In addition, xylazine sensitises the heart to catecholamine induced arrhythmias during halothane anaesthesia (Muir et al., 1975) which is not the case with medetomidine and dexmedetomidine (Flaherty, 2013a). Medetomidine is a racemic mixture consisting of 50% dexmedetomidine and 50% levomedetomidine. Levomedetomidine is an inactive isomer but there is some suggestion from experimental studies that levomedetomidine may worsen the cardiovascular effects of dexmedetomidine (Flaherty, 2013a). Dexmedetomidine is a more recent alpha2-adrenergic agonist which produces dose-dependent levels of sedation and the intensity of these effects is similar to that produced

by twice the dose of medetomidine (Teixeira Neto et al., 2000). Besides the dosage, dexmedetomidine and medetomidine induced similar clinical effects (Granholm et al., 2007). Theoretical pharmacological advantages of dexmedetomidine over medetomidine cannot be denied and future studies may indeed show advantages.

Patients may appear cyanotic and pale following administration of alpha2-adrenergic agonists. This cyanosis and pallor is not generally due to haemoglobin desaturation but rather due to peripheral vasoconstriction. Arterial blood gas analysis and pulse oximetry will generally be relatively normal in animals demonstrating alpha2-adrenergic agonist associated peripheral vasoconstriction, although some pulse oximeters may either fail to detect a pulsatile signal or display erroneous values (Flaherty, 2013a).

Bradycardia occurs in animals that have been sedated or premedicated with alpha2-adrenergic agonists as a compensatory response to the increase in arterial blood pressure induced by the peripheral vasoconstriction. Atropine premedication has been recommended to prevent this bradycardia (Räihä et al., 1989) and, potentially, the reduction in cardiac output. Removal of this compensatory response by administering atropine leads to significant, increases in arterial blood pressure, increased afterload on the heart and potential for cardiac arrhythmias. Therefore most authorities suggest that antimuscarinics be avoided in treating bradycardia that is induced by alpha2-adrenergic agonists (Sinclair, 2003; Flaherty, 2013b).

Hypoventilation occurs with medetomidine sedation in dogs and respiratory depression becomes most significant when given in combination with other sedative or injectable agents (Sinclair, 2003).

No information is available on any possible cardiovascular effects and effects on blood gases in the foetuses of pregnant bitches.

Medetomidine has the advantage that it can be quickly and completely reversed using its specific antagonist atipamizole (Antisedan, Zoetis) in both the bitch and the puppies immediately after delivery. This increases the safety of medetomidine as a choice of sedative and analgesic. Immediately reversing medetomidine with its antagonist atipamizole may also minimize any endocrine effects medetomidine might have (Cullen, 1996) in the peri-parturient bitch. Medetomidine may affect results on thyroid function tests and others

(Cullen, 1996).

Significant dose sparing occurs to both induce and maintain anaesthesia when alpha2-adrenergic agonists are used as premedicant. Reductions of up to 90% may be expected in some cases. This has important implications. Many of the cases of mortality that are reported with alpha2-adrenergic agonists are directly related to anaesthetic overdose because caution is not exerted during induction and maintenance of anaesthesia to prevent overdose and compensate for reduction in induction and maintenance doses (Jones, 2001; Dyson et al., 1998). In dogs, alpha2-adrenergic agonist premedication vastly reduced the minimum alveolar concentrations of isoflurane (Aho et al., 1991; Lawrence and De Lange, 1997; Aantaa et al., 1997) and sevoflurane (Fragen and Fitzgerald, 1999) required to maintain anaesthesia. The induction dose of propofol is vastly reduced to 1 mg/kg body mass when medetomidine is used as premedicant at doses of 20–40 µg/kg body mass (Hammond and England, 1994). In addition to dose-sparing properties when using alpha2-adrenergic agonists (Sinclair, 2003), the anaesthetist should compensate for the so called prolonged "leg-to-brain" circulation time. This is because the bradycardia will increase the time it takes for the induction agent to reach the brain thus appearing to decrease the speed of induction of induction agent. Lack of this awareness and not allowing for sufficient time for the induction agent to exert its full effect before an additional dose is given may lead to accidental overdose. Therefore, when using alpha2-adrenergic agonists as premedication for any intravenous induction agent, the induction agent should be carefully titrated very slowly after enough time has been allowed for the premedication to exert its full effect.

The route of administration of medetomidine is important and the iv route requires a much smaller dose to achieve the same effect as does the im route (Sinclair, 2003). In the current study, the iv route was used.

The dose-dependent sedative and analgesic effects of iv dexmedetomidine in dogs have been clearly demonstrated and are similar to those of medetomidine at equivalent doses, with dexmedetomidine being twice as potent as medetomidine (Kuusela et al., 2001). Micro-doses of 0.4 µg of medetomidine or 0.2 µg of dexmedetomidine/kg as premedicant does not result in profound bradycardia and mild metabolic acidosis during anaesthesia as high doses such as 40 µg of medetomidine or 20 µg of dexmedetomidine/kg do (Kuusela et al., 2001). Likewise, it was shown that the cardiovascular effects of medetomidine in dogs

could be minimised if the dose was less than 5 µg/kg (Pypendop and Verstegen, 1998).

In humans the preoperative and intra-operative administration of dexmedetomidine at 0.4 and 0.6 µg/kg/h iv is effective in attenuating the maternal haemodynamic and hormonal responses to caesarean delivery under sevoflurane anaesthesia without adverse neonatal effects (El-Tahan et al., 2012). The intra- and post-operative analgesic properties of medetomidine have been demonstrated (Barnhart et al., 2000) and reviewed (Murrell and Hellebrekers, 2005) in the dog, and demonstrated in the pregnant woman, before and after CS (El-Tahan et al., 2012).

The analgesic properties of alpha2-adrenergic agonists following a single dose are unquestionable but is relatively short-acting and of significantly less duration than the sedative effect. Reversal by atipamizole will also reverse the analgesic effects. For this reason, the administration of other analgesics during recovery and the immediate post-operative period may be indicated.

The author of the current study deemed it necessary to study the use of medetomidine. This is because we have been using the method, but without sound support from the literature and, hence, saw the need to do a large descriptive study on the method.

2.26.5. Induction of anaesthesia

Studies have cautioned against use of induction agents as thiopentone sodium (Funkquist et al., 1997; Luna et al., 2004) and ketamine (Moon-Massat and Erb, 2002) as their use reduced puppy survival. Similarly, anaesthetic protocols that include xylazine, ketamine (Moon-Massat and Erb, 2002) and midazolam should be avoided because they have been associated with increased maternal and foetal mortality and decreased neonatal vigour (Ryan and Wagner, 2006a; Luna et al., 2004; Moon-Massat and Erb, 2002). Induction using propofol is widely accepted as the agent is associated with good outcome (Funkquist et al., 1997), can be safely recommended for CS (Gabas et al., 2006) and is by far the most widely used protocol for CS in bitches in some regions (Seymour, 1999; Funkquist et al., 1997; Biddle and Macintire, 2000; Brock, 1996; Moon-Massat and Erb, 2002; Moon et al., 2000). Propofol is a short-acting induction agent that has advantages over barbiturates based on its rapid hepatic metabolism and clearance. The reported induction dose for propofol is 6–8 mg/kg iv in dogs that have not been premedicated and this dose may be reduced to

2–5 mg/kg iv with use of premedication (Short and Bufalari, 1999). Propofol should be administered slowly (over about 20 s) to decrease the incidence of apnoea. Because transient dose- and rate-dependent apnoea and respiratory depression are common with propofol use, assisted ventilation in the immediate induction period may be required. Large, rapid boluses are associated with more severe respiratory depression (Glowaski and Wetmore, 1999). These doses may be significantly reduced if sedative premedicants are used. Propofol does not provide analgesia and therefore additional means of analgesia should be provided when using propofol (Short and Bufalari, 1999). Top up doses may be administered (0.5–2.0 mg/kg) but prolonged use may cause depression of the foetuses and metabolic acidosis. There has been a perception in veterinary practice that propofol is a safe drug (Wagner et al., 2003). However, the cardiorespiratory depressive effects of propofol and thiopentone are similar (Rolly and Versichelen, 1985). The safety in using propofol lies in its short action and rapid metabolism which allows for numerous top up doses. In contrast, thiopentone redistributes and will lead to severe suppression and extended recovery times when topped up (Ko et al., 2001). Propofol takes approximately 45–60 s to reach most of its peak clinical effect. Therefore, injecting over a period of longer than 45 s would affect the speed of induction. However shorter injection times may be associated with somewhat longer induction apnoea times (Ilkiw et al., 2002). Speed of induction using propofol was significantly faster than that achieved with various gaseous inhalation agents (Pottie et al., 2008). Induction dose for injectable agents may also be increased in pregnant patients. This is because many anaesthetic agents are highly protein bound and hypoproteinaemia will therefore affect their dosage. For instance, propofol, which in many countries is the most popular obstetric induction agent, is 95% protein bound (Lemke, 2007). Therefore, hypoproteinaemia associated with haemodilution in pregnant bitches (Concannon et al., 1977b; Kimberely et al., 2006) may result in more unbound free propofol to exert its narcotic effects, necessitating lower doses in pregnant bitches. Another factor to consider in the choice of induction agent is the time between induction and delivery of the neonates. A short time between induction and delivery of neonates is desirable (Brock, 1996), but a very short time has not been shown to have a beneficial effect on puppy survival (Moon-Massat and Erb, 2002). This is because if the interval between induction and delivery of the neonates is shorter than the time it takes for the propofol to redistribute and foetuses to metabolize the propofol then significant respiratory depression of neonates may occur (Short and Bufalari, 1999) which in turn may reduce survival of puppies. The critical time in the case of propofol

in dogs was estimated at 15–20 min (Short and Bufalari, 1999).

Mask induction using inhalant agents may have the advantage of exposing the neonates to an anaesthetic agent that can be cleared by simple respiration but this potential benefit must be weighed against the need for restraint and potential catecholamine release in the bitch. It is the need for restraint, induction of hypoxia and the time to effect, that has made mask induction with conventional inhalation agents unpopular (Ryan and Wagner, 2006a).

Some studies have shown that speed of induction was faster when using sevoflurane compared to isoflurane (Hofmeister et al., 2008). Despite this, gas induction is still cumbersome. Intravenous induction is therefore considered the favoured route of induction for CSs in the dog (Ko et al., 2001). In brachycephalic dogs it is generally not advised to use slow induction techniques such as mask or chamber inductions (Greene, 1995).

In concurrence with most theriogenologists and veterinary obstetricians in some regions, (Seymour, 1999; Funkquist et al., 1997; Biddle and Macintire, 2000; Brock, 1996; Moon-Massat and Erb, 2002; Moon et al., 2000) propofol (Fresenius propoven 1%, Fresenius Kabi) will be used in this study for induction of anaesthesia albeit at lower doses than generally used (1–2 mg/kg versus 4–6 mg/kg).

2.26.6. Local anaesthesia

The use of local anaesthetic blocks of the surgical site (Paddleford, 1992; Kramer, 2008) are indicated in anaesthetic protocols that either do not include use of pre-delivery analgesics out of fear of their deleterious effects on the puppies or do not include narcosis. There are some concerns that local anaesthesia that infiltrates in the surgical midline incision may delay wound healing or increase the incidence of post-operative seromas and surgical site infections. Increasing the total surgical time spent performing a CS is considered a risk factor for puppy survival (Moon et al., 2000) and also for complication rates in bitches related to prolonged surgery (Brodbelt et al., 2008a). It is therefore the tendency of experienced veterinary obstetricians to avoid procedures that may lengthen surgical and anaesthetic time. Because of these reasons and personal preference, local anaesthesia will not be employed as a means of achieving analgesia in this study.

2.26.7. Epidural anaesthesia

Epidural anaesthesia in the dog for CS has been used since the early work by Evers (1968). Caesarean section can be performed with either regional or general anaesthesia (Benson and Thurmon, 1984). Even though epidural anaesthesia has the fewest depressant effects on neonates (Ryan and Wagner, 2006a), the survival rate of puppies from bitches induced with and maintained on isoflurane gas-inhalation was similar to that for puppies from bitches receiving epidural analgesia (Funkquist et al., 1997; Luna et al., 2004). Although epidural anaesthesia can be ideal for many cases of dystocia, if the veterinary obstetrician performing the epidural is not proficient with the procedure, administering an epidural block may take a long time and negatively affect outcome (Raffe and Carpenter, 2007). Performing epidural anaesthesia requires proper restraint to prevent the animal from moving during epidural injection and potential injury (Paddleford, 1992; Raffe and Carpenter, 2007). Epidural anaesthesia may be problematic in that if immobilization of the bitch is not achieved, this may necessitate frequent administration of additional narcotics e.g. opioids (Luna et al., 2004). Spinal anaesthesia may be associated with vasodilation caused by sympathetic blockade that leads to hypotension and hypoperfusion (Raffe and Carpenter, 2007). Motor deficits were observed post CS in some dogs that received morphine and bupivacaine by epidural injection that lasted for up to 9 h (Troncy et al., 2002). Urinary retention in a small percentage of dogs may also be encountered as adverse effect following epidural analgesia (Troncy et al., 2002). Epidural analgesia complicates recovery, post-operative ambulation, increases the risk of injury to the newborn due to inability of bitch to move her hind quarters, increases anaesthetic time, delays time of discharge and requires additional skills. It also increases the cost of procedure to owner. Lastly, it may not always be possible to locate the lumbosacral space and insert a needle into the epidural space. This appears to be more common in fat animals. Injection of the agent into spaces not intended may then either be useless or hazardous (Skarda and Tranquilli, 2007). It may be difficult to identify technical failure of the epidural procedure in bitches under full general anaesthesia. This then leaves the veterinary obstetrician with the false impression that their epidural works well in all cases.

For these reasons, general anaesthesia is preferred over epidural-only anaesthesia (Ryan and Wagner, 2006a). Despite this, many academic veterinary hospitals prefer using epidural anaesthesia in combination with general anaesthesia as it provides both narcosis and

analgesia and is considered by them the more ethical approach.

Epidural anaesthesia was not used in this study.

2.26.8. Maintenance of anaesthesia

Numerous anaesthetic agents have been employed to successfully maintain anaesthesia during CS using inhalants as, halothane, isoflurane (Funkquist et al., 1997; Moon-Massat and Erb, 2002; Moon et al., 2000) and more recently sevoflurane (Gabas et al., 2006; Matsubara et al., 2006). Halothane was identified as a factor that if included in anaesthetic protocol for any surgery, increased the risk of mortality in dogs (Brodbelt et al., 2008b).

Pregnancy in ewes caused a general decrease in inhaled anaesthetic requirement of as much as 40% at term (Okutomi et al., 2009). The reasons for this is uncertain, but changes in endogenous PC and endogenous opiate have been implicated (Datta et al., 1989). Therefore, the percentage of anaesthetic agent in the inhaled vapour may have to be reduced in CS anaesthesia. Sevoflurane is a newer agent favoured by human anaesthetists because of its superior safety profile, rapid action and rapid clearance (Yamada et al., 1994). It appears as suitable as isoflurane for maintenance of anaesthesia in canine patients undergoing a range of elective surgical and diagnostic procedures (Bennett et al., 2008). Some claim that sevoflurane has attributes such as a faster and smoother recovery but this is not conclusively proven (Pottie et al., 2008; Bennett et al., 2008). Given these advantages, it is likely that as more veterinary hospitals gain access to sevoflurane vaporisers, its use will gain popularity. In North America, administration of propofol for induction followed by administration of isoflurane for maintenance was the most common (Moon et al., 1998).

In the years before sevoflurane became available, isoflurane was used in the practice of the primary researcher following premedication with medetomidine and propofol induction. The outcome of this anaesthetic protocol appeared to be no different from the one used in the current study where isoflurane was replaced with sevoflurane. This concurs with the finding that isoflurane and sevoflurane behaved similarly when used in combination with an array of premedicants for various procedures (Bennett et al., 2008).

Due to the slightly better safety profile of sevoflurane above isoflurane (Johnson et al., 1998; Galloway et al., 2004) and availability of anaesthetic equipment in the hospital of the primary researcher, this study only used sevoflurane to maintain anaesthesia at 1% to 2% in

oxygen. It is recommended that the time from induction to delivery of puppies be minimized to reduce respiratory depression as a result of their exposure to inhalant anaesthetics (Brock, 1996). All equipment and personnel required for anaesthetic induction and maintenance, perioperative management, surgery and neonatal resuscitation should be prepared and available before anaesthetic induction.

2.26.9. Preoxygenation prior to caesarean sections

The term oxygenation refers to delivery of oxygen to a patient by inhalation of a gas mixture that contains oxygen at a higher percentage (approximating 100%) than that normally inhaled during breathing of atmospheric air.

The term "preoxygenation" is the delivery of oxygen to a patient by inhalation of a gas mixture that contains oxygen at a higher percentage than that normally inhaled during breathing of room air prior to the induction of general anaesthesia. The technique of preoxygenation is typically used in human patients and was initially developed to prevent desaturation following induction of anaesthesia (Weingart and Levitan, 2012). During preoxygenation, the air occupying the functional residual capacity or "reservoir" in the lungs is replaced by near 100% oxygen. In the event of a partial airway obstruction, complete airway obstruction or apnoea, it takes about 3–4 min for patients that have been preoxygenated to become hypoxemic, compared with only 1½ min for patients that were breathing room air. Desaturation to below 70% puts patients at risk for dysrhythmias, haemodynamic decompensation, hypoxic brain injury and even death (Weingart and Levitan, 2012). Uncompromised patients with an initial pulse oximetry reading of 100% on room air have very low risk of desaturation after adequate preoxygenation (Weingart and Levitan, 2012). Pregnant women and very obese humans are more likely to desaturate quickly (Tanoubi et al., 2009) because their functional residual capacity of their lungs are than those of non-pregnant counterparts or non-obese individuals. This decrease reduces the lung's oxygen reservoir and further aids to shortening the interval between the start of an apnoeic event (induction apnoea) and desaturation. The purpose of preoxygenation is to increase the oxygen reserves so as to prevent desaturation in the time period until the airway is secured (Tanoubi et al., 2009). For preoxygenation to be successful, close to 100% oxygen must be delivered, there must be no leaking between mask and face of patient, the flow of oxygen must exceed maximum inspiration flow rate and the patient must breath in pure oxygen for a minimum of 3 min at tidal volume breaths (at rest breathing) to replace all

alveolar and bronchial airspace with pure oxygen (Tanoubi et al., 2009). In humans, a delay between induction of anaesthesia and securing open airways following intubation is anticipated (Weingart and Levitan, 2012). This is because of the necessity to paralyse the gag reflex in humans and prevent laryngeal spasm, using depolarising agents which also renders the patient unable to breathe without assisted ventilation (Gavel and Walker, 2013). Intubation in humans is also more complicated as the laryngeal positioning within the oral cavity (anatomical arrangement) does not allow for direct view of the larynx and plica vocalis (Boedeker et al., 2007). Given these circumstances with human anaesthesia, preoxygenation makes perfect sense. In contrast, intubation in dogs is easily and quickly achieved (within seconds) due to ability to intubate under direct unaided visual inspection immediately following induction. Anaesthetic induction agents may cause transient apnoea in the dog that may result in desaturation. Polytraumatised dogs with varying degrees of dyspnoea and existing desaturation will benefit from oxygenation following injury and pre oxygenation prior to anaesthesia and surgery (Crowe, 2006). Deep sedation or light anaesthesia may be employed to achieve mask aided preoxygenation in these patients (Greene, 2002).

Preoxygenation is often provided prior to CS in the dog to decrease the risk of hypoxia in bitch and foetuses (Ryan and Wagner, 2006a; Von Heimendahl and Cariou, 2009; Groppetti et al., 2010). Bitches in late pregnancy are more prone to hypoxemia because they have a decreased functional reserve capacity and increased metabolic rate (Pascoe and Moon, 2001) relative to non-pregnant dogs.

All the reports on preoxygenation in the dog prior to CS are based on extrapolation from experience in the human where the risk profile is vastly different. To date, there are only two reports that critically evaluated preoxygenation in the dog (Marsico et al., 1997; McNally et al., 2009). In the one study, it was determined that the time to reach the desaturation point (SpO2 equalled 90% or less) following induction with propofol, averaged 70 s (Mean ± SEM 69.6 ± 10.6 s). In contrast, dogs that were preoxygenated, took four times longer to desaturate (McNally et al., 2009). This proves not only that preoxygenation increases the desaturation time but also that preoxygenation becomes relevant only if the delay between induction, intubation and connection to oxygen at 100% takes longer than around 60 s. In both preoxygenation studies in dogs, sedation was required to deliver the preoxygenation to the patient (Marsico et al., 1997; McNally et al., 2009). Sedation of

pregnant bitches may further result in desaturation in particularly brachycephalic breeds (Greene, 2002). The choices of breed in these studies (mongrels) are also of great clinical relevance as brachycephalic breeds have a greater potential for rapid desaturation (Fasanella et al., 2010). In many countries, the number of CS performed on brachycephalic dog breeds far outnumber those done on other breeds. Brachycephalic breeds are known to suffer from brachycephalic airway obstructive syndrome (Fasanella et al., 2010; Bernaerts et al., 2010). Brachycephalic airway obstructive syndrome affected dogs have present at least one but usually several of brachycephaly-related anatomic components such as stenotic nares, elongated soft palate, everted laryngeal saccules, everted tonsils, hypoplastic trachea, partial collapse of main bronchus, epiglottic cysts, laryngeal granulomas and reduced space surrounding nasopharyngeal turbinates (Bernaerts et al., 2010). These factors compromise affected dogs' ability to adequately ventilate and it also reduces their functional residual capacity or oxygen reservoir predisposing them to more rapid desaturation. Although not proven it is suspected that the ambient temperature may also play a role in whether a dog displays signs of brachycephalic airway obstructive syndrome or not. Dogs with brachycephalic airway obstructive syndrome are known to have lower arterial saturation compared to other breeds (Hoareau et al., 2012). Rapid tracheal intubation is required to prevent hypoxia (Senn et al., 2011). Sedation and muscle relaxation is known to lead to a compromised airway in brachycephalic dogs. A technique of nasotracheal oxygen supplementation has been used to prevent post-operative hypoxia in brachycephalic airway obstructive syndrome dogs (Senn et al., 2011).

The factors mentioned in the previous paragraph compromise the ability of affected breeds to adequately ventilate and these factors also reduces their functional residual capacity or oxygen reservoir. Irrespective of breed, pregnancy reduces functional residual capacity compared to non-pregnant bitches. This is because during pregnancy there is a progressive increase in minute ventilation (Ciliberto and Marx, 1998). This hyperventilation is due to an increase in both, respiratory rate and tidal volume and is a physiological adaptation to compensate for a decrease in reserve volume caused by an increased abdominal pressure and compression atelectasis of lung tissues (Robertson and Moon, 2003). If this compensatory mechanism is lost during anaesthesia, it will cause an immediate decrease in arterial oxygen pressures and saturation (Shnider, 1978). This further loss in functional residual capacity in pregnancy increases the risk of desaturation in these patients. Furthermore, at the end of gestation, 25.8% of English Bulldog bitches suffered from

respiratory problems and partial anorexia (Wydooghe et al., 2013). These respiratory problems may in some cases lead to severe anoxia and death in the days leading to parturition and leaving the pregnancy at peril. In exceptional cases, it may be indicated to perform a preparturient CS or even preterm CS. These cases are those where the pregnancy impedes the respiration of the bitch to the extent of cyanosis and potential for death of the dam. Furthermore, hot conditions predispose some of these bitches suffering from brachycephalic airway obstructive syndrome to heat exhaustion, leaving the pregnancy at peril or causing death.

All the above-mentioned factors strongly support the practice of preoxygenation prior to induction of bitches for CS and even more so in brachycephalic bitches. For this reason, most reports on CS in the bitch are in support of preoxygenation.

Preoxygenation may only be beneficial in animals that are not stressed by a face mask (Kushnir and Epstein, 2012). Patient compliance is required for successful preoxygenation in dogs (McNally et al., 2009). Dogs that are not sedated, frequently resist the restraint required to deliver sufficient flow of oxygen to achieve proper preoxygenation. Preoxygenation “attempts” may indeed be counterproductive based on the observation that it frequently results in dyspnoea and cyanosis in the struggling uncooperative patient. This observation is made more often in brachycephalic breeds than in others. Significant desaturation is suspected to occur during these attempts. It seems more likely that patients suffering from brachycephalic airway syndrome, such as the English Bulldog, would undergo desaturation (Greene, 2002). Because of these difficulties, research on preoxygenation in the dog was performed following sedation (Marsico et al., 1997; McNally et al., 2009). From this, it appears that sedation may be a prerequisite in preoxygenation of healthy dogs that do not tolerate restraint and positioning of facemasks for any length of time.

The brachycephalic dog may require special preanaesthetic considerations (Greene, 2002). When premedicating these dogs, it is preferable to avoid deep sedation as this may be associated with excessive relaxation of upper airway muscles and worsened obstruction again leading to the clinical display of cyanosis. In addition to this concern, the dangers of premedicating bitches prior to CS have been adequately discussed.

The same difficulties encountered with preoxygenation are observed in bitches where gas

inhalation induction is attempted prior to CSs. The risks of inhalation induction prior to CS in bitches include stress and hypoxaemia that adds to catecholamine release, hypoxia and acidosis of the foetuses, as well as regurgitation and aspiration (Ryan and Wagner, 2006a).

Veterinary obstetricians are aware of the difficulties associated with preoxygenation in heavily pregnant brachycephalic bitches. For this reason, they often attempt preoxygenation by holding the anaesthetic tube in near proximity of the patient's nose as this requires no restraint. The efficacy of this technique is questioned as it does not allow for a tight fit; the dog frequently moves its head away from the hose and the gas expelled from the anaesthetic machine mixes with room air before being inhaled. This practice does not achieve the desired goal.

This study will not make use of preoxygenation but intubate the bitch immediately and connect the bitch to oxygen as soon as possible.

2.26.10. Positioning of bitch during CS

During pregnancy there is a decrease in lower oesophageal sphincter tone and gastric motility which, together with increased intra-abdominal pressure, may predispose the pregnant bitch to reflux, vomiting and aspiration (Paddleford, 1992). This accentuates the need to intubate pregnant bitches in preparation for CS. The increased intra-abdominal pressure may compress the caudal vena cava especially when placed in dorsal recumbency in humans (Marx, 1979) but it is unknown whether the same applies to the pregnant bitch. Also, pregnancy has a significant effect on arterial blood pressure, arterial blood gases, pH, base excess and respiratory rate in bitches (Probst and Webb, 1983). Despite this, dorsal recumbency had no significant ($P < 0.05$) effect on any measured parameters in bitches before or after parturition (Probst and Webb, 1983; Probst et al., 1987).

Because it is more convenient to place bitches in dorsal recumbency during CS and because no adverse events have been reported when doing so (Probst and Webb, 1983; Probst et al., 1987), all bitches in the current study will be placed in dorsal recumbency during CS.

2.26.11. Respiratory support during anaesthesia

Pregnancy reduces functional residual capacity and total lung capacity, and increases the likelihood of atelectasis of the lung tissues in bitches (Probst and Webb, 1983; Probst et al., 1987). They compensate for this by hyperventilating and increasing tidal volume. These

compensatory mechanisms are lost during induction apnoea and may be reduced during anaesthesia. Simply increasing the percentage of oxygen inhaled by these patients may not be sufficient. Especially in a brachycephalic bitch, induced "sighing" is recommended during surgery as it may decrease the degree of atelectasis. This may be achieved by closing the pop-off valve on the anaesthetic circuit and squeezing the reservoir bag briefly for 1–2 s at a pressure of 15–20 cm H_2O (Robertson and Moon, 2003). Sighing may become of increased importance if the surgery for whatever reason takes a very long time. In severe cases, mechanical intermittent positive pressure ventilation may be required.

Overzealous maternal ventilation may be as detrimental as maternal hypoventilation. This is because respiratory alkalosis increases uterine vascular resistance and a left-shift of the oxygen dissociation curve resulting in reduced exchange of oxygen in the foetus (Robertson and Moon, 2003). This accentuates the need for anaesthetic monitoring using pulse oximeters and (or) capnometers. In this study, no mechanical assistance to ventilation will be employed unless an emergency arises but bitches will be manually sighed during the procedure.

2.26.12. Anaesthetic monitoring

Accurate, continuous monitoring of cardiovascular and ventilation parameters, temperature and depth of anaesthesia is important (Moon-Massat and Erb, 2002).

Anaesthetic mortality in small animal practice was one in 1849 for healthy dogs and one in 75 for sick dogs (Brodbelt et al., 2005). In comparison, mortality associated with anaesthesia in humans appears to be approximately one in 10,000 to one in 100,000. The lower mortality in humans may in part be due to the administration of anaesthesia primarily by specialist anaesthetists and access to advanced electronic monitoring equipment (Flaherty and Musk, 2005).

a) Cardiovascular Monitoring

Heart rate can be measured by digital pulse detection, oesophageal stethoscope, electrocardiography or pulse oximeter. The main disadvantage of pulse rate alone is that it gives no indication of cardiac output, blood pressure, or tissue perfusion (Wagner and Brodbelt, 1997).

b) Blood pressure monitoring

Blood pressure can be measured by direct or indirect techniques. Direct blood pressure monitoring using an arterial catheter and aneroid manometer or strain gauge transducer is the gold standard and provides the most accurate information on blood pressure status, giving systolic, diastolic, and mean blood pressure readings (Waddell, 2000). It provides continuous information and accurate readings in hypo-, normo-, and hypertensive states. Arterial catheter placement also allows serial arterial blood sampling for blood gas analysis—the gold standard for assessing ventilation. Placing an arterial catheter can be technically demanding and time-consuming and can delay delivery of puppies; therefore, invasive blood pressure monitoring is not routinely recommended for CS. Non-invasive blood pressure monitoring using a Doppler flow detector and sphygmomanometer as well as osillometric techniques provide acceptable estimates of the blood pressure in dogs.

c) Cardio-respiratory monitoring

Respiration can be monitored by direct observation of thoracic wall movement and subjective assessment of breathing bag excursions. Electronic respiratory monitors can also be used as additional aids but do not replace the need for direct monitoring of respiration and do not provide information about the effectiveness of ventilation (Dyson, 1997).

Cardiac function can be evaluated using oesophageal stethoscopes, pulse oximetry, electrocardiography, R-wave monitors and monitors of arterial blood pressure. Respiratory function may be evaluated using respiratory rate monitors, pulse oximetry, capnography and haemoximetry. Pulse oximetry and haemoximetry are discussed in greater detail because both will be used in this study. Arterial blood gas analysis is the gold standard for assessing ventilation, arterial oxygenation, and acid-base status. The technique for arterial sampling and interpretation is well described (Proulx, 1999). Capnography can be used to assess ventilation by measuring the end-tidal carbon dioxide concentration, which approximates the alveolar carbon dioxide concentration, which should in turn approximate the partial pressure of arterial carbon dioxide (PaCO2).

d) Pulse oximetry

Pulse oximetry provides a simple non-invasive means of monitoring arterial oxyhaemoglobin saturation (SpO2) of the dam during anaesthesia. It also provides

information on the pulse (heart) rate. The relationship between SpO2 and arterial blood gas measurements of PaO2 is not linear. Most animals remain sufficiently oxygenated if SpO2 is 90% or greater, corresponding to a PaO2 of greater than 60 mm Hg. Oxygen saturation measurements are best used as a real-time trend indicator of arterial oxyhaemoglobin saturation during surgery but do not replace arterial blood gas analysis as the gold standard of assessing arterial oxygenation. Although arterial blood gas analysis remains the gold standard of assessing arterial oxygenation, SpO2 measurements are used as a real-time trend indicator of arterial oxyhaemoglobin saturation during surgery. A haematocrit of greater than 15% is required for accurate pulse oximeter readings in dogs (Lee et al., 1991). There are drugs and inhalation anaesthetics that cause decreased cardiac output, or systemic vascular resistance or both, that, in turn, result in hypotension and potentially poor tissue perfusion. This may impair the ability of pulse oximeters to receive signal (Waddell, 2000).

Blood contains different species of haemoglobin. Functional haemoglobin consisting of oxyhaemoglobin and deoxyhaemoglobin, can carry oxygen while non-functional haemoglobin, consisting of carboxyhaemoglobin and methaemoglobin cannot.

As almost all oxygen carried by blood (98%) is bound to haemoglobin. Measuring the amount of haemoglobin that is saturated with oxygen (oxyhaemoglobin) indicates of how well oxygenated the patient is. Deoxygenated and oxygenated haemoglobin absorb different wavelengths of light and this forms the basis of pulse oximetry. The word pulse in pulse oximeter refers to the fact that the device only measures haemoglobin in pulsating (moving arterial) blood and not the haemoglobin that is static in muscle or other tissues. Pulse oximetry may be affected by pigmentation, hair and movement at the probe. Premedication with alpha-2-adrenergic agonists causes peripheral vasoconstriction and may impair the pulse oximeter's ability to detect a signal. Vasoconstriction caused by hypothermia and other causes may have the same effect. In a clinical setting pulse oximetry is relatively cheap to use, provides continuous information and is generally accepted as reliable and an invaluable monitoring tool at saturation levels (SpO_2) of 80% and above (Ortega et al., 2011). All medical devices that measure oxygen saturation do so with a certain error (Gehring et al., 2007). However, the pulse rate (equivalent of heart rate) monitoring ability of pulse oximeters are regarded as very reliable (Burns et al., 2006).

The World Health Organization recommends 88% for marked desaturation and 94% for mild desaturation. This number is also used in the intensive care unit literature for ventilation

where the oxygen is targeted between 88% and 94% for acute respiratory distress syndrome. The reason for targeting this range is to prevent hyperoxic injuries. This seems to be a reasonable basis on which to assess saturation (WHO., 2011)

Site of placement of probe influences results. In uncooperative and non-anaesthetised dogs the probe cannot be placed on the lip or tongue respectively. The vulva proves a suitable site in these bitches (Matthews et al., 2003). The model and make of pulse oximeters may vary in ability to accurately read or fail to read saturation (Huss et al., 1995).

In this study, pulse oximetry was used to monitor and record oxygen saturation during surgery for CS.

e) Haemoximetry

Haemoximetry is used to assess the adequacy of ventilation and involves measuring the partial pressure of carbon dioxide (PaCO2) in the arterial blood by blood gas analysis. Haemoximetry discontinuously measures haemoglobin oxygen saturation and dyshaemoglobins from blood samples. It is an essential component of blood gas analyser systems. The partial pressure of oxygen dissolved in arterial blood is termed (PaO_2). The percentage saturation of oxygen bound to haemoglobin in arterial blood is termed (SaO2). When the latter is measured by a pulse oximeter, this value is called (SpO2) (Ortega et al., 2011).

The main advantage of haemoximetry has always been that it can distinguish between the functional and non-functional haemoglobin. Its main disadvantages are that it is discontinuous and invasive. It appears that technology has made progress in correcting one of the chief weakness of pulse oximetry namely accuracy in the presence of abnormal haemoglobin species (Barker and Badal, 2008). This was achieved by the development of a multi-wave pulse oximeter. Currently, haemoximetry is regarded as the “gold standard” and is used both to calibrate pulse oximeters (Gehring et al., 2007) as well as the method of choice for use in studies where oxygen saturation data is crucial. Therefore, in time, the use of the multi-wave pulse oximeter may dethrone haemoximetry as the “gold standard” method of monitoring as it provides a continuous measurement of saturation and is non-invasive.

Haemoximetry is invasive, technically demanding and the equipment required is expensive.

Therefore, it is not commonly used in veterinary clinical practice and was not used for this study.

2.26.13. Recovery from anaesthesia

In the brachycephalic dog, special attention should be paid to airway patency during recovery and the period immediately following extubation due to their propensity to airway obstruction. The time of extubation requires discussion. Some veterinary obstetricians prefer to extubate the dog before it wakes up to prevent biting and destruction of the tube. This practice may expose the brachycephalic patient at a greater risk of upper airway obstruction (Greene, 2002). Greene (2002) advocates the procedures stated in the remainder of this paragraph for brachycephalic breeds. The patient should be almost totally awake and react to the presence of the endotracheal tube by coughing and making swallowing movements before removing it. Once extubated, the dog should be under supervision until it can lift its head and able to stand. Correct positioning of the patient is also crucial for adequate ventilation. The dog should be kept sternal with the head in extension and if the dog is not ventilating properly the tongue may be pulled forward to relieve the obstruction.

2.27. Hydramnios and hydrallantois

Excessive fluid build up in the amniotic and allantoic sacks is respectively referred to as hydramnios and hydrallantois. Hydrallantois is much more common than hydramnios, although the latter is frequently seen in association with specific foetal abnormalities in the cow (Pereira et al., 2011). Hydrallantois leads to severe respiratory distress in bitches, presumably due to acute dilatation of the abdomen and compression of the diaphragm and splenic vessels (Feliciano et al., 2013; Smith, 1972). The significance of these conditions is that it may place canine pregnancies at peril and necessitate preterm or preparturient CS.

2.28. Caesarean section

2.28.1. Indications for caesarean sections

The criteria for examining of the dam for possible CS has been tabulated (Gendler et al., 2007).

- Prolonged gestation confirmed by ovulation timing using the preovulatory progesterone rise above 6 nmol/L or the LH peak

- Temperature decreases below 37.6°C for a consistent 12–24 h without signs of parturition
- Vulvar discharge for more than 2–3 h
- Lack of progression to stage 2 parturition after 6–8 h
- Strong, active abdominal contractions for 30 min without expulsion of a foetus
- Stage 2 of parturition taking longer than 12 h
- Prolonged parturition lasting longer than 24 h
- Membranes or part of foetus protruding from the vagina
- Signs of systemic illness in the dam

It is problematic to put specific time limits to the criteria above. This is because the starting point of stage 1 of parturition may be impossible to determine. Observation for foetal demise using ultrasound is therefore a valuable tool in this regard and should be used to guide the veterinary obstetrician to make decisions.

Though good general guidelines, the literature review in this document will challenge some of these criteria. The review will show that following these criteria religiously in all circumstances may lead to increased puppy mortalities.

For bitches included in the current study, criteria for a CS were:

- Earliest sign of dilation of cervix on visual inspection of the vagina through a speculum
- Very first sign of strong, active abdominal contractions in high risk breeds
- Intra-partum presentation of any bitch belonging to high risk breeds
- Any sign of foetal membrane rupture in high risk breeds
- Signs of foetal distress on ultrasonography (fewer than 140 foetal heart beats per minute)
- High risk pregnancies

For the singleton litter, additional criteria apply:

- Response to oxytocin administration displayed as either contractions, increased uterine tone palpated trans-abdominally and restlessness and discomfort of the bitch, characterised by her looking at her flank and licking her vulva
- Hyperventilation

- Known D0

When advising clients on the likelihood of puppy and dam survival associated with CS, knowledge of associated mortality rates is essential. Survival rates immediately, 2 h and 7 d after delivery were 92%, 87%, and 80%, respectively, for 3410 puppies delivered by CS and 86, 83, and 75%, respectively, for 498 puppies born naturally (Moon et al., 1998). In the same study, all puppies in 76% of the litters delivered by CS were born alive. The maternal mortality rate was 1%. It is important to note that there might be a difference in outcome when surgery was performed on an elective basis or in an emergency. In the bitch the likelihood of all the puppies being alive if the CS was performed on an emergency basis or if the bitch delivered puppies naturally was lower than for elective CS (Moon et al., 2000). Likewise in humans, the neonate survival of an intrapartum CS is less than in planned CS (Ben-Meir et al., 2005). This is an important factor to consider and certainly supports performing elective CS in breeds prone to dystocia.

The most common breeds of dogs that underwent emergency surgery were English Bulldog, Labrador Retriever, Boxer, Corgi, and Chihuahua whereas the most common breeds of dogs that underwent elective surgery were English Bulldog, Labrador Retriever, Mastiff, Golden Retriever and Yorkshire Terrier (Moon et al., 1998). The 10 breeds with the highest percentages of litters born by CS were the Boston Terrier, English Bulldog, French Bulldog, Mastiff, Scottish Terrier, Miniature Bull Terrier, German Wirehaired Pointer, Clumber Spaniel, Pekingese and Dandie-Dimont Terrier. More than 80% of Boston Terrier, English Bulldog and French Bulldog litters are delivered by CS (Evans and Adams, 2010). These lists of breeds may reflect true predispositions but they may also be influenced by breed representation in the surveyed geographical area of the trial and as well as clients and practices that participated. It is evident that most reports covering risk of CS in the bitch will include either brachycephalic breeds or English Bulldogs only and these breeds should thus be regarded as very high risk. In brachycephalic breeds initiation of surgery before natural parturition may be important, although there are no published data on the incidence of problems during natural delivery in these breeds (Concannon, 2011). Whether an ovariohysterectomy is performed at the time of the CS depends on the findings during surgery and on the owner's request (Michel and Reichler, 2008a) and does not appear to affect puppy survival or lactation.

2.28.2. Indications for preterm caesarean sections in bitches

Current indications for planned preterm delivery by CS (elective preparturient CS) in women are pre-eclampsia, preterm breech, intrauterine growth restriction, macrosomia, chorioamnionitis, placenta previa, placenta accrete and vasa previa (Oyelese and Smulian, 2006; Kolås et al., 2003). In many of the above conditions the aim of preterm delivery is to pre-empt terminal hypoxaemia and other foetal and maternal complications (The GRIT Study, 2003).

Although less common and not well described, there are indications for preparturient CSs (preterm) in bitches at a time where the likelihood of puppy survival is restricted. These are bitches that suffer from pre-existing undetected medical conditions that only present themselves in late pregnancy when their physiological reserve capacity becomes exhausted. The combined effect of decreased functional residual capacity and increased oxygen consumption makes dogs in late gestation very susceptible to hypoxemia (Seymour, 1999). When combined with brachycephalic airway obstructive syndrome (Fasanella et al., 2010; Bernaerts et al., 2010) and hydramnios or hydrallantois, fatal consequences are possible (personal observation De Cramer, 2015).

Bitches with known or unknown cardiac disease that were previously stable or well controlled on medications can become decompensated and develop heart failure during pregnancy and parturition (Thurmon et al., 1996) with potentially fatal consequences.

Idiopathic preterm labour in the bitch may occur as result of inappropriately timed myometrial activity accompanied by cervical dilatation and subsequent abortion in the presence of adequate PC in the blood to support pregnancy (Davidson, 2015). Idiopathic preterm labour has no metabolic, infectious, congenital, traumatic, or toxic cause identified (Davidson, 2015). In contrast, another worker hypothesized that hypoluteodism is likely to be the cause of preterm labour in the bitch based on low progesterone measurements at the time of pregnancy loss (Johnson, 2008a).

There may also be an indication for selective removal of one or more foetuses prematurely from one or both horns and leaving other foetuses intact in the gravid uterus. This may be necessary in cases of uterine torsion (Smith, 2007) or in cases where preterm foetal death has occurred with evidence of cervical dilatation characterised by a vaginal discharge (personal observation). It is not clear whether cervical incompetence leads to or follows

foetal demise in the bitch. Cervical incompetence is a well-recognised complication of pregnancy in man which may necessitate preterm CS (Coussins, 1980; Hager, 2003).

2.28.3. Incidence of caesarean section in bitches

In a survey conducted in 151 breeds represented by 13,141 bitches which had whelped 22,005 litters, the percentage of litters that were reported to be delivered by CS in three brachycephalic breeds (French Bulldogs, English Bulldogs and Boston Terrier) was 85.7% versus 18% for the other breeds (Evans and Adams, 2010). The latter statistic is substantially higher than the 5% incidence of dystocia (dystocia which often occurs before CS) in all breeds as suggested by others (Linde-Forsberg and Eneroth, 2000). In one study, the CS ratio for bitches was defined as the frequency of CSs estimated as the percentage of litters that were reported to be born by CS for each breed (Evans and Adams, 2010). This definition does not consider the number of repeat CS that may be required in the same bitch and therefore may lead to an overestimation of the CS ratio in a breed. This is particularly true as repeat CS is suspected to be common. Another estimate pertaining to CS in bitches of a breed may be the ratio or percentage of bitches of a particular breed that required one or more CSs in their lifetime. Therefore, if the CS ratio in a breed is determined it should be specified whether repeat CS were considered or not.

The clinical relevance of the high incidence of CS in certain breeds provides evidence for the need to manage parturition in these breeds (Evans and Adams, 2010; Bergström et al., 2006b).

Dystocia may occur in all dog breeds, but the English Bulldog is predisposed because of its conformation and specific problems such as anasarca puppies (Wydooghe et al., 2013). In the English Bulldog, CS was performed in 94.8% of the cases (Wydooghe et al., 2013), with natural delivery in only 5.2% of the bitches and was the most frequently represented breed for both elective and emergency CSs (Eneroth et al., 1999). The narrow pelvic inlet predisposes Scottish terriers and Boston terriers to dystocia and subsequent CS (Moon-Massat and Erb, 2002).

Knowledge of incidences of CS in the various breeds helps form a consensus regarding what breeds belong to high risk breeds and what an appropriate CS rate for that breed is. It also helps bring about awareness about the need for corrective measures that are required in affected breeds.

2.28.4. Repeat caesarean sections in woman and vaginal birth after caesarean section

Having undergone a previous CS is the most common obstetric indication for a repeat CS. In humans prior CS is the indication for repeat caesarean in 28% of births in the United Kingdom (RCOG, 2001) and over 40% of births in the United States (Curtin et al., 2000). In woman the most common reason for repeat CS is patient request and not medical indication (Lyerly and Little, 2010). Vaginal birth after CS success rates are variably quoted as between 56% and 80%, although the proportion of women attempting vaginal birth varies considerably (Flamm, 1997; McMahon, 1998; Stone et al., 2000). In women who have had any previous CS, the chance of successful vaginal birth was only 33% (RCOG, 2001). Numerous reports highlight an increased risk of morbidity, including uterine rupture (Hibbard et al., 2001; Lydon-Rochelle et al., 2001), which may be potentially fatal to mother and cause perinatal death (Smith et al., 2002) associated with vaginal birth after CS. In a very large study vaginal birth after CS was attempted by 17,898 women, and 15,801 (88.28%) women underwent elective repeated caesarean delivery without labour (Landon et al., 2004). In this study, uterine rupture occurred in 124 women (0.7%) and hypoxic-ischaemic encephalopathy occurred in 12 infants from 2097 (0.57%) successful vaginal birth after CS. In contrast no uterine ruptures or encephalopathy occurred in mothers who underwent elective repeated caesarean delivery (Landon et al., 2004). Also the rate of endometritis was higher in women undergoing a trial of labour vs repeat CS (2.9% vs 1.8%), as was the rate of blood transfusion (1.7% vs 1.0%) (Landon et al., 2004). This study concluded that in mothers that had a previous CS, the chance for maternal adverse events in trial of labour vs repeat CS was 5.5% vs 3.6% (odds ratio 1.56, 95% CI, 1.41–1.71) (Landon et al., 2004).

Repeat CS has associated problems including bleeding, the need for blood transfusion, infection, damage to the bladder and bowel, and deep venous thrombosis in the legs (Hemminki and Meriläinenb, 1996). As the numbers of CSs in a woman increases, so does the difficulty in performing surgery due to adhesions, and the risk of damage to the bladder or bowel during surgery. There may also be difficulties in conceiving again or a subsequent placenta may develop over the scar in the uterus (*placenta praevia*) (Hemminki and Meriläinenb, 1996). Occasionally the placenta may continue to develop into the muscle wall of the uterus (*placenta accreta* or *placenta percreta*). This may cause difficulties with the placenta being delivered after birth and sometimes excessive bleeding. Babies delivered by

CS may develop some difficulties with breathing (called transient tachypnoea of the newborn), and may need to spend time in a special care nursery (Hemminki and Meriläinenb, 1996). Ongoing efforts to refine the evidence base for assessing and comparing risks of vaginal birth after CS versus scheduled caesarean have led to pendulum swings in the recommendations for clinical practice. During this, the women's autonomy needs to be respected as well.

2.28.5. Repeat caesarean sections in bitches and vaginal delivery in bitches after caesarean section

To date no reports on trial of labour after CS or vaginal birth after CS or repeat CS could be found or formal reports on problems associated with repeat CS in dogs.

2.28.6. Performing caesarean section in the presence of a closed cervix

When the cervix has not opened, the bitch may be considered to either not be in parturition at all or in the early part of stage 1 of parturition. In humans when an elective CS is performed, the cervix will always be closed. The surgeon then routinely manually dilates the cervix with their index finger either from the uterus towards the vagina or through the vagina into the uterus to allow for blood and debris to escape from the uterus. In the reports where preparturient CS was performed in bitches, it was not mentioned whether the cervices were open or not. The danger exists that if the cervix does not dilate adequately to allow for blood and debris to escape following CS, complications may arise. It is important in the design of a study in which CS are performed in the presence of a closed cervix, to take note of whether uterine content can easily escape from the uterus after surgery and whether complication associated with the closed cervix may arise during the 7-d following CS. The seven-day clause is specifically elected because in all the experiments in the current study the occurrence of adverse events in both the puppies and bitches are recorded up to day 7 following CS.

2.28.7. Using cervical dilatation as indicator for readiness for caesarean section

During stage 1 of parturition progressive cervical dilatation occurs, the uterine tone increases but no uterine contractions are detected (Linde-Forsberg and Eneroth, 2000). This stage usually lasts 6–12 h or possibly 24 h (Linde-Forsberg and Eneroth, 2000). When regular visual inspection of the vagina through a speculum is performed (6-hour intervals),

dilatation of the cervix will not go by undetected and allows one to perform a CS before strong abdominal and uterine contractions characteristic of stage 2 of parturition commences. The moment uterine contractions occur, potential for foetal distress exists more readily than early in stage 1 when those contractions are absent. It is speculated that if the CS is delayed until uterine contractions commence, a planned elective CS may become an emergency CS and foetal compromise in high risk bitches may already be present at that stage. Therefore, because cervical dilatation precedes uterine contractions it can be used as a safe indicator for readiness for CS. Care needs to be taken when visual inspection of the vagina through a speculum is performed to evaluate the true state of the cervix as the cervix may not be visible because of the gravid uterus that pulls it ventrally over the pelvic brim. In these cases, the abdominal wall needs to be lifted to advance the speculum sufficiently deep so that the cervix can be visualised. It is not known whether the dilatation of the cervix is synchronised with decline in preparturient PC or not and whether PC decline consistently precedes cervical dilatation and if so by what number of hours.

2.28.8. Using paracervical dilatation as indicator for readiness for caesarean section

The paracervix has been described by Lindsay (1983). The opening to its crescentic lumen appears as a narrow tunnel, into the distal extremity of which projects the vaginal portion of the cervix. The dorsal median fold is a permanent, single longitudinal mucous membrane on its dorsal median wall. Cranially, the dorsal median fold or post-cervical fold, attaches to the tubular vaginal cervix. The caudal tubercle demarcates the fold from the rugose area behind. This caudal tubercle may by some be confused as the opening to the cervix. The internal dimensions of the paracervix vary between individual bitches and is usually between 20 and 50 mm in length and seldom exceeding 5–7 mm in intraluminal diameter (Lindsay, 1983) with dimensions depending on the stage of the oestrous cycle. A greyish transparent mucus originating from the cervix may hinder the examiner to clearly see the entrance to the paracervix. Even though the mucus can be suctioned in most cases there may still be some uncertainty in some cases.

There is no specific literature that describes the temporal relationship between opening of the paracervix and parturition.

2.28.9. Elective caesarean section in humans

Elective CS refers to a CS that is performed on a pregnant woman or animal due to

obstetrical (Rothenberg, 2006) or non-medical reasons (Lavender et al., 2012; Kwee et al., 2004) or upon the request of the pregnant woman (Ben-Meir et al., 2005; Bergholt et al., 2004). The elective CS is therefore also a "planned CS". By implication it also means that it is scheduled in advance rather than performed because of an unscheduled emergency. Human obstetricians will therefore commonly perform the operation at a scheduled time, rather than waiting for the onset of parturition. The period of safety wherein a human foetus may be delivered prior to the time that natural parturition would have occurred is well defined in humans (Zegers-Hochschild et al., 2009). Planned CSs in women are therefore possible and practical and lead to good outcome (Husslein and Wertaschnigg, 2002). By definition, at the time an elective CS is performed, the woman is not parturient (“in labour”) yet and the surgery is thus performed on a preparturient patient. In bitches, by contrast, the surgery is usually performed at parturition as the timing of the surgery is based on exhibiting signs of onset of parturition. This is because it is difficult to time CS in the bitch, an ill-defined period of safety in the bitch and because the delivery of non-viable preterm foetuses is a reality. A CS performed when the bitch is already in the early stages of parturition is no longer an elective CS but a parturient CS or an emergency CS depending on whether an emergency exists or not. Also at the time of a parturient CS the preparturient decline in the concentration of progesterone in the blood plasma or serum will have occurred and the cervix will have dilated. Elective CS in woman are performed for many reasons, including history of previous CS (Vernon, 2005), placenta previa, abnormal presentations, multiple pregnancy, known obstructions of the birth canal and medical conditions. The advantages of performing the delivery at a scheduled time include use of daytime services when both, hospital resources and the ability to plan and prepare for the event, which are crucial factors that may affect outcome, are optimal. Critics of elective CS in humans argue that because obstetricians and institutions may benefit by reducing night time and weekend work, that an inappropriate incentive exists to suggest elective surgery (Vernon, 2005). Elective CS in humans also carries with it the risk in that the surgery may be scheduled too early resulting in premature or compromised delivery. Prenatal testing mitigates this risk considerably in woman. However, fear of litigation and legal issues are cited to drive the elective CS rate in woman higher (Kwee et al., 2004). There should be no doubt that a major advantage and motivation for elective CS in both the human medical and veterinary professions is the ability to ensure optimal operative and staffing conditions (Ryan and Wagner, 2006b).

2.28.10. Timing of caesarean section in bitches

The timing of a CS in the bitch may be performed at different stages of gestation or parturition. They are preterm CS, preparturient CS, parturient CS and emergency CS.

a) Preterm caesarean section

In humans a premature infant is a baby delivered by a preterm CS, which means delivery before 37 completed weeks of gestation or more than 3 weeks before the due date (Fleischman et al., 2010a; Engle and Kominiarek, 2008). Preterm CS is therefore a CS performed before the safe period of intervention or "term". For the purposes of this thesis, it is suggested that preterm in the dog should mean the same. Because we do not know what the extent and duration of the safe period of intervention is, preterm in the bitch will be that undefined time before term before the foetuses have sufficiently matured and during which there is increased risk of reduced puppy survival when removed from uterus.

b) Preparturient caesarean section

In contrast to a parturient CS, a preparturient CS in the bitch involves the delivery of the foetuses before P0 occurs, before dilatation of the cervix and in some cases, may also be before preparturient decline in PC (Levy et al., 2009; Vannucchi et al., 2012; Fontbonne et al., 2009). As defined above, a preterm CS always results in the delivery of premature offspring with reduced survival rates whereas a preparturient CS need not necessarily result in premature offspring provided the preparturient CS is performed in that critical period before the onset of P0 wherein the foetuses have similar survival rates than when delivered at P0. Therefore performing preparturient CS requires some knowledge of the range of the safe period of intervention in the bitch. It was assumed in the current study that a foetus can be delivered and remain viable without assistance at 48 h before P0 based on the studies on parturition induction using either aglepristone priming (Baan et al., 2005; Levy et al., 2009) or betamethasone priming (Vannucchi et al., 2012) and unpublished data of the authors on CSs done without priming. It is not known how long before 48 h prior to the time for natural parturition a CS can be done safely. The motives for research into parturition induction protocols (Fontbonne et al., 2009; Fieni and Gogny, 2009) and preparturient CS are similar. In both, the objective is to allow for delivery of puppies during a shorter period of supervision and to expect delivery or perform CS at a predictable time. The ideal is that this time falls within normal working hours when there is a full complement of staff available to

assist and supervise. This notion concurs with what the suggestion that a significant proportion of pregnant brachycephalic bitches undergo elective caesareans before natural parturition begins (Evans and Adams, 2010).

The studies that did use predicted parturition dates to time CS in the bitch or induce parturition, all used progesterone either on its own or together with LH during the peri-oestrous period, which is sometimes referred to as "upfront timing" (Baan et al., 2005; Levy et al., 2009; Vannucchi et al., 2012; Fontbonne et al., 2009). Unfortunately, none of these studies are large or convincing enough to conclude that performing CS based on their timing method is safe for routine use in clinical practice. Also, these studies do not show whether it is safe to omit priming. Priming refers to the administration of either aglepristone of betamethasone to simulate the foetal maturation process that supposedly occurs during the preparturient period. No study thus far, used D0 to time preparturient elective CS nor did any study omit priming using either aglepristone or betamethasone. As is the case in women, a preparturient CS in the bitch may later also be referred to as a term CS but this can only be when the extent and range of term is fully defined in the bitch.

It has been suggested that eutocic vaginal deliveries result in puppies that display stronger signs of vitality compared to newborns delivered via emergency or elective CS and that the absence of a compressive force in the foetal thoracic area while passing through the birth canal reduces the breathing reflex in neonates delivered by caesarean (Silva et al., 2015; De Luca et al., 2009). It has also been speculated that performing a CS prior to the onset of uterine contractions likely prevents the rise in foetal catecholamine concentrations and, ultimately, blunts the final stimulus for lung maturation (Silva et al., 2015).

c) Emergency caesarean section in dogs

Emergency CSs are often performed during parturition by necessity because an emergency exists or because there is foetal demise due to being overdue or to other causes. The only advantage of emergency CS over preparturient CS is that in the former the veterinary obstetrician is assured that the bitch is parturient. It is known that emergency CS is associated with reduced survival of the offspring in the bitch (Moon et al., 2000) and in man the neonatal survival of an intrapartum CS is less than in planned CS (Ben-Meir et al., 2005; Häger et al., 2004). It is assumed that in all cases of emergency CS, the preparturient decline in PC will have already occurred. The only exception is suspected to be some singletons and

bitches that suffer from incomplete preparturient luteolysis. The outcome of emergency CS in the dog has not been researched and this study will explore it.

d) Post-term caesarean section in dogs

In woman some pregnancies progress to beyond term with increased risk of perinatal death (Smith, 2001a). Although speculated to be a rare occurrence, bitches may be "overdue" or beyond term for a variety of reason. Severe illness in the bitch, inappropriate treatment with long-acting gestagens, singletons and failure of the parturient luteolysis are some possible causes of the duration of gestation going beyond the normal range.

2.28.11. Pre-anaesthetic assessment

Preoperative assessment should include a complete history and a physical examination (Ryan and Wagner, 2006a). In this study, minimum laboratory database included a blood smear evaluation, haematocrit and blood glucose in small breeds. If the clinical examination or history raises concerns, then the minimum database should also include serum concentrations of urea, creatine, albumin, alanine aminotransferase, and alkaline phosphatase, as well as diagnostic imaging (Ryan and Wagner 2006a).

2.28.12. Fluid therapy during anaesthesia for CS in the dog

Fluid therapy is recommended as a standard for CS (Moon et al., 2000; Robertson and Moon, 2003; Ryan and Wagner, 2006b; Traas, 2008b; Von Heimendahl and Cariou, 2009; Kushnir and Epstein, 2012; Smith, 2012). Fluids are given to correct any fluid and electrolyte deficits, acid-base balances, the hypotensive effects of anaesthesia, maintain cardiac output and uterine blood flow (Mazzaferro and Wagner, 2001; Ryan and Wagner, 2006a). In a review of the literature, fluid rates of 10–30 ml/kg/h with additional boluses under conditions specified below have been suggested to maintain perfusion (Robertson and Moon, 2003; Ryan and Wagner, 2006b; Smith, 2012). Fluid therapy has the potential to change the haematocrit.

Crystalloid fluid therapy should be administered during anaesthesia for CS in pregnant dogs and cats (Gilroy and DeYoung, 1986; Paddleford, 1992; Pascoe and Moon, 2001; Moon et al., 2000; Robertson and Moon, 2003; Traas, 2008b; Von Heimendahl and Cariou, 2009; Kushnir and Epstein, 2012; Smith, 2012) and should preferably begin preoperatively (Kudnig et al., 2003). Fluid therapy with crystalloids at the time of CS is recommended to

maintain systemic blood pressure, cardiac output and ultimately uterine perfusion and oxygenation of the foetuses. Fluid therapy also helps counteract the hypotensive effects of anaesthetics (Kudnig et al., 2003). The crystalloid of choice is lactated ringer's solution. Although no specific work has been published regarding perioperative fluid rates for CS, fluid rates commonly recommended are 5–20 ml/kg/h in the perioperative period, but may be increased when emergency volume resuscitation is required (Ryan and Wagner, 2006a; Kudnig et al., 2003; Robertson and Moon, 2003; Smith, 2012). One author suggests that fluid administration be adjusted according to the bitch's blood pressure which should be maintained above 60 mm Hg. If the bitch becomes hypotensive it is advised to decrease anaesthetic concentration and administer an iv fluid bolus (Smith, 2012).

In the current study 35 ml/kg of ringer's lactate was administered to each bitch undergoing CS beginning at time of induction and ending not before 1 h after surgery and not exceeding 2 h after surgery. Fluid therapy will be administered through standard fluid administration sets (20 or 60 drops/ml) and the total volume administered was controlled by calculating the total amount the bitch requires by multiplying the bitch's body mass with 35 ml from a drip bag containing known quantities.

2.28.13. The surgical technique of a CS

The surgical technique to perform CS has been well described (Gilson, 2003; Bebchuk and Probst, 1998; Hedland, 2002). Efficient preparation of the abdomen and aseptic scrub is recommended. Briefly a midline approach is followed incising the *linea alba*, a single incision is made in the body of the uterus through which all foetuses are manipulated for removal. Whether the placenta should be removed or the foetal membranes broken open and the umbilicus ligated requires discussion. It is general practice in some veterinary teaching hospitals to leave the placenta whilst others will remove them but only if the placenta is partially released already and it dislodges easily. Also, some veterinary teaching hospitals tie off the umbilical cord whilst others do not.

The main reason for leaving placentas is the fear of post-operative haemorrhage from the placental sites. In woman it was demonstrated that manual removal of the placenta during CS was associated with greater blood loss than placentas delivered spontaneously (Hidar et al., 2004). There are veterinary obstetricians that remove placentas to avoid risk of retained placentas and metritis that may ensue. It is the author's experience that placentas detach

easily in carefully timed CS and that haemorrhage is not a problem but that the problems of post-operative metritis and toxic milk syndrome are. Once all the foetuses have been removed the uterus must be palpated from both ovaries to cervix and cranial vagina to ensure that all the foetuses have been delivered. The uterine incision must be closed with absorbable suture in standard fashion and the *linea alba* and skin using non-absorbable sutures. Intravenous fluids may be administered at the intraoperative rate (10 ml/kg/h) during preparation for surgery to help improve intravascular volume, correct electrolyte shifts and counter hypotension secondary to anaesthesia (Gilroy and DeYoung, 1986).

2.28.14. Intra-operative uterotonics and anti-fibrinolytic agents

Prophylactic uterotonics are administered as a routine during human CS to cause placental separation and ensure uterine contraction to reduce risk of postparturient haemorrhage (Cotter et al., 2010). The uterotonic for which there is most evidence of efficacy and least side effects is 10 iu oxytocin im (Cotter et al., 2010). Recently, attention has focused on the use of tranexamic acid, an anti-fibrinolytic agent, to reduce blood loss if given prophylactically at CS (Halder et al., 2013) or administered if there is evidence of excessive blood loss during or after parturition (Sentürk et al., 2013).

2.28.15. Complications of caesarean sections

Many complications of CS, relating to both anaesthesia and surgery, may affect the bitch and puppies.

a) General surgical complications

The complications in humans included haemorrhage, haemoperitoneum, abdominal wall haematoma and emergency ovariohysterectomy due to uncontrolled haemorrhage (Ko et al., 2011). In addition, wound site infection, wound dehiscence with herniation or evisceration, seroma formation and reduced puppy survival and bitch fatalities are possible following CS in the bitch (Ryan and Wagner, 2006b; Michel and Reichler, 2008a). Maternal mortality associated with CS has decreased significantly in the past 30 years from about 15% to about 1% (Mitchell, 1966; Moon et al., 1998). Fatalities of up to 1% have been recorded as complication for CS in the bitch (Moon et al., 1998) and brachycephalic breeds such as Pekinese and English Bulldog were highlighted as having a higher risk of complications and death with anaesthesia (Clarke and Hall, 1990; Dyson et al., 1998; Brodbelt et al., 2008b).

b) Haemorrhage as complication of caesarean sections in dogs

The bitch is already severely anaemic (Concannon et al., 1977b) or marginally anaemic at the time of CS (Kimberely et al., 2006; Dimço et al., 2013). Further blood loss can be assumed with surgery, the extent of which is unknown. Blood loss with concomitant fluid replacement (maintaining patient blood volume via administration of crystalloid or colloid solutions), results in normovolaemic haemodilution (Prittie, 2010). In a study of trauma patients in humans it was shown that during ongoing fluid resuscitation, the change in haematocrit is a reliable indicator of continuing blood loss (Thorson et al., 2013). In this study a change in haematocrit of 6% (sensitivity 89%, specificity 95%) or greater during initial resuscitation is highly suspicious for ongoing blood loss, but even smaller changes have predictive value (Thorson et al., 2013). In another study in dogs it was shown that in acute normovolaemic haemodilution, the ratio between oxygen tension in skeletal muscle and cardiac index started decreasing in healthy dogs at the threshold haematocrit of 25% (Freitag et al., 2002). When evaluating bitches before and after CS, no published thresholds are available and therefore further research is required regarding these thresholds. Until we know what the thresholds are, a threshold haematocrit of 25% is important because it provides a safety net for those bitches which are approaching a haematocrit close to where they are known to become compromised (Freitag et al., 2002).

c) The blood transfusion trigger in bitches undergoing caesarean section

The blood transfusion trigger proposed in this study requires discussion. Clearly, red cell transfusion has its main place in maintaining the oxygen carrying capacity of blood and there is much debate as to what is the optimal haematocrit for various clinical settings and what the target should be during resuscitation (Isbister, 1997). The ideal target haematocrit for pregnant dogs is also not known. There were no advantages in raising the haematocrit above 30% in the post-injury state following acute haemorrhage (Fortune et al., 1987). Historically, the widely accepted clinical standard in humans was to transfuse patients when the haemoglobin content dropped below 100 g/L (Calder et al., 1997) or haematocrit ≤ 30% (Zander, 1999), but this practice is no longer universally acceptable (Carson et al., 2002). A consensus conference (1988) reported that the evidence did not support a single criterion for transfusion in man but rather advised that a range of haemoglobin content values between 60 g/L and 100 g/L can be used, depending on the presence of serious comorbidity (Calder et al., 1997). This corresponds roughly to a haematocrit of 18%–30%. The transfusion

trigger for women that had vaginal delivery in some human clinics was a haematocrit below 27% (Nicol et al., 1997). Currently the transfusion trigger at the Onderstepoort Veterinary Animal Hospital for dogs with anaemia due to babesiosis is a haematocrit of below 20%.

For the purposes of this study a haematocrit of below 28% was considered a risk factor and these bitches were not immediately discharged. Given an adequate cardiac output, a haematocrit of 25% may be a better lower cut-off because the oxygen tension in skeletal muscle only decreases once the haematocrit decreases below 25% following normovolaemic haemodilution (Freitag et al., 2002). Possibly, the haematocrit of bitches may still decrease somewhat during the 2 h after CS due to blood loss from surgery or from the placental sites, uterine auto-transfusion and fluid administration. Given these reasons, a higher cut-off value than the 25% above appears justified. In the current study bitches were kept in for observation and blood transfusion was administered if the haematocrit decreased below 28%. This haematocrit is higher than the 20% which is mostly used in clinical practice for dogs, corresponds with the haematocrit below which oxygen delivery to tissues may become compromised and agrees with general practice in humans (Rohilla et al., 2011). In one study it was suggested that blood loss during CS should be replaced with 3 ml of crystalloid for every 1 ml of blood lost (Smith, 2007). The author did not explain how the amount of blood lost should be estimated or determined. In order to achieve and maintain normovolaemia, 3–4 times the blood volume deficit needs to be replaced with crystalloid (Isbister, 1997). However, blood loss cannot only be compensated for by crystalloid fluid administration. The lack of maintenance of cardiac output following resuscitation may play an important role in the development of multiple organ failure after severe haemorrhage (Wang and Chaudry, 1991).

Carson et al. 2002 advocates restrictive rather than liberal transfusion thresholds (triggers) because of the many potential dangers associated with transfusions in man. Restrictive triggers are those in the lower haemoglobin content range whereas liberal triggers are in the higher range of–100 g/L (Carson et al., 2002). Many of these dangers (infectious disease) are not present in animals and therefore direct extrapolation of a trigger established in man may not be appropriate for dogs. Therefore, every blood transfusion needs to be considered carefully and the identification of a critical transfusion trigger is important. Transfusion reactions are described in dogs and it is advisable to determine the blood type and cross match before a blood transfusion is given (Jutkowitz et al., 2002; Holowaychuk et al., 2014;

Tocci, 2010).

Knowledge of very specific guidelines in man (target haemoglobin levels) may prevent unnecessary blood transfusions (So-Osman et al., 2010). Human medical health institutions need a transfusion trigger and institutional guidelines to reduce unnecessary transfusions (Ismail et al., 2014). Such guidelines may be helpful in veterinary science as well. In this study, bitches with a haematocrit below 28% will only be discharged when it is confirmed that their haematocrit is stable and not decreasing. In this study a haematocrit of below 28%was used as transfusion trigger.

d) Amniotic fluid embolus

Amniotic fluid embolus occurs in women when amniotic fluid containing foetal cells and debris enter the maternal circulation through open uterine veins (Fahy, 2001). Classically, amniotic fluid embolus presents shortly after birth and affected women become dyspnoeic and shocky and develop cardiorespiratory arrest. Most patients die from this syndrome despite treatment (Fahy, 2001; Davies, 2001). There are theories supporting amniotic fluid embolus as an embolic event and as an anaphylactoid reaction. When performing research that may have serious ethical consequences, the researcher should have in depth knowledge of all possible adverse events that may occur and influence results. There are anecdotal reports of amniotic fluid emboli in bitches.

2.28.16. Lactation and transfer of colostral antibodies following CS

Breast milk is superior to all milk substitutes because it possesses highly digestible nutritional constituents and immunologic properties (Hurley and Theil, 2011). Maintaining normal lactation following CS should therefore be a priority. There is no literature on lactation following CS or parturition induction in the bitch. Ovariohysterectomy during a CS does not adversely affect lactation (Gaudet, 1985). We also do not know whether CS influences colostrum uptake in the puppies or whether puppies delivered after parturition induction but before natural parturition was due can absorb colostrum or even whether the bitch has normal amounts of colostrum in her milk at that stage. Lactation and colostrum uptake is supposedly normal in these instances because puppy growth and survival is comparable to that of puppies delivered naturally (Baan et al., 2005). Parturition induction with prolactin inhibitors may affect or abolish lactation (Baan et al., 2005). Administration of corticosteroids to the preparturient bitch may interfere with colostral uptake in the puppies

(Gillette and Filkins, 1966). Anecdotal evidence of veterinary obstetricians administering antenatal steroids to pregnant bitches suggest that it does not affect colostral uptake significantly. In dogs, it is known that colostral transfer accounts for approximately 90% of the maternally derived antibodies (Burtonboy et al., 1991; Stoffel et al., 2000), and newborn puppies and kittens must obtain passive immune protection through the ingestion of colostrum within the first hours of life (Day, 2007). Failure to obtain passive transfer of immunity via colostrum can be detrimental to the health and survival of a young puppy (Poffenbarger et al., 1991). The time of colostrum absorption after birth is important. IgG absorption rate was significantly affected by the time of colostrum administration, and the IgG concentrations in puppies' serum 48 h after administration were significantly higher when colostrum was ingested during the first 4 h after birth compared to first ingestion 8–12 h or 16–24 h after birth (1.68 ± 0.4, 0.79 ± 0.07 and 0.35 ± 0.08 g/L, respectively; $p < 0.001$) (Chastant-Maillard et al., 2012). In the current study, all efforts were made to ensure discharge of the bitch within 2 h of surgery to ensure arrival at home before this critical time cut-off.

Efficacy of colostral antibody transfer can be measured in serum (German et al., 1998). Lack of sufficient immunoglobulins may be remedied by administration of oral adult dog serum as a source of protective immunoglobulins (Poffenbarger et al., 1991).

Although many theriogenologists use various galactagogues (substances that can induce, maintain and increase milk production), their efficacy has not been evaluated in bitches. Much of the information on agents used as galactagogues in bitches is an extrapolation of what is known in the human literature. In woman, low doses of acepromazine (0.01–0.02 mg/kg) has been used postoperatively to allow milk let-down and promote lactogenesis (Zuppa et al., 2010). Metoclopramide (Ehrenkranz and Ackerman, 1986) and sulpiride (Gabay, 2002) are proven galactagogues in humans and are ones used by veterinary surgeons for the same reason.

Bitches are thought to voluntarily inhibit parturition due to excessive stress and excitement (Wykes and Olson, 2003) and that it may negatively affect lactation. Stress and pain are also suspected to suppress lactogenesis. It is therefore suggested that controlling post CS pain in the bitch is important (Gendler et al., 2007). Some galactagogues have anxiolytic properties as well and are therefore preferred over those that do not. In this study sulpiride (50 mg/bitch

total dose for bitches of 20–40 kg and 100 mg/bitch total dose for bitches over 40 kg) per os was used bid for 5 days routinely.

2.28.17. Post-operative care

Postoperative care of the bitch is crucial. Since the bitch will be disconnected from the electronic monitoring devices upon leaving the theatre, direct visual inspection of the bitch in the recovery room is vital. Respiration rate, mucous membrane colour and capillary refill time should be assessed during this time (Flaherty and Musk, 2005). Fluid therapy should continue until the bitch is considered haemodynamically stable characterised by demonstration of normal heartbeat, capillary refill time and colour of mucous membranes. When the coughing reflex returns the endotracheal tube should be removed and an increased vigilance by the observer until the bitch is breathing without difficulty (Greene, 2002). This is of extreme importance in brachycephalic breeds. The bitch should be monitored for postoperative haemorrhage. It was shown that administration of oxytocin during and after CS in women decreases the potential of post-operative haemorrhage and promotes the expulsion of the placenta (Casey and MacDonald, 1993). Likewise, some veterinary surgeons administer oxytocin during and after CS in bitches, believing that doing so has the same effects as in women CS (Smith, 2012). Postoperative NSAID on the day of surgery only (Mathews, 2008) and antibiotics (5 d) may be dispensed. The routine use of antibiotics for routine surgery where there exists no prior infection however is controversial. Once the bitch is considered stable and ambulatory the bitch may be discharged and placed in the care of the owner within 2 h of surgery.

2.28.18. Placental separation

Many questions regarding placental separation remain unanswered. The exact mechanism by which placental separation (placental release) is facilitated in the bitch is unknown but a decrease in progesterone and an increase in PGF2α precedes placental separation. Changes in the concentrations of oestrogen, progesterone, relaxin, cortisol and others are thought to play a role (Steinetz et al., 1987; Klonisch et al., 1999). The temporal relationship between placental separation and parturition as well as puppy maturation is also not known. It is however known that erroneous (premature) timing of CS intervention results in delivery of preterm non-viable puppies, failure of placental release and increased risk of serious uterine haemorrhaging (Smith, 2007). In prolonged gestation, puppies may be found dead with

placentas already detached (Irons et al., 1997). It can be speculated that the canine placenta has a predetermined exact lifetime (whereas the canine corpus luteum may have a lifetime more variable), after which it separates or becomes dysfunctional. It is of interest to know whether pharmaceutical intervention may aid in placental separation, puppy maturation and survival. It is speculated that betamethasone will be useful in this respect. In the study where premature puppies were delivered following parturition induction (Fontbonne et al., 2009), the aglepristone administration before parturition was neither able to prevent prematurity nor able to induce the release of lung surfactant (induce maturation), as indicated by the post-mortem examination.

2.28.19. Removal of placentas during caesarean sections

In woman, it is standard procedure to remove the placenta during CS in all cases except for cases where abnormal placentation is encountered. In such cases the conservative management involves leaving the placenta (complete) in situ. This can be effective and fertility can be preserved but should only be considered in highly selected cases when blood loss is minimal and if there is desire for fertility preservation (Timmermans et al., 2007), otherwise hysterectomy is advised (Fawcus and Moodley, 2013). In woman it was demonstrated that manual removal of the placenta (by introducing a hand into the uterine cavity, creating a cleavage plane between the placenta and uterine wall and then grasping the placenta and removing it) during CS was associated with greater blood loss than when placentas were delivered "spontaneously" (by gentle traction on the umbilicus, massage of the uterine wall and rapid iv infusion of oxytocin placed in ringer lactate) (Hidar et al., 2004; Anorlu et al., 2008). Unfortunately, the word spontaneous as used by Hidaret al., (2004) has been misinterpreted by others (Traas, 2008b) as if the placenta was left inside the uterus of the women and allowed to pass spontaneously through an open cervix sometime in the post-operative period.

The mean decline in haematocrit during CSs in women was greater in the perioperative period when placentas were removed manually compared to "spontaneous" passing of placentas (5.57 ± 3.86 and 2.65 ± 2.67, respectively; $P < 0.01$) (Hidar et al., 2004; Anorlu et al., 2008). Endometritis occurs less frequently in women when placentas were allowed to pass "spontaneously", instead of being removed manually during surgery (Baksu et al., 2005; Hidar et al., 2004; Morales et al., 2004). Hidar et al. (2004) showed that the incidence of postpartum infectious morbidity was significantly greater in the manual group than in the

"spontaneous" group (relative risk 15.8, 95% CI 2.2–117.5). Amongst veterinary specialist theriogenologists, there appears to be consensus (CAFE-Reprod, Canine and Feline reproductive internet forum) that if the placentas are tightly adhered to the uterine wall, they should be left to pass spontaneously. However, it has been speculated that because placental types differ between humans and domestic carnivores, a randomized clinical trial is needed to provide definitive advice regarding removal of tightly adhered foetal membranes in the bitch (Traas, 2008b).

Routine removal of placentas increases the chances of diagnosing placenta abnormalities and identifying peculiar placental configurations, such as foetuses sharing a placental site.

Another important consideration regarding whether to leave the placenta *in situ* during CS in the bitch is whether the cervix is open at the time of surgery or not. It is suggested that if the cervix is not open, the membranes should be removed gently and slowly with uterine massage. If the membranes are tightly adhered then removal should be delayed until all neonates have been delivered from both horns but nevertheless removed (Traas, 2008b). It is not known what the effect is of failing to remove the placenta in bitches of which the cervix is closed at the time of surgery for CS.

In women, retained placental tissue and membranes after birth is associated with serious complications including post parturient haemorrhage, infections and mortalities (Chandraharan, 2012; Carroli et al., 2008). It was suggested that retained placentas prevent adequate uterine contraction leading to uterine atony and post parturient haemorrhage (Oyelese and Ananth, 2010). In the current study, all placentas were removed with each foetus irrespective of timing of CS.

2.29. The neonate

2.29.1. Neonate survival from delivery until weaning

The rate of stillbirth and neonatal death is relatively high in dogs with mortality ranging from 17% to 30% during the first 8 weeks of life (Indrebo et al., 2007) and asphyxia being the leading cause of death (Andersen, 1957; Van der Beek et al., 1999). Stillbirths and deaths during the first week were responsible for most these losses. In a large study involving 10,810 litters, perinatal mortality occurred in 24.6% of litters (Tønnessen et al., 2012). In the same study, 4.3% of puppies were stillborn and another 3.7% died within a week,

totalling 8% in the first week, and only another 1% died from 8 d to 8 weeks of age. This indicates that the 7-day survival rate, which is used in the current study, is a good parameter to use as it is well correlated to the puppy survival rate at weaning age. Another study indicated that the majority (69.2%) of all puppy losses occurred during the first 3 d of life (Böhm and Hoy, 1999). The puppy death rate in other studies was 7.6%–11.9% (Linde-Forsberg and Forsberg, 1989; Linde-Forsberg and Forsberg, 1993; Gavrilovic et al., 2008; Böhm and Hoy, 1999).

One study evaluated influence of method of delivery on neonate survival. In 193 puppies from 42 litters, 65 born by spontaneous delivery, 66 by assisted delivery and 62 by CS, the percentage of stillbirths were 14%, 20% and 8% respectively (Veronesi et al., 2009). In another study, survival rates immediately, 2 h and 7 d after delivery were 92%, 87%, and 80%, respectively, for puppies delivered by CS (n = 3,410) and 86%, 83%, and 75%, respectively, for puppies delivered naturally (n = 498) (Moon et al., 1998). In 76% of litters delivered by CS, all the puppies were delivered alive (Moon et al., 1998). The maternal mortality rate was 1% (Moon et al., 1998).

It is important to note that there may be a difference in outcome when surgery was performed on an elective basis or in an emergency. In the bitch the likelihood of all the puppies being alive if the CS was performed on an emergency basis or if the bitch delivered puppies naturally was decreased (Moon et al., 1998; Moon et al., 2000) but no specific data were provided by these authors. Likewise, in humans, the neonate survival of an intrapartum CS is less than in planned CS (Ben-Meir et al., 2005). This is an important factor to consider and certainly supports performing elective CS in dystocia-prone breeds. CS are often required in obstetrical emergencies in bitches (Biddle and Macintire, 2000). From this it is clear that the expected puppy survival rate following carefully planned and timed CS is similar or better than the puppy survival rate following natural delivery.

Breed has important implications in the current study. This is because the current study focuses on breeds predisposed to dystocia. The current study will almost exclusively use English Bulldogs and Boerboels, which is a very large mastiff like breed with adults frequently reaching body mass of 60–86 kg. The Boerboel also frequently has very large litters. In large breeds, the litter size was positively correlated to stillbirth rate and stillbirth rate was 10.9% and mortality rate of live born puppies till 3 weeks of age was 6.7% (Indrebo

et al., 2007). Similar findings were made in a medium-sized breed (beagle). Puppy losses were 4.8% in litters with up to five live-born puppies and 12.1% in litters with a size of 9–12 alive-born puppies (Böhm and Hoy, 1999).

Primary uterine inertia can result because of lack of initiation of parturition as is suspected to be the case in singletons (Johnson, 2008a; Munnich and Kuchenmeister, 2009), or because of overstretching of the uterine wall in very large litters (Bennett, 1980) as is suspected to be the case with the Boerboel breed (personal observation).

Although one study reported that English Bulldogs had similar a stillbirth rate (1.5%) and death rate (7%), resulting in 8.5% mortality before 1 week of age as those of other breeds (Tønnessen et al., 2012), there are numerous studies showing that English Bulldog puppies are at increased risk of being stillborn or being born with defects requiring euthanasia or having poorer survival rates at 2 h and at weaning age compared to other breeds. In a study involving English Bulldogs, 13% of the puppies were stillborn and 8.2% of the puppies delivered alive were deformed (mainly palatoschisis and anasarca) (Wydooghe et al., 2013). In addition, Wydooghe et al. (2013) reported that 10% of the puppies died before the age of weaning. This study is consistent with others studies showing that English Bulldog puppies were at increased risk compared to all other breeds for suffering from poor vigour after CS (Moon-Massat and Erb, 2002), having lower survival rates at 2 h than other breeds following CS delivery (Moon et al., 2000) and also having higher puppy mortality (Moon et al., 2000) than other breeds.

2.29.2. Puppy vigour and vigour scoring

Foetal depression following dystocia and CS has two primary causes; the first (and often most important) cause is hypoxia and the second is depression from anaesthetic agents given to the bitch (Traas, 2008a) as is the case in women (Downing et al., 1976).

It has been observed that Apgar scores from puppies delivered by CS are lower than those in eutocia and that puppies delivered by CS initially present with depression (Crissiuma et al., 2010; Silva et al., 2015). It was also suggested that performing a CS prior to the onset of uterine contractions, thus performing a preparturient CS, likely prevents the rise in foetal catecholamine concentrations and, ultimately, blunts the final stimulus for lung maturation (Silva et al., 2015).

To have a base for objective comparison, assessment of puppy vigour and survival associated with a CS, a protocol that includes the method and duration of anaesthesia should be established. This allows for the measurement of differences in puppy vigour and survival in relation to changes in protocol. It is also important to establish these baselines so that our results may be compared to those published. In one such study, puppy survival at birth, 2 h after birth and at 7 d was elected (Moon et al., 2000). These specific times were selected because they were considered to more accurately reflect effects of perioperative conditions or factors on puppy vigour and survival and exclude causes of death related to infectious causes and other factors which are common in the neonatal period. The current study elected identical specific time periods for the same reasons.

It is important to establish whether all the foetuses are alive and well prior to intervention. This will avoid a false overestimation of foetal deaths due to the intervention. Fontbonne et al. (2009) used ultrasonography to assess the presence of any dead foetuses before the start of treatments or CS. It is however not sure how accurate this method is but attempts to assess presence of dead foetuses will also be made in this study using ultrasonography.

The Apgar score was initially developed to assess viability and prognosticate survival in infants (Apgar, 1966) and has since been used to assess the condition and prognosis of newborn infants throughout the world for almost 50 years. (Casey et al., 2001).

The Apgar scoring system is an easy and reliable method for evaluating both human and animal neonates and correlates reasonably well with umbilical lactate which in turn is well correlated to neonatal survival (Groppetti et al., 2010). The score reflects a temporary condition of the neonate and, therefore, is useful in verifying the effectiveness of interventions. Apgar score is however not used widely in veterinary medicine. One study described a modified Apgar scoring system (Veronesi et al., 2009). This scoring system evaluates heart rate, respiratory effort; reflex irritability, motility and mucous membrane colour and a score of 0, 1 or 2 is allocated to each of these parameters according to Table 2.1, which are summed, thus ending up in a score between zero and 10.

The time after birth that such evaluations are performed is important. Although in early studies a one-min score was used in humans (Apgar, 1966), it was later demonstrated that the five-min score is more predictive of survival than the one-min score (Casey et al., 2001). Apgar score correlated well to short-term survival in puppies: Puppies that died within 2 h

after birth more frequently had scores of 4–6 than 7–10 ($P < 0.01$) or zero to three than 7–10 ($P < 0.0001$) (Veronesi et al., 2009). In most studies in puppies, the evaluations were performed 5 min after birth (Doebeli et al., 2013; Veronesi et al., 2009; Groppetti et al., 2010) but another evaluated Apgar scores at 5, 15 and 60 min after delivery (Doebeli et al., 2013). This differs from the 2-min evaluation performed on puppies by Moon-Massat and Erb (2002). In humans, this scoring method is easily performed as there is usually only one foetus to evaluate. In polytocous species, this is not so simple. There is the question of when to start the evaluations. In the ideal world, one could perform the Apgar scores individually starting the count when each puppy is delivered. This is however a practical problem in large litters because there is a considerable time delay between the delivery of the first puppy and the last puppy. For the purposes of this discussion the delivery time refers to the time it takes to free the puppy from the uterus and remove its placenta. In the two groups combined, the average time it took to deliver an individual puppy was 42.6 s. The delivery times increased depending on how far the puppy was removed from the incision line in the uterus. What this implies is that in the litter that produces say 10 puppies, the time delay between freeing the first puppy until the last puppy can be around 10 min. In English Bulldogs the delivery times may be increased if there are several anasarca puppies to be delivered. Assigning Apgar scores to individual puppies of a large litter at a specific time after delivery would require a very large staff. Further, the puppies would have to be kept separately and the exact times that they were delivered recorded so that the Apgar score can be evaluated at the correct time for each puppy without getting confused amongst a group of wriggling puppies and lots of assisting hands. There is no mention of how other workers overcame this problem in dogs (Doebeli et al., 2013; Veronesi et al., 2009; Groppetti et al., 2010). In the current study this problem was overcome by defining the point of evaluation as 15 min after delivery of the last puppy. This is a convenient time because by then the surgeon will have completed surgery, the bitch is awake and all the puppies have been processed. The processing of puppies refers to administration of atipamizole (Antisedan, Zoetis) to the puppies immediately after birth to take effect to reverse the effects of medetomidine premedication of the bitch, tying off the umbilicus, disinfection of umbilicus, drying of puppies, and placing them in an incubator.

Table 2.1

Apgar score chart adopted from Veronesi et al. (2009)

Parameter	Score		
	0	1	2
Heart rate	<180 bpm	180–220 bpm	>220 bpm
Respiratory effort	No crying and respiratory rate <6	Mild crying and respiratory rate 6–15	Crying and respiratory rate >15
Reflex irritability	Absent	Grimace	Vigorous
Motility	Flaccid	Some flexion's	Active motions
Mucous colour	Cyanotic	Pale	Pink

It is proposed that newborn puppies require a short period of time to adapt to extra uterine life, during which spontaneous respiration and organ adaptation of the functions previously performed by the placenta can be established (Johnston et al., 2001b) and this explains why poor scores taken at birth may vastly improve when taken again at 60 min (Vassalo et al., 2015). The time from induction to delivery of the first puppy may also impact on puppy vigour. This is because if this time is less than 15–20 min, the foetus has not had enough time to diminish the effect of the induction agent by redistribution (Short and Bufalari, 1999; Funkquist et al., 1997). This is another reason for only performing Apgar scores after 15 min following delivery.

In this study, the Apgar scores were assessed 15 min after the last puppy was delivered and notes on status of puppies were made regarding presence of birth defects, meconium or uteroverdin staining and stillbirths as well as survival at 2 h post-delivery and 7 d post-delivery.

2.29.3. Estimating neonatal maturity

Puppies born naturally or delivered by CS before they were full-term are premature. Estimation of neonatal maturity is based on the subjective assessment of the amount and extent of hair cover on the ears, face, feet and trunk (Mosier, 1978; Smith, 2007). These puppies are usually weak and have difficulty breathing. There may however be degrees of prematurity that are not easily identifiable by these criteria. In the absence of accurate parturition dates it is not possible to categorically confirm that a litter is full-term. There is an accelerated growth rate towards the end of gestation in dogs (Evans and Sack, 1973; Salazar and Yllera, 1991; Moriyoshi et al., 1996). From this follows that premature puppies

might be smaller than full-term puppies.

Comparison of birth weights, crown-rump lengths and other parameters may give important clues in humans but are not accurate enough to allow for establishing maturity or prematurity in puppies (Jabin et al., 2007). Premature delivery is a significant cause of perinatal and neonatal mortality in humans and is defined by the World Health Organization as birth before 37 weeks (Dodd et al., 2008). This stage of pregnancy in woman was speculated to be analogous to 58 d after the LH surge in canine pregnancy (Johnson, 2008a). In humans, the risk of neonatal death is greater at the earlier gestational ages of 32–36 weeks. Again this was speculated to be analogous to 50–57 d after the LH surge in canine pregnancy (Johnson, 2008a). Puppies born that early are usually stillborn or die shortly after birth and show difficulty breathing. In people, the birth of a dead foetus after mid-gestation (20 weeks) is called stillbirth (Fretts, 2005), whereas the same circumstance is often called abortion in veterinary medicine (Kustritz, 2005; Volkmann et al., 2006).

2.29.4. Dysmaturity

If it is assumed that ovulation is almost synchronised (within less than 36 h and probably 12 h), all the puppies in a litter should be of similar age. If this could not be assumed asynchrony of ovulation in a polytocous species would provide a reason for foetuses of discordant ages within the same litter. At birth, dysmature puppies have less developed hair cover that does not extend to the ear tips and extremities and are usually, but not always, smaller than those that are not dysmature. These puppies will be of the same age but their foetal development did not progress at the same rate as that of their litter mates or of those in a normal pregnancy. Dysmaturity is speculated to be caused by some uterine or other factor that impaired the oxygen or nutrient supply of these puppies. Dysmature puppies are also not premature because they were born following a gestation of normal duration. These small and or immature looking puppies are termed dysmature puppies (not fully developed but yet full-term). Desmature foetuses were described by Dewhurst et al. (1972) in man. In man it was established that there was a positive correlation between smaller size as adult and being small at birth (Sann et al., 1986). It is not known whether the same is true for puppies. It is the observation of the author that dysmature puppies have vastly decreased survival rates when compared to mature puppies.

2.29.5. Runts and weaklings

All observations on runts are anecdotal. Runts are small looking puppies that seem to be weaker, grow slower, mature slower and are usually in poor condition. The disparity in size is frequently exaggerated as time goes by, as the stronger litter mates bully the weakling at feeding times. These puppies should not be confused with dysmature or premature puppies. Most important is that runts should not be confused with normal small puppies. One worker found that the small size of runts in pigs was correlated to small placental size suggesting placental insufficiency (Wootton et al., 1977). There is no information from the literature about the variation (or lack of variation) of foetal size before or at the time of birth.

2.29.6. Neonatal care of puppies and its effect on puppy vigour following CS

Following CS, newborns are severely depressed in contrast to normal delivery and may require resuscitation (Traas, 2008a).

To have a base for objective comparison, assessment of puppy vigour should be defined post CS. Depression in neonates at the time of CS may result from anaesthetic drugs and hypoxia of the foetus (Pascoe and Moon, 2001; Kramer, 2008). The hypoxia may be induced by the procedure, due to dystocia or lack of lung maturation at birth due to prematurity of the puppies.

With the onset of spontaneous respiration, any inhalant anaesthetic in a neonate will be quickly eliminated (McLeod et al., 1998). Administration of particularly opioids is known to cause neonatal depression. Their effects may be treated effectively by the administration of naloxone immediately following CS delivery (Schmid and Russe, 1987). The extent of neonate depression associated with the CS procedure is both time and dose dependent, accentuating the need for keeping surgery and anaesthetic time to the minimum. Drying and rubbing puppies after delivery not only helps with maintaining heat but also aids in stimulating the puppies respiratory and cardiovascular systems and helps reduce periparturient neonatal mortality (Ryan and Wagner, 2006b). However, newborns remained hypothermic regardless of the type of delivery, whether or not subjected to artificial heating (Vassalo et al., 2015).

The airways should be cleared of fluids (Traas, 2008a). Swinging puppies to void fluids from the airways is frequently practiced but has no benefit over careful suction and can be

detrimental if the head and neck are not correctly supported (Moon et al., 2001). Vocalization is a good sign that the lungs are well expanded. Oxygen tents or masks in cyanotic puppies may be helpful (Jutkowitz, 2005; Traas, 2008a) and will be used for puppies that need this. Oxygen induced retinopathy is a well-recognised adverse effect of oxygen supplementation to human neonates (McLeod et al., 1998). It is not known whether sustained oxygen supplementation has adverse effects in puppies. Routine use of doxapram hydrochloride in neonatal puppies delivered by CS has been and is still practiced by many today (Holladay, 1971) but was not used in this study. To all the CSs reported in this thesis, atipamizole (Antisedan, Zoetis) was administered to the puppies sc immediately after birth to reverse the effects of medetomidine premedication of the bitch. The umbilical cord was tied off 6–9 mm away from the abdominal wall using catgut and disinfected using 10% povidone-iodine solution.

The need to address hypothermia in the puppy is well recognised (Smith, 2007; Raffe and Carpenter, 2007; Johnson, 2008b). Transfer to incubators set at 32.2°C has been advocated (Traas, 2008a; Lennoz-Roland, 1998). In this study the temperature of the incubators was set at 34–35°C and no adverse effects are seen therewith

Puppies should be weighed because newborns with a low birth weight require greater attention from both the veterinary obstetrician and the owner to ensure survival (Vassalo et al., 2015).

Finally, the puppies and bitch should be reunited as soon as clinically possible (Kushnir and Epstein, 2012) provided continued monitoring is possible until it becomes evident that there is good maternal care.

2.29.7. Meconium stained puppies and meconium aspiration syndrome

Meconium is the earliest stool of a mammalian infant. Unlike later faeces it is composed of materials such as intestinal epithelial cells, mucus, amniotic fluid, bile and water (Warren and Anderson, 2010). Any factor that results in foetal stress can be a risk factor for passing meconium in utero (Warren and Anderson, 2010). Chorioamnionitis, enhancement of growth of bacteria in amniotic fluid by serving as a growth factor, inhibiting bacteriostatic properties of amniotic fluid and meconium aspiration syndrome are all sequelae (Siriwachirachai et al., 2010). It was established that preventative antibiotic therapy of

woman suffering from meconium stained amniotic fluid helped prevent maternal and neonatal infections (Siriwachirachai et al., 2010). In man, meconium aspiration syndrome is a well-recognised syndrome leading to fatalities and lung disease in neonates (Warren and Anderson, 2010; Harris et al., 2010). Experimental meconium aspiration causes significant cell death and inflammation in rabbit newborns and the lesion of which are more severe than that caused by aspiration of milk (Zagariya et al., 2010). Treatment of meconium aspiration syndrome in man involves antibiotic therapy, corticosteroid therapy (Yang, 2010), surfactant therapy (Khammash et al., 1993), oxygen therapy (Speer et al., 1990; Dunn et al., 1990) and pentoxifylline (Siriwachirachai et al., 2010) therapy.

Puppies were used in an experimental induction of meconium aspiration syndrome to evaluate the efficacy of corticosteroid (prednisolone) therapy. In this study, it was shown that prednisolone reduced physiological and histological changes in puppies with meconium aspiration syndrome and that a 30 mg/kg dose was more effective than 2 mg/kg (Kirimi et al., 2003)

Foetal stress in the bitch can also be assessed by examining the foetal fluids and foeto-placental units (Lopate, 2008). Increases in the echogenicity of the foetal fluids may indicate passage of meconium (Zone and Wanke, 2001) or haemorrhage into the foetal fluids due to premature placental separation (Lopate, 2008). Meconium-stained amniotic fluid and meconium-stained puppies are common in dogs and is more prevalent in breeds where the risk of CS is higher (personal observation).

Considering that extensive meconium staining of puppies and amniotic fluids is a very frequent finding in intra-partum CS, meconium aspiration syndrome in puppies may have been overlooked in veterinary medicine. Many of these puppies are hypoxic, weak, suffer from respiratory distress, do not cry soon after delivery and frequently die (personal observation). The incidence of this syndrome in naturally born puppies will be difficult to assess as the bitch lick the puppies after birth. Based on the observation that meconium staining of foetuses and amniotic fluid is more common following dystocia, it is speculated that peristaltic movements are accelerated and passing of meconium is stimulated in these stressed puppies. In man, meconium excretion in the amniotic fluids lead to various deleterious effects. This discussion was necessary because there is no information on meconium aspiration syndrome in the dog and the syndrome is likely to exist also in this species.

2.29.8. Respiratory distress syndrome and surfactant replacement therapy

Respiratory distress syndrome is a serious complication in preterm babies and the primary cause of early neonatal mortality in man (Stableman, 1975; Roberts and Dalziel, 2006). The more premature the infant, the less the surfactant production and the higher the probability for respiratory distress syndrome (Von Neergaard, 1929). Respiratory distress syndrome develops because of insufficient surfactant development in immature foetal lungs. Lung surfactant is produced by type II alveolar cells as a mixture of phospholipids, surfactant proteins, and neutral lipids (Jobe, 1986). Surfactant lowers alveolar surface tension and is crucial for the prevention of alveolar collapse and improves mucocilliary clearance (Von Neergaard, 1929; Avery and Meads, 1959). Surfactant deficiency therefore causes neonatal respiratory distress syndrome (Jobe, 1986). It is possible to test for surfactant from amniotic fluid to assess foetal lung maturity (Hallman et al., 1993; Merritt et al., 1991). In contrast to human medicine, use and success of surfactant testing or surfactant replacement therapy remain limited in veterinary medicine (Christmann et al., 2009). Lung surfactant has been studied in large animals as models of human disease. However, only a few reports exist on lung surfactant alterations in naturally occurring respiratory disease in large animals (Christmann et al., 2009; Walters, 1984). In small animals, there are only some publications in laboratory animals (Halliday, 1996) and no information on the use of surfactant in puppies. The composition of surfactant from the lungs of neonatal puppies dying from “fading puppy complex”' differs from that of neonates dying from other causes. Surfactant from fading puppies contained significantly less phosphatidylcholine than did the surfactant from other puppies and therefore abnormal surfactant may be implicated in fading puppy complex (Blunden et al., 1987). Abnormal surfactant has been implicated in the pathogenesis of sudden infant death syndrome in human infants (Blunden et al., 1987).

The treatment of respiratory distress syndrome with surfactant in humans has been extensively reviewed and proven to vastly improve neonate survival (Berry, 1991; Stableman, 1975; Zola et al., 1993; Yuksel et al., 1993; Kattwinkel et al., 1993; Abbasi et al., 1993; Gizzi et al., 2010). Likewise, administration of corticosteroids to the mother antenatally improves outcome in human neonates with respiratory distress syndrome (Roberts and Dalziel, 2006). It was also established that antenatal treatment with corticosteroids does not increase the risk of chorioamnionitis, puerperal sepsis or death of the mother (Roberts and Dalziel, 2006). Therapeutic or rescue administration of surfactant

is indicated even in full-term infants with respiratory distress (Horbar et al., 1993) as is oxygen supplementation (Speer et al., 1990; Dunn et al., 1990). It is not known whether this practice may be of value in puppies. It is important to note that administration of surfactant without clinical merit is not advised because there may be procedural complications as plugging endotracheal tube by surfactant (Berry, 1991), haemoglobin desaturation (Merritt et al., 1991) and lung trauma because of mechanical ventilation and over inflation (Goldsmith et al., 1991).

This discussion was necessary because as with meconium aspiration syndrome in the dog, no research on respiratory distress syndrome has been conducted in the dog. It is unknown whether these syndromes exist in the domestic bitch. It is also unknown whether respiratory distress syndrome may be expected in premature puppies delivered by CS, parturition induction that is performed preterm or in dysmature puppies and whether respiratory distress syndrome may play a role in the fading puppy enigma as reported (Blunden et al., 1987). Respiratory distress syndrome should be considered a field of interest for future research in dogs.

2.29.9. Uteroverdin-stained puppies

Uteroverdin in the dog was first described by Lemberg et al. (1931) and refers to the green pigment of the marginal haematomas from the placenta. Little is known about the causes of uteroverdin staining of puppies. The observation that these puppies frequently suffer from respiratory distress syndrome, have poor post-delivery Apgar scores and are suspected to have a higher mortality rate than their unstained litter mates, indicates that its occurrence is a pathological syndrome associated with poor outcome. This notion is supported but not confirmed by the anecdotal observation that many stillborn puppies delivered by CS have green discolouration.

This discussion was necessary because this uteroverdin stained puppies and fluids are a frequent phenomenon at CS and no information on its causes or treatment is available.

2.29.10. The antenatal administration of corticosteroids

a) The antenatal administration of corticosteroids in pregnant women

Caesarean section is a risk factor for the development of mainly two neonatal respiratory complications, mostly respiratory distress syndrome but also transient tachypnoea of

newborn babies. Both these conditions may occur in term and preterm infants (Morrison et al., 1995). Infants born at term by caesarean delivery are more likely to develop respiratory morbidity than infants born vaginally and this risk increases furthermore for the subgroup of children delivered after elective CS before onset of parturition (Gerten et al., 2005). This risk also decreases with advancing gestational age. In woman, elective CS should normally be performed at or after the 39th week of gestation to reduce respiratory morbidity and, if done before the onset of the 39th week, corticosteroids should be administered to all the mothers to reduce the risk of respiratory morbidity in the babies (Hansen et al., 2008). During parturition and at birth, the mature lung switches from active chloride and fluid secretion to active sodium and fluid absorption (O'Brodovich, 1996). The reduced incidence of transient tachypnoea in the steroid group is consistent with the hypothesis that corticosteroids allow the lung to switch from fluid secretion to fluid absorption. Without another source of corticosteroid, elective CS will disrupt this process (Rimmer and Fawcitt, 1982).

Foetuses that were not exposed to the process of parturition lack the alveolar fluid drainage mechanism due to a failure of lung epithelial sodium channel activation (Jain and Eaton, 2006). Glucocorticoids appear to increase the number and function of sodium channels as well as the responsiveness to catecholamines, thus providing a rationale for the exogenous administration of glucocorticosteroids in cases of elective caesarean (Jain and Eaton, 2006).

Both the administration of antenatal betamethasone to the pregnant mother as well as delaying delivery until the 39 nth week, reduce admissions to a special care baby unit with respiratory distress after elective CS at term (Stutchfield et al., 2005). Liggins et al. (1973) was the first to report the beneficial pulmonary effects in foetuses after maternally administered corticosteroids. Since then betamethasone and dexamethasone have been employed for accelerating foetal lung maturation for women at risk of preterm birth (Brownfoot et al., 2007).

A period of 24 h to 7 d should lapse after woman received the second antenatal administration of corticosteroids to have the maximum effect in reducing respiratory distress syndrome (Roberts and Dalziel, 2006). Antenatal corticosteroid use still reduces neonatal death within the first 24 h and therefore should still be given even if delivery is expected within this time (Roberts and Dalziel, 2006). This may be of practical significance in the dog. Antenatal corticosteroids have no known benefits for the mother (Roberts and Dalziel,

2006) nor is a single course of antenatal corticosteroids (two doses 12–24 h apart) associated with any significant short-term adverse effects in the mother or the foetus. However, caution should be exercised when giving corticosteroid therapy to women with systemic infection including tuberculosis or sepsis. Experiments with animals and cohort studies have provided a body of evidence suggesting that repeated doses may further improve lung maturation but may be accompanied by deleterious effects on the developing brain and other organs in man (Newnham and Jobe, 2009). Therefore, antenatal corticosteroid treatments should be restricted to single-course treatments (Roberts and Dalziel, 2006; Mwansa-Kambafwile et al., 2010; Newnham and Jobe, 2009; Kumar, 2007). One researcher reported promising results for prophylactic corticosteroid administration prior to full-term elective CS. This trial was conducted because human infants delivered at term by elective caesarean delivery are more likely to develop respiratory morbidity than infants born vaginally (Sotiriadis et al., 2009). Two antenatal betamethasone doses of 12 mg each, administered intramuscularly 24 h apart during the 48 h before elective CS was effective in reducing incidence of respiratory distress syndrome at term (Stutchfield et al., 2005). Betamethasone 12 mg given twice intramuscularly, 24 h apart or dexamethasone 6 mg given intramuscularly four times 12 h apart are the most commonly used protocols used (Stutchfield et al., 2005).

In women, minor side effects have been reported in a small number of patients receiving betamethasone. They included generalised flushing, nausea, injection site tenderness and insomnia (Stutchfield et al., 2005). The scientific basis for use of prenatal steroid therapy in preparing the human foetus for premature delivery (before the 37th week) is extensive and convincing (Ballard and Ballard, 1995). Similarly, in numerous animal species, it has been documented that precocious maturation of lung and other tissues occurs after administration of glucocorticoids to the dam (Liggins, 1976). Therefore, prenatal steroid therapy may mimic the effect of endogenous glucocorticoids in animals as it does in man.

b) The antenatal administration of corticosteroids in the bitch

It has already been established that maternal cortisol (and probably also foetal cortisol) concentration and maternal PGF2α concentration increase before parturition in the bitch (Concannon et al., 1978; Kowalewski et al., 2010). The same mechanism has been confirmed in several other mammalian species (Challis and Thorburn, 1975; Currie and Thorburn, 1977; Flint et al., 1978). Whilst glucocorticosteroids are effective abortifacients

in some species (Anderson et al., 1975), they are not in the bitch. In this species, bitch lengthy treatments with dexamethasone were required and worked better if given twice a day (Zone et al., 1995; Wanke et al., 1996). In contrast one worker administered betamethasone (single dose of 0.5 mg/kg) (Celestone Soluspan®) at 55 d after ovulation and then a CS was scheduled for day 63 (Vannucchi et al., 2012). However, in this study the treated dams showed precocious signs of parturition 3 d (76 h) after the corticosteroid treatment and CS were performed at 58 d after ovulation. Despite apparent preparturient birth, the puppies showed acceptable Apgar scores 120 min after birth albeit slightly lower than the controls (Vannucchi et al., 2012). It might be that the bitch may be more sensitive to the effects of glucocorticosteroids as parturition approaches or other factors may have played in role in the study by Vannucchi et al. (2012).

Development of the foetal respiratory system includes both pulmonary growth and maturation. One study concluded that saccular phase of lung development in canine foetuses begins around 57 and 60 d of pregnancy in which surfactant production is believed to occur (Sipriani et al., 2009). In the study by Sipriani et al. (2009) the gestational age was determined according to foetal crown rump measures.

Although the possible value of pre-surgical administration of dexamethasone has not been reported in the bitch, there is anecdotal evidence of its successful use in some practices (Concannon, 2000). The intention is apparently to mimic the natural rise in corticosteroid that likely occurs at normal parturition (Concannon, 2011).

One potential adverse effect of maternal antenatal administration of corticosteroids in bitches is that it does reduce colostral uptake of antibodies in newborn puppies (Gillette and Filkins, 1966), a factor that has no significance in man because of significant trans-placental immunoglobulin transfer. The significance of this is unknown in puppies.

This discussion was necessary because in performing preparturient elective CSs, the delivery of a premature non-viable puppy may not be excluded. Knowledge of use of administration of antenatal steroids could be helpful in these cases. In this study, CSs were performed prior to the onset of parturition. Although the study by Vannucchi et al. (2012) was very small (3 treatment and control bitches each) it showed that in their three treated bitches, induced premature signs of parturition 5 d before the estimated day of delivery and resulted in puppies that had acceptable Apgar scores 120 min after CS.

Chapter 3. Factors affecting stillbirths and the need for caesarean section in bitches

The content of this chapter will be submitted for publication in a different format as an article by K G M De Cramer and J O Nöthling and under the title "Factors affecting stillbirths and the need for caesarean section in bitches".

3.1. Introduction

Management of parturition in bitches is a frequent request by dog breeders in clinical practice and often stems from experience of considerable puppy losses due to dystocia. Dystocia may be due to maternal and foetal causes (Linde-Forsberg and Eneroth, 2000; Wykes and Olson, 2003) and may be due to a cause specific to a particular bitch e.g. pelvic fracture or a specific foetus, e.g. incorrect disposition that was not the result of an obstruction (Linde-Forsberg and Eneroth, 2000). The aim of this study was not to investigate factors affecting individual bitches, litters or foetuses but to look at generic factors (affecting any breed) active at the population level and breed-specific factors that affect stillbirths and the need for CS in bitches. Dystocia occurs in approximately 5% of all parturitions in dogs of a variety of breeds (general obstetric population), but may be considerably higher in some dog breeds (Linde-Forsberg and Eneroth, 2000). Conservative obstetric treatment does not always succeed in delivering all the foetuses and may be associated with stillbirths (Lennoz-Roland, 1998; Munnich and Kuchenmeister, 2009). Bitches treated conservatively for dystocia may still require CS (Michel and Reichler, 2008a; Pretzer, 2008). Retrospective studies showed that 60% to 75% of cases with dystocia required a CS (Stolla et al., 1999; Darvelid and Linde-Forsberg, 1994; Bergstrom et al., 2006; Polster et al., 2005). Only 19.9% (Stolla et al., 1999) or 27.6% (Darvelid and Linde-Forsberg, 1994) of bitches suffering from uterine inertia that were treated with oxytocin and calcium, whelped without further intervention such as whelping forceps delivery or CS. The proportion of puppies born dead during dystocia was 31.9% (Stolla et al., 1999) and in other studies it was suggested that a prompt decision for CS will considerably improve the prognosis for survival of bitch and puppies (Stolla et al., 1999; Michel and Reichler, 2008a,b). Therefore, a strong argument can be made for CS at first indication of dystocia in high-risk pregnancies. High-risk pregnancies are considered those occurring in English Bulldog bitches (Wydooghe et al., 2013), those with a history of dystocia (Stolla et al., 1999), those with known large litters

(Bennett, 1974), those with singletons (Lopate, 2008; Johnson, 2008a), those with small litters (Darvelid and Linde-Forsberg, 1994) and anecdotally, those with a history of prior CS. These arguments are at the core of the decision to plan an elective CS.

The aims of this study were to (1) determine the proportion of bitches that ever required a CS in South Africa in various dog breeds other than English Bulldogs and German Shepherd Dogs, (2) determine the prevalence of CS in German Shepherd Dogs in South Africa, (3) determine the odds of stillbirths in Boerboel bitches allowed to whelp naturally until an emergency CS became necessary (if it did) (4) compare the odds of a bitch requiring another CS after having undergone a CS in her previous pregnancy with those of bitches that did not and compare the odds of stillbirth in these bitches as well, (5) compare the odds of stillbirth of puppies born by emergency CS to that of puppies born by elective CS.

3.2. Materials and Methods

The part of the project pertaining to CSs was approved by the Animal Ethics Committee of the Faculty of Veterinary Science of the University of Pretoria (protocol number V048-14). Permission was obtained from the Faculty of Veterinary Science, University of Pretoria, to publish the survey part of the current study. All respondents including the German Shepher Dog (GSD) Federation of South Africa granted the authors permission to publish the results of the data obtained from them on the condition that kennel name, owner and bitch anonymity is maintained. Respondents were requested to respond only if they had accurate written or electronic records of the data requested. Questionnaires were sent (May 2015) by email to 948 breeders in South Africa for survey 1 and survey 4. A template of the table with examples of how to respond was sent together with the survey question per email. Potential respondents were encouraged to partake in the surveys by awarding book prizes on a random draw basis. Potential respondents were dog breeders representative of all the provinces in South Africa and were all breeders that had voluntarily subscribed to a dog breeders information group. The GSD Federation of South Africa allowed access to their registration records following approval by their executive committee.

3.2.1. Experimental animals

All the animals in surveys 1–5 were privately owned purebred bitches. All bitches hospitalised for management of parturition (Elective CS-group and Emergency CS-group) were housed at an obstetric clinic owned by the author of the thesis and were fed commercial

dry pellet rations and ad-lib water. They were taken out for short walks twice daily and, weather permitting, put in outside dog runs during the day. Most dogs stayed in hospital for 4–6 days. Unless there was a complication, all bitches and their litters were discharged on the same day that the CS was performed but not earlier than 2 hours following surgery.

3.2.2. Survey 1: Prevalence of bitches in South Africa that ever required a caesarean section in various dog breeds other than English Bulldogs and German Shepherd Dogs

In survey 1, the survey question was "List for the breeds (excluding English Bulldogs and German Shepherd Dogs) you have bred with, the number of bitches owned and the number of them that required one or more caesarean sectionss in their reproductive lifespan. You may go back as far you can, provided you have written record of them."

English Bulldogs were excluded as the vast majority, if not all, breeders of English Bulldogs in South Africa, do not attempt natural whelp. German Shepherd Dogs were excluded as they were already included in another survey and including them here as well may have caused double representation. Breeds were classified arbitrarily by the author in size categories as large, medium or small and as brachycephalic or not based on the Wikipedia classification of brachycephalic breeds (Wikipedia, 2017).

A mixed-effects logistic regression was used to assess the effect of size category and being brachycephalic or not on the odds of a bitch ever having undergone a CS. Large dogs and being non-brachycephalic were used as base-line groups to which the others were compared. Breed was used as a as cluster variable. The interaction between size category and being brachycephalic was initially included in the model but then removed.

3.2.3. Survey 2: Prevalence of caesarean section in German Shepherd Dogs in South Africa

Data were extracted from the registration papers of all litters registered with the German Shepherd Dog Federation of South Africa from 1 January 2012 until 31 October 2015. Data extracted were the number of litters registered, whether a CS was required with the delivery and the size of each litter.

A mixed-effects logistic regression model, with method of delivery (CS or natural birth) as response variable and the sequential number of the litter in the data set for each bitch (1

being the first litter of a bitch in the data set, 2 the second, and so on) as factor, and bitch as cluster variable. The first litter was used as baseline group.

Considering only the first and second litters of bitches with two or more litters in the data set, logistic regression was used to compare the odds of the second litter being born by CS vs. being born naturally if the first litter was born naturally, to the odds of the second litter being born by CS if the first litter was born by CS.

3.2.4. Survey 3: Odds of stillbirths in Boerboel bitches allowed to whelp normally

This survey involved a single Boerboel kennel of 156 Boerboel bitches (256 pregnancies) in which the owner only presented a bitch for CS when the owner was concerned that parturition failed to progress normally. Maiden bitches were bred after having reached 14 months of age. This breeder's objective was to select against the high prevalence of CS in the Boerboel breed by not ever again breeding from a bitch that required a CS and selecting prospective breeding bitches exclusively from progeny not stemming from CS. This breeder preferred to breed most bitches only once. Data in this group were recorded over a period of 12 years. The data recorded for each litter were the identity of the bitch, whether the litter was her first or later, number of puppies in the litter, the number of stillborn puppies and the method of delivery (CS or natural whelp).

Litter size was categorised as follows: Category 0 consisted of litters with 8–11 puppies, Category 1 of litters with 1–7 puppies and Category 2 of litters with more than 11 puppies. A mixed-effects logistic regression was used to assess the effect of litter size category on the odds of stillbirth, with Bitch as a cluster variable. Parity, with two levels, namely primipara and multipara, and its interaction with litter size category were included in the model as covariates but then removed.

3.2.5. Survey 4: Effect of method to deliver the previous litter on the odds of caesarean section and stillbirth in the current

This survey involved bitches in which the owners wished to pursue a trial of labour after the bitch had undergone a CS to deliver her previous litter. Respondents had to reply in tabular form by email. A table—see Table 3.1— was sent to the dog breeders information group. Not shown in Table 3.1 are its first six rows, which were populated with various examples of how to respond. The request was: "Complete the table given below for any bitch in which

you attempted natural whelp after she had had a caesarean section in her previous pregnancy. Please do not list the outcome of the first caesarean section.".

Table 3.1
The table sent to breeders to complete, showing only the column headings and the first row for data entry

Name of bitch	Breed	Number of puppies born alive by natural whelp	Number of puppies stillborn by natural whelp	Number of puppies born alive by CS	Number of puppies born dead by CS

TOLAC (trial of labour after caesarean section) was defined as an attempt to allow a bitch to whelp naturally after her previous litter was delivered completely or in part by CS. For each bitch the first or only litter appearing in the data set was delivered by TOLAC and the litter before that (not shown in the data set) by CS. For bitches with more than one litter in the data set the following two criteria applied in addition to the one stated above for TOLAC:

i. The birth of the second or later litter was not by TOLAC (termed "non-TOLAC") if it was born naturally after the previous litter had also been born naturally.

ii. If two or more litters were delivered by CS without any puppy having been born naturally, each of these litters were deemed impossible to classify as TOLAC or non-TOLAC because it was uncertain whether any attempt was made to let the bitch deliver naturally. These litters were termed "uncertain". TOLAC and non-TOLAC were considered successful if all puppies were delivered naturally, irrespective of whether they were born alive or not. TOLAC and non-TOLAC failed if part or all of the litter had to be delivered by CS.

A mixed-effect logistic regression was used to compare the odds of TOLAC births requiring CS to that of non-TOLAC births doing so. Breed was used as a cluster variable. Body size of the breed and brachycephalic status as well as the interaction between them were initially included in the model but then removed.

A mixed-effect logistic regression was used to determine the effect of TOLAC, non-TOLAC and deliveries classified as uncertain on the odds of puppies being born dead. Breed was used as a cluster variable. Size of breed and brachycephalic status as well as the interaction between them were initially included in the model but then removed.

3.2.6. Survey 5: Odds of stillbirth when delivery method is by elective caesarean section as opposed to emergency caesarean section

This survey consisted of two groups of bitches. They were the Elective CS-group and the Emergency CS group. The Elective CS-group bitches were selected from the general obstetric population because of increased obstetric risk. A trial of labour (attempt at spontaneous unassisted parturition) was declined by all the owners of the bitches in this group. The decision on when to perform a CS was based upon the first appearance of any degree of dilatation of the cervix. The management of parturition, anaesthetic protocol, surgery, post-operative care and processing of the puppies was performed as described in Chapter 8. The number of stillborn puppies were recorded. The data in this group was collected over a period of 4 years at one private veterinary clinic acting as an obstetric clinic. The elective CS-group included 390 CSs, of which 120 were performed on English Bulldogs, 176 on Boerboels and 94 on other purebred bitches and 2233 puppies were delivered in total. Singleton pregnancies were excluded from this group because preparturient CSs were performed on them.

The Emergency CS-group included purebreds of any breed which were presented to a private obstetric clinic for emergency CS. The CS was considered an emergency CS based upon exhibiting at least one of the following: green or black vaginal discharge, delivery of a dead puppy prior to presentation, presence of a dead foetus or foetus in distress (heartbeat < 120 beats per minute with an open cervix) on ultrasonography and sustained tenesmus for longer than 0.5 h. The CS was performed as soon as it was established that there was an existing emergency and the anaesthesia and surgery was performed as with the Elective CS-group. The Emergency CS-group included 72 CSs, eight were performed on English Bulldogs, 21 on Boerboels and 43 on other purebred bitches and combined, 458 puppies were delivered from them.

A mixed-effect logistic regression model was used to compare the odds of stillbirth of puppies born by emergency CSs to that of puppies born by elective CS. Method of assistance (elective CS or emergency CS) was included in the model as a factor, and breed was used as cluster variable.

Data analysis was done using Stata 14 (Stata Corp College Station, Texas, USA.). All analyses were done using to avoid negative values for the lower limit of the 95% CI or values

about 1.0 for the upper limit of the probability of CS (Survey 1) or the failure of TOLAC (Survey 4) or the probability of stillbirth (elective and emergency CS) for individual breeds, these limits were calculated according to Wilson (1927) as cited by Michael Thrusfield (2005) Veterinary Epidemiology, Third Edition. Blackwell Science Ltd, Oxford, UK, Page 316.

3.3. Results

3.3.1. Survey 1: Prevalence of bitches in South Africa that ever required a caesarean section in various dog breeds other than English Bulldogs and German Shepherd Dogs

From Table 3.2 it appears that some breeds had a high probability of bitches undergoing a CS at least once during their reproductive life times. These breeds, with the probability between parentheses, are the St. Bernard (1.00), Scottish Terrier (0.75), Boston Terrier (0.62), Chihuahua (0.60), Pug (0.56), Boerboel (0.54), Chow Chow (0.50), Pekingese (0.47), Bull Mastiff (0.47) and Bull Terrier (0.43). Some non-brachycephalic breeds having a higher probability of CS than brachycephalic breeds (English Bulldog excluded). The Scottish Terrier and Boerboel were such breeds.

The last six rows of Table 3.2 summarise the odds and probability of bitches ever having had a CS by breed, size category and being brachycephalic or not. Size category and being brachycephalic did not interact in their effects on the odds of a bitch ever having had a CS. After accounting for the correlation within breeds and controlling for brachycephalic status, medium-sized bitches had 2.33 (95% CI 1.01–5.38) times more bitches ever having had a CS per bitch that did not than was the case in large bitches (P = 0.048). The odds of small bitches ever requiring a CS was the same as the odds for large bitches (odds ratio 1.16, 95% CI. 0.63–2.14, P = 0.63).

After accounting for the correlation within breeds and controlling for size category, brachycephalic bitches had 2.25 (95% CI 1.19–4.27) times more bitches ever having had a CS per bitch that did not, than was the case for non-brachycephalic bitches (P = 0.013).

Table 3.2
Odds and probabilities of caesarean section for dog breeds (other than the English Bulldog and the German Shepherd Dog) and breed categories in South Africa

Breed	Rank[a]	Respondents	Number of bitches		Cephalic index[b]	Size[d]	Odds of CS	Probability of CS
			All	Had a CS				
Airedale		2	18	1	0	L	0.06 (0.01–0.47)	0.06 (0.01–0.26)
Beagle	11	2	30	9	0	M	0.43 (0.19–0.95)	0.30 (0.17–0.48)
Boerboel	1	12	265	142	0	L	1.15 (0.91–1.47)[d]	0.54 (0.48–0.59)[e]
Boston Terrier		3	21	13	1	S	1.63 (0.66–4.01)	0.62 (0.41–0.79)
Bouvier des Flandres		1	35	6	0	L	0.21 (0.08–0.50)	0.17 (0.08–0.33)
Bull Mastiff		2	15	7	1	L	0.88 (0.31–2.50)	0.47 (0.25–0.70)
Bull Terrier	6	10	163	70	0	M	0.75 (0.55–1.03)	0.43 (0.36–0.51)
Cairn Terrier		2	31	7	0	S	0.29 (0.12–0.69)	0.23 (0.11–0.40)
Chihuahua		2	72	43	1	S	1.48 (0.27–1.67)	0.60 (0.48–0.70)
Chow Chow		7	86	43	1	L	1.00 (0.65–1.53)	0.50 (0.40–0.60)
French Mastiff		1	7	3	1	L	0.75 (0.15–3.78)	0.43 (0.16–0.75)
German Shorthaired Pointer		2	15	3	0	L	0.25 (0.07–0.93)	0.20 (0.07–0.45)
Golden Retriever	8	2	30	5	0	L	0.20 (0.08–0.53)	0.17 (0.07–0.34)
Great Dane		9	84	20	0	L	0.31 (0.19–0.52)	0.24 (0.16–0.34)
Irish Setter		3	22	1	0	L	0.05 (0.01–0.37)	0.05(0.01–0.22)
Irish Wolfhound		3	20	8	0	L	0.67 (0.27–1.67)	0.40 (0.22–0.61)
Labrador Retriever	7	8	119	26	0	L	0.28 (0.18–0.43)	0.22 (0.15–0.30)
Miniature Doberman		3	33	6	0	S	0.22 (0.09–0.55)	0.18 (0.09–0.34)
Miniature Schnauzer	12	3	49	6	0	S	0.14 (0.06–0.33)	0.12 (0.06–0.24)
Miniature Wirehaired Dachshund		1	12	4	0	S	0.50 (0.14–1.75)	0.33 (0.14–0.61)
Newfoundland		4	42	12	1	L	0.40 (0.20–0.79)	0.29 (0.17–0.44)
Pekingese		5	75	35	1	S	0.88 (0.55–1.38)	0.47 (0.36–0.58)
Pomeranian	9	5	50	18	0	S	0.56 (0.31–1.01)	0.36 (0.24–0.50)

(Continued)

(Table 3.2, Page 2)

Breed	Rank[a]	Respondents	Number of bitches		Cephalic index[b]	Size[c]	Odds of CS[d]	Probability of CS
			All	Had a CS				
Pug		2	41	23	1	S	1.28 (0.68–2.39)	0.56 (0.41–0.70)
Rottweiler	4	10	125	26	0	L	0.26 (0.17–0.41)	0.21 (0.15–0.29)
Scottish Terrier		2	28	21	0	S	3.00 (1.26–7.17)	0.75 (0.57–0.87)
Shih Tzu		1	23	3	1	S	0.15 (0.04–0.52)	0.13 (0.05–0.32)
St. Bernard		1	7	7	0	L		1.00 (0.65–1.00)
Staffordshire Bull Terrier	5	5	98	30	0	M	0.44 (0.29–0.68)	0.31 (0.22–0.40)
Swiss Shepherd		1	3	1	0	0	0.50 (0.03–9.46)	0.33 (0.06–0.79)
West Highland White Terrier		1	5	2	0	S	0.67 (0.09–4.93)	0.40 (0.12–0.77)
Yorkshire Terrier	10	9	169	73	0	S	0.76 (0.56–1.03)	0.43 (0.36–0.51)
Categories of breeds combined								
Brachycephalic		27	382	182			0.91 (0.74–1.11)	0.48 (0.43–0.53)
Non-brachycephalic		97	1411	492			0.54 (0.48–0.60)	0.35 (0.32–0.37)
Large breeds		68	893	311			0.53 (0.30–0.96)	0.35 (0.22–0.48)
Medium breeds		19	319	130			0.69 (0.45–1.06)	0.41 (0.30–0.51)
Small breeds		37	581	233			0.67 (0.46–0.97)	0.40 (0.31–0.49)
All breeds		124	1793	674			0.60 (0.45–0.81)	0.38 (0.31–0.45)

[a] Rank of breed with regards to number of puppies registered in South Africa during 2016 with the GSD Federation of South Africa, South African Boerboel Breeding Society or Kennel Union of South Africa.

[b] Brachycephalic (1) or non-brachycephalic (0).

[c] Size categories: Large (L), medium (M), small (S)

[d] Odds followed by its 95% CI between parentheses

[e] Probability followed by its 95% CI between parentheses

3.3.2. Survey 2: Prevalence of caesarean section in German shepherd Dogs in South Africa

Table 3.3 shows the odds and probability of German Shepherd Dog bitches undergoing a caesarean section with consecutive litters.

Accounting for the correlation within bitches, the odds of CS is similar for the first and second litter during the recording period (P = 0.095). From the second litter onwards there is a general trend for the odds of CS tend to increase (P ≤ 0.05) relative to the odds in the first litter with every increase in number of litters up to the 5th litter (Table 3.3). There is only one instance of a 6th litter and the odds can therefore cannot be compared to the first litter.

Table 3.3
Odds and probability of German Shepherd Dog bitches undergoing a caesarean section (CS) with consecutive litters

Litter sequence	Natural whelp	CS	Total	Odds of CS	Probability of CS	P
1	521	78	599	0.15 (0.12–0.19)[a]	0.13 (0.10–0.16)[a]	
2	229	40	269	0.17 (0.12–0.24)	0.15 (0.11–0.19)	0.10[b]
3	86	18	104	0.21 (0.13–0.35)	0.17 (0.10–0.25)	0.05
4	26	9	35	0.35 (0.16–0.75)	0.26 (0.11–0.40)	0.05
5	5	4	9	0.80 (0.2–30.23)	0.44 (0.10–0.79)	0.03
6	0	1	1			
All litters	867	150	1017	0.17 (0.14–0.22)	0.15 (0.12–0.18)	

[a] Odds and probability, each followed by its 95% CI between parentheses

[b] The odds of CS of the 2nd to 5th litters were each compared to the that of the first

Fifteen of 26 bitches that had undergone CSs to deliver their first litter again underwent CS to deliver their second litter. Twenty five of 243 bitches that had delivered their first litter naturally underwent CS to deliver their second. The odds of a GSD bitch requiring a CS to deliver the second litter was 11.8 (95% CI 4.9–28.7) times higher in bitches where the first litter was born by CS than it was for bitches of which the first litter was born naturally (P < 0.001).

The 14.8% probability of CS reported in the German Shepherd Dog bitches in Survey 2 was significantly lower than the 37.6% in all breeds and the 47.6% in brachycephalic breeds reported in Survey 1 (P < 0.001).

3.3.3. Odds of stillbirth in Boerboel bitches allowed to whelp normally

Figure 3.1 suggests a curvilinear relationship between the odds of stillbirth and litter size—that is between the number of stillborn puppies per live-born puppy and litter size: Litters of 8–11 puppies seem to have lower numbers of stillborn puppies per live-born puppy than smaller or larger litters.

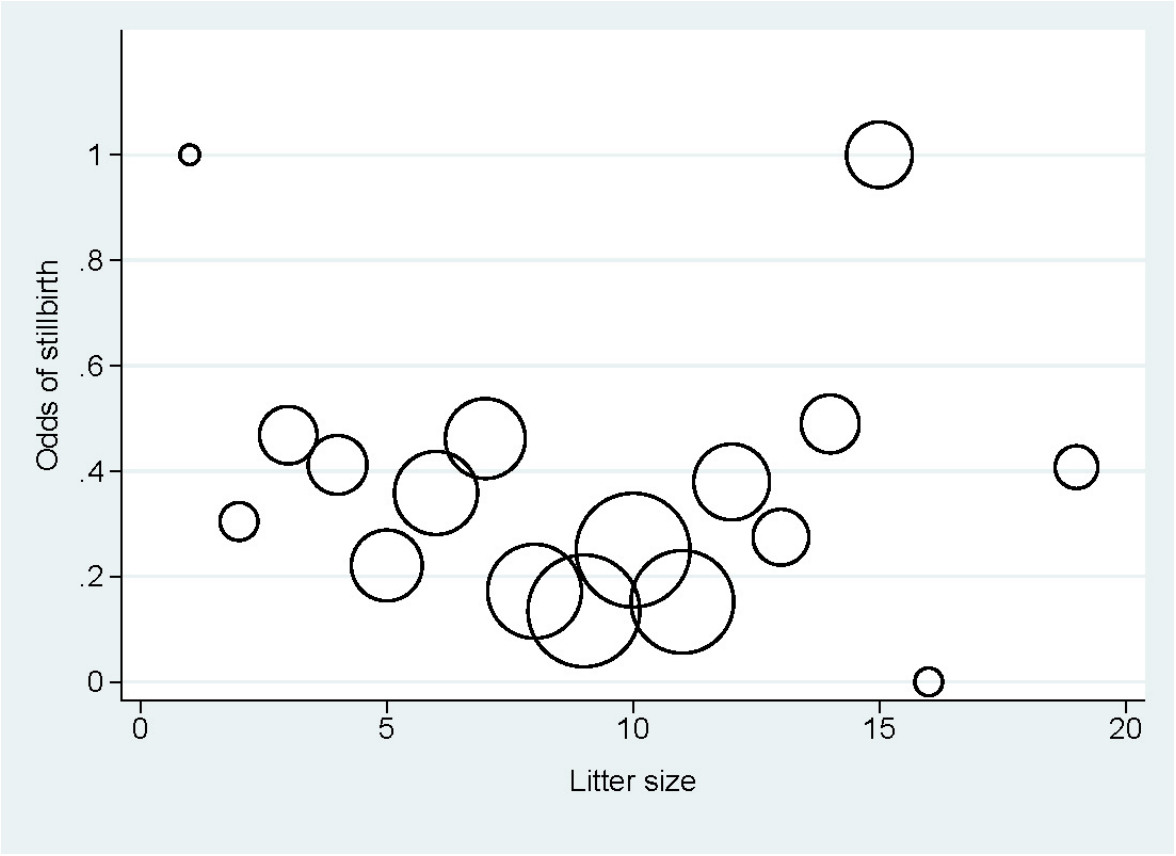


Figure 3.1
Curvilinear relationship between odds of stillbirth and litter size in Boerboel bitches allowed to whelp naturally. The size of each circle is proportional to the number of litters it represents.

Without considering the effect of Bitch, Table 3.4 shows that naturally-born Boerboel litters of 8–11 puppies had an average of 0.18 stillborn puppies per live-born puppy and an average probability of 15% stillborn puppies. Litters smaller than eight puppies had an average of 0.38 stillborn puppies per live-born puppy and an average probability of 27% of stillborn puppies whereas, for litters larger than 11 puppies, these figures were 0.46 and 32%.

Controlling for the effect of Bitch, the odds of puppies being stillborn is 2.19 times higher

(95% CI 1.50–3.21) in litters smaller than eight than in litters having 8–11 puppies ($P < 0.001$) and the odds of puppies being stillborn is 3.65 times higher (95% CI 2.30–5.79) in litters larger than 11 than in litters having 8–11 puppies ($P < 0.001$).

There were 93 uniparous bitches and 63 multiparous bitches in the sample (Table 3.4). The odds of stillbirth was independent of parity ($P = 0.66$) and parity did not interact with litter size in its effect on the odds of stillbirth ($P > 0.22$).

3.3.4. Survey 4: Effect of method to deliver the previous litter on the odds of caesarean section and stillbirth in the current litter

The responses include the birth of 458 litters from 279 purebred bitches of 29 breeds. All 279 of the bitches in this group had undergone at least one CS and the owners elected natural whelp as the preferred method of delivery in their subsequent pregnancy or pregnancies and the owners chose to present the bitches for CS only when they were concerned that parturition failed to progress normally. Data submitted by various breeders spanned a period of up to 20 years.

a) The effect of TOLAC and non-TOLAC on the need for a caesarean section

Each of the 279 bitches had at least one TOLAC. Of the 458 litters, 321 were TOLAC. Seventy-seven litters from 54 bitches of 19 breeds were non-TOLAC. Forty-five bitches from 18 breeds delivered litters classified as uncertain. Two hundred of 321 (62%, 95% CI 57% to 68%) of TOLACs were resolved by CS (Table 3.5), compared to 40 of 77 (52%, 95% CI 41% to 63%) of non-TOLACs.

The random effect of breed on the odds of delivery requiring a CS was significant ($P = 0.03$). Neither size nor being brachycephalic nor the interaction between them affected the odds of a delivery requiring a CS ($P > 0.25$). After accounting for the correlation within breeds, the ratio between the odds of requiring a CS or not of TOLACs and non-TOLACs was 1.32 (95% CI 0.78–2.25, $P = 0.30$). This means that TOLACs (current litters from bitches of which the previous litter was delivered by CS) required 1.32 times more CSs per naturally born litter than non-TOLACs (current litters from bitches of which the previous litter was born naturally), but this effect was not significant ($P = 0.30$).

Table 3.4
Odds and probability of stillborn puppies of various litter size categories and parity in the Boerboel

	Bitches	Litters	Live-born puppies	Stillborn puppies	Total number of puppies	Odds of stillborn puppies (CI 95%)	Probability of stillborn puppies (CI 95%)
Litter size							
Below 8	74	128	408	153	561	0.38 (0.28–0.50)	0.27 (0.22–0.33)
8–11	58	99	794	141	935	0.18 (0.13–0.24)	0.15 (0.11–0.19)
Above 11	24	29	273	126	399	0.46 (0.29–0.73)	0.32 (0.22–0.41)
Parity							
Uniparous	93	93	545	163	708	0.30 (0.22–0.40)	0.23 (0.18–0.28)
Multiparous	63	163	930	257	1187	0.28 (0.21–0.36)	0.22 (0.17–0.26)
Overall	156	256	1475	420	1895	0.28 (0.23–0.35)	0.22 (0.19–0.26)

Table 3.5

Odds and the probability of a trial of labour after caesarean section (TOLAC) failing in various breeds

			TOLAC				
Breed	Size	Brachycephalic	Total	Succeeded	Failed	Odds of failure	Probability of failure
Basset Hound	Large	No	5	4	1	0.25 (0.03–2.24)[a]	0.20 (0.04–0.62)[b]
Boerboel	Large	No	66	14	52	3.71 (2.06–6.70)	0.79 (0.68–0.87)
Border Collie	Large	No	1	0	1		
Boston Terrier	Small	Yes	4	1	3	3.00 (0.31–28.84)	0.75 (0.30–0.95)
Bouvier des Flandres	Large	No	6	0	6		1.00 (0.61–1.00)
Bull Mastiff	Large	Yes	16	6	10	1.67 (0.61–4.59)	0.63 (0.39–0.160
Bull Terrier	Medium	No	12	3	9	3.00 (0.81–11.08)	0.75 (0.47–0.91)
Cavalier King Charles	Medium	Yes	4	2	2	1.00 (0.14–7.10)	0.50 (0.15–0.85)
Chihuahua	Small	Yes	13	5	8	1.60 (0.52–4.89)	0.62 (0.36–0.82)
Chow Chow	Large	Yes	12	5	7	1.40 (0.44–4.41)	0.58 (0.32–0.81)
Cocker Spaniel	Medium	No	1	0	1		
German Shorthaired Pointer	Large	No	7	5	2	0.40 (0.08–2.06)	0.29 (0.08–0.64)
Golden Retriever	Large	No	16	6	10	1.67 (0.61–4.59)	0.63 (0.39–0.81)
Great Dane	Large	No	5	1	4	4.00 (0.45–35.79)	0.80 (0.38–0.96)
Husky	Large	No	4	2	2	1.00 (0.14–7.10)	0.50 (0.15–0.85)
Labrador Retriever	Large	No	54	26	28	1.08 (0.63–1.84)	0.52 (0.39–0.65)
Miniature Schnauzer	Small	No	8	4	4	1.00 (0.25–4.00)	0.50 (0.22–0.78)
Min. Wirehaired Dachshund	Small	No	2	2	0		0.00 (0.00–0.66)

(Continued)

(Table 3.5, Page 2)

Breed	Size	Brachycephalic	TOLAC				
			Total	Succeeded	Failed	Odds of failure	Probability of failure
Pekingese	Small	Yes	7	1	6	6.00 (0.72–49.84)[a]	0.86 (0.49–0.97)[b]
Pomeranian	Small	No	12	5	7	1.40 (0.44–4.41)	0.58 (0.32–0.81)
Rottweiler	Large	No	3	2	1	0.05 (0.05–5.51)	0.33 (0.06–0.79)
Scottish Terrier	Small	No	8	2	6	3.00 (0.61–14.86)	0.75 (0.41–0.93)
Shetland Sheepdog	Medium	No	1	0	1		
St. Bernard	Large	No	6	2	4	2.00 (0.37–10.92)	0.67 (0.30–0.90)
Staffordshire Bull Terrier	Medium	No	19	12	7	0.58 (0.23–1.48)	0.37 (0.19–0.59)
Standard Poodle	Large	No	5	0	5		1.00 (0.57–1.00)
Swiss Shepherd	Large	No	5	3	2	0.67 (0.11–3.99)	0.40 (0.12–0.77)
Welsh Terrier	Small	No	1	0	1		1.00 (0.21–1.00)
Yorkshire Terrier	Small	No	18	8	10	1.25 (0.49–3.17)	0.56 (0.34–0.75)
Total			321	121	200	1.65 (1.32–2.07)	0.62 (0.57–0.67)

[a] Odds and its 95% confidence interval between parentheses

[b] Probability and its 95% confidence interval between parentheses

Table 3.5 shows that the odds and the probability of a TOLAC failing vary widely among the breeds represented in the survey.

b) The effect of TOLAC and non-TOLAC on the odds of stillbirths

Table 3.6 shows the frequency, odds and probability of stillbirths in TOLAC, non-TOLAC and uncertain cases. The status (live or dead) of puppies at birth was highly correlated within breeds ($P < 0.001$), justifying the inclusion of breed as cluster variable. Neither body size of the breed nor brachycephalic status nor the interaction between them affected the odds of puppies being stillborn ($P > 0.6$). After accounting for the correlation within breeds with respect to survival status of puppies at birth, TOLAC litters resulted in 40% (95% CI 4% to 90%) more stillborn puppies per liveborn puppy than non-TOLAC litters ($P = 0.029$), whereas uncertain litters resulted in 29.4% fewer (95% CI 55% fewer to 10% more) stillborn puppies per live-born puppy than non-TOLAC litters ($P = 0.12$). TOLAC litters that failed (ended in CSs) and litters that were completely delivered by CS (unsure whether any attempt at natural birth was made or not) had similar numbers of stillborn puppies per live-born puppy as non-TOLAC litters that ended in CS ($P > 0.05$). TOLAC litters that succeeded (all puppies were born naturally) had similar numbers of stillborn puppies per live-born puppy than non-TOLAC litters that succeeded (all puppies were born naturally), $P = 0.10$.

Table 3.6
Frequency, odds and probability of stillbirths in TOLAC, non-TOLAC and uncertain cases

	Born alive	Born dead	Total	Odds of stillbirth (CI 95%)	Probability of stillbirth (CI 95%)
TOLAC	334	63	397	0.19 (0.15–0.23)	0.16 (0.13–0.19)
non-TOLAC	1463	425	1888	0.29 (0.22–0.38)	0.23 (0.18–0.27)
Uncertain	262	39	301	0.15 (0.08–0.29)	0.13 (0.06–0.20)
Total	2059	527	2586	0.26 (0.20–0.32)	0.20 (0.17–0.24)

3.3.5. Odds of stillbirth when delivery method is by elective caesarean section as opposed to emergency caesarean section

Table 3.7 summarises the numbers, odds and probability of stillbirths by breed and across breeds.

After accounting for the correlation within breeds, the number of dead puppies per live

puppy born by emergency CS was 7.3 (95% CI 5.16–10.36) times higher than was the case for elective CSs ($P < 0.001$).

Table 3.7

Odds and probability of stillbirth when delivery method was by elective caesarean section and emergency caesarean section

	Elective caesarean section				Emergency caesarean section			
Breed	Born alive	Stillborn	Odds of stillbirths	Probability of. stillbirths	Born alive	Stillborn	Odds of stillbirth	Probability of stillbirths
Beagle	5[a]	0[b]		0 (0–0.43)[c]				
Boerboel	1342	37	0.03 (0.02–0.04)[d]	0.03 (0.02–0.04)	145	40	0.28 (0.17–0.46)	0.22 (0.16–0.28)
Bull Mastiff	11	0		0 (0–0.26)				
Bull Terrier	52	0		0 (0–0.07)	29	3	0.10 (0.03–0.3)	0.09 (0.03–0.24)
Chihuahua					3	5	1.67 (0.16–17.51)	0.63 (0.31–0.86)
Chow Chow	19	3	0.16 (0.02–1.45)	0.14 (0.05–0.33)	9	5	0.56 (0.26–1.17)	0.36 (0.16–0.61)
Cocker Spaniel					7	2		0.22 (0.06–0.55)
Dachshund					13	1	0.08 (0–1.22)	0.07 (0.01–0.31)
English Bulldog	523	18	0.03 (0.02–0.06)	0.03 (0.02–0.05)	35	13	0.37 (0.11–1.27)	0.27 (0.17–0.41)
French Bulldog	1	0		0 (0–0.79)	0	8		1 (0.68–1)
French Mastiff	3	1		0.25 (0.05–0.7)				
Golden Retriever					5	1		0.17 (0.03–0.56)
Great Dane	4	2		0.33 (0.1–0.7)				
German Shepherd Dog	59	2	0.03 (0.01–0.11)	0.03 (0.01–0.11)	29	6	0.21 (0.08–0.51)	0.17 (0.08–0.33)
German Shorth. Pointer	6	1		0.14 (0.03–0.51)				
Irish Wolfhound	10	0		0 (0–0.28)	7	0		0 (0–0.35)
Jack Russel					0	2		1 (0.34–1)

(Continued)

(Table 3.7, Page 2)

	Elective caesarean section				Emergency caesarean section			
Breed	Born alive	Stillborn	Odds of stillbirths	Probability of. stillbirths	Born alive	Stillborn	Odds of stillbirth	Probability of stillbirths
Labrador Retriever	28[a]	10[b]	0.36 (0.09–1.36)[d]	0.26 (0.15–0.42)[c]	12	4	0.33 (0.24–0.46)	0.25 (0.1–0.49)
Miniature Doberman					2	0		0 (0–0.66)
Miniature Schnauzer					0	3		1 (0.44–1)
Neapolitan Mastiff					9	2		0.18 (0.05–0.48)
Pekingese	19	0		0 (0–0.17)	6	1		0.14 (0.03–0.51)
Rottweiler	1	0		0 (0–0.79)				
Schipperke	4	0		0 (0–0.49)				
Scottish Terrier	4	0		0 (0–0.49)	5	1		0.17 (0.03–0.56)
St Bernard					4	3		0.43 (0.16–0.75)
Staffordshire Bull Terrier	24	3	0.13 (0.06–0.27)	0.11 (0.04–0.28)	10	2	0.20 (0.02–1.71)	0.17 (0.05–0.45)
Pomeranian	8	0		0 (0–0.32)	8	1	0.13 (0.08–0.2)	0.11 (0.02–0.44)
Unknown	29	4	0.14 (0.06–0.34)	0.12 (0.05–0.27)	4	0		0 (0–0.49)
Yorkshire Terrier					11	2	0.18 (0.03–0.96)	0.15 (0.04–0.42)
Overall	2152	81	0.04 (0.02–0.06)	0.04 (0.02–0.05)	353	105	0.30 (0.23–0.38)	0.23 (0.18–0.27)

[a, b] Numbers of puppies born alive and dead, respectively

[c] Probability and its 95% confidence interval between parentheses

[d] Odds and its 95% confidence interval between parentheses

3.4. Discussion

Within the canine obstetric population, there exist subpopulations of bitches with pregnancies in which the prevalence of dystocia is likely to be higher than that of the general obstetrical population. These may be defined as high risk pregnancies and include pregnancies of high risk breeds (Munnich and Kuchenmeister, 2009; Evans and Adams, 2010; Linde-Forsberg and Eneroth, 2000; Davidson, 2008; Trautmann and Nolte, 2003; Johnson, 2008a; Moon et al., 1998). The proportion of litters born by CS in a very large sample (22005 litters from 151 breeds in the UK) was 19% (Evans and Adams, 2010). Survey 1 shows that 38% of bitches required one or more CS during their reproductive lifetime. This percentage can not be directly compared to the 19% of Evans and Adams (2010) because different methods to calculate the prevalence of CSs and different breeds were examined. However, both studies confirm the existence of a high proportion of deliveries requiring CS. A weakness of survey 1 of the current study was that neither age or parity of the bitches were recoreded or considered and thus the effect of age or parity on prevalence of CS could not be evaluated.

In agreement with Evans and Adams (2010), Survey 1 shows that cephalic index strongly affects the probability of a bitch requiring a CS. Survey 1 shows that the odds of CS associated with brachycephaly is expected to be 2.25 times higher than is the case for non-brachycephalic bitches. Considering that the English Bulldog—which was ranked third in South Africa in terms of the number of puppies registered during 2016—was not represented in the data set, the effect of brachycephaly on the odds of CS may have been more profound had they been included. Although this study shows the 15% probability of CS in a classic dolichocephalic dog breed such as the German Shepherd Dog is lower than the 38% in the general dog population other than English Bulldogs or the 48% in the general population of brachycephalic breeds other than English Bulldogs in South Africa, cephalic index is not the only risk factor. In survey 2, the litter sequence was used wich is not the same as parity. Although it is clear that the probability of CS increases with increase in litter sequence, the effect of age on probability of CS cannot be separatedly evaluated from the effect of increase in parity on probability of CS.

Our data showed that breed had a strong effect on the probability of having to undergo a CS with some non-brachycephalic breeds having a higher probability of CS than brachycephalic breeds. In the current study the Scottish Terrier and Boerboel were such breeds. This suggests

that other features than cephalic index, may strongly influence the probability of bitches requiring CS. The limited number of respondents and bitch numbers included in survey 1 for some of the breeds however limits the inference that can be drawn for those breeds.

Effective selection methods against dystocia and thereby the need for CS, is needed in many breeds. In order to reduce the high proportion of bitches in many breeds requiring CS, some kennel clubs only allow two CSs in a bitch and do not allow registration of puppies thereafter from these bitches (The Kennel Club UK, 2012). Some breeders have similar self-imposed selection protocols by not breeding a bitch again if she has required one prior CS. It is doubtful if such selection protocols would work. This is because selection against the occurrence of a CS may not be as effective as selection against the underlying anatomic- and physiologic traits within the breed that are the true causes of the need for a CS in the first place. It is suggested that one such anatomic trait is the cephalic index of a breed (Johnson, 1986; Evans and Adams, 2010; Linde-Forsberg and Eneroth, 2000; Wydooghe et al., 2013; Farrell et al., 2015). Selecting against CS without selecting against brachycephaly and (or) other anatomical traits (and also adopting a change in breed standards) may be futile as such selection may in any event change the morphology of the breed away from the preferred morphology as embedded in the existing breed standards. Such selection may therefore threaten the existence of some affected breeds in their currently preferred morphological standards—something breeders may be unwilling to accept.

Survey 2 shows that the probability of CS in the German Shepherd Dog breed tends to increase with parity. Because age data were lacking in this survey, it was not possible separate the effects of parity and age. It therefore remains unknown whether increasing age, independent of parity, may also have influenced the odds of CS.

The average litter size for the Boerboel litters was 7.40 puppies per litter. This makes the Boerboel breed used in Survey 3 an ideal breed to evaluate the effect of litter size on variables of interest. Survey 3 shows that there was a curvilinear relationship between the odds of stillbirth and litter size in the Boerboel. This agrees with suggestions by others that both large litters (Bennett, 1974), and small litters (Lopate, 2008; Johnson, 2008a; Darvelid and Linde-Forsberg, 1994) of other breeds are associated with reduced puppy survival at birth. It also supports the notion by breeders that middle of the range litter sizes result in best outcome for offspring.

There are no reports on the obstetric risks associated with repeat CS or TOLAC in bitch populations. Survey 4 in the current study shows that 62% of TOLACs required a repeat CS and Survey 2 showed that German Shepherd Dog bitches that had undergone CSs to deliver their first litter required 11.8 times more CSs per natural delivery for their second litters than German Shepherd Dog bitches of which the first litter was born naturally. This is of clinical importance. Firstly, it should act as a deterrent for opting too quickly for CS in bitches that never before had undergone a CS in at least those breeds with a low-risk for CS. Secondly it identifies bitches having undergone a CS in their prior pregnancy, as high-risk for CS in their next pregnancy. Therefore, in high risk breeds and in bitches that underwent a CS in their prior pregnancy, our results suggest that a repeat CS may be preferable to a trial of labour. This notion is supported by the high proportion of puppies born alive (> 95%) associated with well managed elective CS in the current study and the high proportion of stillbirths associated with TOLAC litters (40% more than non-TOLAC litters). We do not know why there is an increased risk of CS in the next pregnancy if the prior litter was delivered by CS. It could be speculated that either individual proneness of a bitch to dystocia or the direct effect of surgery, influenced the prevalence of repeat CS. It is also not known which one of the two stronger influences repeat CS, if any.

It may be speculated that the number of stillbirths associated with unsuccessful TOLAC may vary depending on how the decision was made to either continue with a trial of labour or to perform a CS. This could not be standardised in the current study and may have varied by owner and veterinary obstetrician. It appears logical that the proportion of stillbirths following an unsuccessful TOLAC, would be higher than that of elective CS. This is because it might be difficult to distinguish between eminent dystocia or delayed eutocia, leading to a delay to timeous intervention by CS and to performing an emergency CS.

Survey 5 showed that the number of dead puppies born by emergency CS was significantly higher than was the case for elective CSs ($P < 0.001$). This finding confirms those previously reporting that the likelihood of all the puppies being alive if the CS was performed on an emergency basis was decreased compared to elective surgery (Moon et al., 2000) and concurs with findings in human obstetrics (Ben-Meir et al., 2005). Avoiding an emergency CS should therefore be a priority for the veterinary obstetrician. Elective CS is therefore an important tool in the management of high-risk pregnancies. This study proved that in such subpopulation of bitches carrying high-risk pregnancies, a carefully planned elective CS may be considered

the best-justified method of delivery and results in very few stillbirths. This study confirms that the probability of stillbirths in high-risk pregnancies may be so high that, if neonatal survival is paramount, it may be a questionable practice to proceed with CS only after medical therapy has been attempted and failed as this may lead to further delays and puppy mortalities as previously described (Munnich and Kuchenmeister, 2009).

3.5. Conclusions

This study identified Boerboel litters larger or smaller than the optimum for the breed (8–11) as obstetric risk factors. This study identified previous CS in a bitch as a risk for a CS in her next pregnancy. It also showed that a large proportion of attempts at natural whelp after a previous CS delivery, fails and are associated with considerable risk for stillbirths. It further showed that these obstetric risks may be circumvented by planned elective CS. Bitches with a history of CS in their prior pregnancy represent a subpopulation of the general obstetric population in which the risk of dystocia is high. Therefore in this subpopulation, not unlike in the English and French Bulldog breeds, the request by owners for a properly planned CS in such bitches should be considered justified. Avoiding unnecessary first time CSs should be the clinicians priority and selection in breeds to preclude excessive caesarean statistics should always be advocated but further research is required to identify effective selection criteria in this respect.

Chapter 4. Puppy survival and vigour associated with the use of a low dose medetomidine premedication, propofol induction and maintenance of anaesthesia using sevoflurane gas-inhalation for caesarean section in the bitch

The content of this chapter has been submitted for publication in a different format as an article by K G M De Cramer, K E. Joubert, J O Nöthling and under the title "Puppy survival and vigour associated with the use of a low dose medetomidine premedication, propofol induction and maintenance of anaesthesia using sevoflurane gas-inhalation for CS in the bitch" and is currently under review

Abstract

The safety of an anaesthetic protocol consisting of medetomidine hydrochloride (7 µg/kg iv) as premedicant, propofol, (1–2 mg/kg iv) as induction agent and sevoflurane, at 2% in oxygen for maintenance of anaesthesia was studied in 292 elective CSs and 2232 puppies delivered. Medetomidine effects were reversed using atipamezole hydrochloride at 50 µg/puppy sc immediately following delivery and in the bitch iv immediately following surgery. The protocol's safety for puppies was expressed using survival immediately-, 2 h- and 7 d after delivery, and Apgar scores (measurement starting 15 min after delivery of the last puppy). The maternal survival rate was established immediately-, 2 h- and 7 d after CS. The CSs included 148 on Boerboel-, 84 on English Bulldog- and 60 on other purebred bitches, which resulted in 1378, 541 and 313 puppies, respectively. Boerboel, English Bulldog and other purebred bitches yielded 97.39%, 96.67% and 91.69% live puppies at delivery, 95.43%, 88.35% and 89.78% alive by 2 h and 89.19%, 79.11% and 84.03% alive by 7 d. Sixteen (1.16%), 32 (5.59%) and four (1.28%) malformed Boerboel-, English Bulldog- and other purebred puppies were euthanized. Thirty-five, 18 and 26, Boerboel-, English Bulldog- and other purebred puppies were stillborn respectively, of which 12, 9 and 15, respectively had been discovered dead upon ultrasound examination immediately before CS. After correction for foetuses found dead on ultrasound examination and malformed euthanized puppies, 98.21%, 95.60% and 94.30% of Boerboel-, English Bulldog- and other purebred puppies survived until 2 h and 91.78%, 87.17% and 88.26% until 7 d. Two-hour survival rates were negatively correlated to the proportion of puppies in a litter with scores of eight or below

(r = 0.14, P = 0.01, n = 292 litters) and tends to be positively correlated to the lowest Apgar score in a litter (r = 0.11, P = 0.05, n = 292 litters). This study shows that medetomidine hydrochloride in the protocol used is a safe premedicant in bitches prior to CS and is associated with good puppy vigour as well as 2 h and 7 d puppy survival rates. The use of medetomidine as premedicant permitted use of less than half the dose of propofol usually required as induction agent.

Keywords*:* caesarean section; medetomidine; propofol; Apgar score, puppy survival; dog

4.1. Introduction

The ideal anaesthetic protocol for CS should provide adequate muscle relaxation, analgesia and narcosis for optimal operating conditions, be safe for the bitch (Benson and Thurmon, 1984) and should not affect the viability and survival of the puppies (Robertson and Moon, 2003; Gabas et al., 2006). Additional recommendations include using drugs with a short duration of action (Pascoe and Moon, 2001) and using drugs that are reversible (Ryan and Wagner, 2006a). Induction using propofol and maintenance of anaesthesia using isoflurane is widely accepted and associated with good outcome (Gabas et al., 2006; Seymour, 1999; Funkquist et al., 1997; Biddle and Macintire, 2000; Brock, 1996; Moon-Massat and Erb, 2002; Moon et al., 2000). The use of alpha2-adrenergic agonists before anaesthesia for CS, however, is controversial. The alpha2-adrenergic agonist xylazine, is not recommended in patients undergoing CS because it was identified as a risk factor for increased puppy mortality (Navarro and Friedman, 1975; Moon et al., 2000), associated with increased risk of death in the dog (Clarke and Hall, 1990; Dyson et al., 1998) and caused severe maternal and neonatal cardiovascular depression (Traas, 2008b).

The greatest objection to the use of the alpha2-adrenergic agonists is the cardiopulmonary effects that include transient hypertension followed by mild hypotension, bradycardia, increased systemic vascular resistance, reduced cardiac output, and respiratory depression (Pypendop and Verstegen, 1998). In more recent surveys of anaesthetic mortality in the dog however, premedication with medetomidine prior to anaesthesia for routine surgery, was not identified as an increased risk factor for mortality (Brodbelt et al., 2008b) and data from human literature has shown that dexmedetomidine is associated with a reduction in all causes of mortality when used for non-cardiac, cardiac and vascular surgery (Wijeysundera et al., 2003; Biccard et al., 2008). No studies in veterinary medicine have been conducted on the use

of medetomidine for CS.

This study assessed puppy vigour and survival following medetomidine, propofol and sevoflurane anaesthesia for elective CS.

4.2. Materials and Methods

The protocol was approved by the Animal Ethics Committee of the Faculty of Veterinary Science, University of Pretoria, (Onderstepoort, South Africa) (Project numbers v048-14 and v048-14 amend 1). During hospitalization, all experimental animals were housed and fed commercial dry pellets twice daily and had access to water ad-lib. All the bitches were taken out twice daily for walks.

This is a retrospective, descriptive study that included 292 CSs in 256 privately owned bitches that underwent a CS and were selected from the general obstetric population because of increased obstetric risk. High-risk pregnancies in the current study were considered those occurring in bitches from breeds with a high risk of complicated parturition (Wydooghe et al., 2013), with a history of dystocia (Stolla et al., 1999), or with known very large litters (Bennett, 1974). A trial of labour (attempt at spontaneous unassisted parturition) was declined by all the owners of the bitches in the current study. From this subpopulation of bitches, only those destined for elective CS and for which the day of onset of cytological diestrus (D0) had been determined, were included. For all the CSs, the bitches were admitted 3–4 d prior to the predicted parturition date calculated as 57 d following D0. During these days, the bitches were observed for signs of impending parturition (panting, inappetence, nesting behaviour, tenesmus) and by 6 hourly vaginal speculum examinations to assess the cervix. The decision on when to perform a CS was based upon the first appearance of any degree of dilatation of the cervix. To ensure that no obstetric emergency existed at the time of CS, bitches that presented with a green or black discharge signifying some degree of placental detachment or that presented when already in stage 2 of parturition evidenced by foetal membranes that had ruptured were excluded from the current study. All but two singleton pregnancies were excluded from the current study and were assigned to another study. This was done because some singleton pregnancies fail to progress normally (Johnson, 2008a). Once the decision to perform a CS was made, an ultrasound examination of the abdomen was performed to establish if there were any dead foetuses (absence of detectable heartbeat). The bitches were weighed before surgery. In all bitches, fluid administration (Ringer lactate, Fresenius Kabi,

Midrand, South Africa) commenced starting at induction and continued for 1½–2 h following induction for surgery until the set amount of fluids (35 ml/kg body weight) had been infused and the haematocrits before and after CS determined as previously described (De Cramer et al., 2016). The anaesthetic protocol used in the current study included the alpha2-adrenergic agonist medetomidine hydrochloride (Domitor®, Zoetis Animal Health, Sandton, South Africa) at 7 µg/kg iv as premedicant, followed one min later by propofol (Fresenius propoven®1%, Fresenius Kabi, Midrand, South Africa), (1–2 mg/kg iv) as induction agent. The propofol was administered as follows, the calculated dose of 2 mg/kg was drawn up in syringe and 1 mg/kg/iv was administered as a bolus. The remaining propofol was used as top-up if required. This was followed by immediate intubation and inhalation of room air. Following surgical preparation (averaging 3–5 min), the bitch was connected to a closed circuit anaesthetic machine with 2% sevoflurane (Sevoflo®, Safeline Pharmaceuticals, NorthCliff, South Africa) in oxygen for maintenance of anaesthesia. The CS was performed in standard fashion as described (Gilson, 2003). Meloxicam (Metacam®, Boehringer Ingelheim, Randburg, South Africa) was administered iv (0.1 mg/kg) intra-operatively as proposed by Mathews (2008), immediately after delivery of the last puppy. For 10 English Bulldog- and 10 Boerboel litters, the exact time it took to deliver all the foetuses from the uterus (delivery time) and the total surgery times were recorded. The delivery time was the time measured in seconds that it took the surgeon to migrate all the foetuses to the uterine incision on the dorsal aspect of the uterine body, deliver them via the incision, remove the foetal membranes from their faces, sever their umbilical cords and hand them to an assistant (The delivery time starts with the onset of manipulation of the first foetus and ends when the last foetus is handed to the assistant.). The surgical time was the time it took from making the first incision through the abdominal skin until the last suture was placed in closing the skin. The processing of puppies following delivery involved immediate administration of atipamezole hydrochloride (Antisedan®, Zoetis Animal Health, Sandton, South Africa) at the dose of 50 µg/puppy sc, tying off of the umbilicus and applying 10% povidone iodine thereto, drying the puppies, shaking fluids from their airways and placing them in an air-heated incubator set at 35°C. No oxygen support was offered to the puppies after delivery. Also, immediately after surgery, atipamezole hydrochloride at the dose of 20 µg/kg was administered iv (extra label) to the bitch, following which the remainder of the 35 ml/kg ringers lactate was allowed to infuse. The bitch was observed until the coughing reflex returned and was then extubated and observed until it was established that she was breathing

comfortably whilst in sternal recumbency, sitting upright or standing. It was recorded whether the bitches were fully ambulatory 15 min following extubation. After delivery of the puppies the following records were made; total number of puppies delivered, live puppies, dead puppies, deformed puppies and puppies euthanized. The Apgar scores were assessed starting with the first puppy 15 min after the last puppy was delivered according to the method adapted by Veronesi et al. (2009) and later used by Doebeli et al. (2013). The bitch and puppies were discharged usually 2–3 h following surgery.

The puppy survival rate was established immediately after delivery, at 2 hours post CS and 7 d post CS and the maternal survival rate was established after delivery of the last puppy, at 2 h and at 7 d post CS. The Glasgow pain scale evaluation was performed at the time of discharging the bitch according to Glasgow Composite Measure Pain Scale to ensure adequacy of pain management (Reid et al., 2007).

4.3. Results

A total of 292 CSs were performed, 148 on 133 Boerboel bitches, 84 on 68 English Bulldog bitches and 60 on 55 other purebred bitches, which resulted in a total of 2232 puppies (1378, 541 and 313, respectively, per breed). Thirty-six bitches underwent more than one CS in this study. The ages of 138 of the bitches were known and ranged from 1 year to 8 years of age (mean = 3). The parity of 141 were known and ranged from zero to five previous litters (mean = 1). The percent live at delivery for the Boerboel, English Bulldog and other purebred's puppies respectively was; 97.39%, 96.67% and 91.69%. The 2-h survival rate respectively was 95.43%, 88.35% and 89.78%. The 7-day survival rate was respectively 89.19%, 79.11% and 84.03%. The numbers of puppies euthanized due to malformation were 16/1378 (1.16%), 32/541 (5.59%) and 4/313(1.28%) respectively for Boerboels, English Bulldogs and other purebreds.

Thirty-five, 18 and 26 Boerboel-, English Bulldog- and other purebred puppies were stillborn. Of these, 12, 9 and 15 had, respectively, been found dead on ultrasound examination immediately before the CS was performed.

After correction for foetuses discovered dead on ultrasound and malformed euthanized puppies, the survival rates for Boerboel-, English Bulldog- and other purebred puppies were 98.21%, 95.60% and 94.30%, respectively, at 2 hours and 91.78%, 87.17% and 88.26% at 7 d.

The total delivery time varied between 88 s and 621 s in the 10 Boerboel bitches and from 21 s to 424 s in the 10 English Bulldog bitches. In the two groups combined, the average time it took to deliver an individual puppy was 42.6 s. The average surgery time for the Boerboels and English Bulldogs was 38 min (range 21–41 min) and 33 min (range 28–46 min) respectively.

The Apgar scores averaged 9.66 for all the breeds combined, 9.77, 9.35 and 9.68 for Boerboel, English Bulldogs and other purebred breeds respectively. The 2-hour survival rates of 292 litters were negatively correlated to the proportion of puppies in a litter with scores of eight or below (Spearman's rank correlation coefficient (r) = 0.14, P = 0.01), tended to be positively correlated to the lowest Apgar score in a litter (r = 0.11, P = 0.05) but was not correlated to the mean Apgar score of litters (r = 0.094, P = 0.11). The maternal survival rate was 291/292. One Boerboel bitch died from gastric dilatation and volvulus 2 days following surgery. The average Glasgow pain scale for bitches at discharge was 6.4 (S.D. 0.65, with a minimum of five and a maximum of eight, n = 292). No bitch had a haematocrit of below 30% after surgery and all bitches were fully ambulatory 15 min after extubation.

4.4. Discussion

The prime objectives for using premedication in any patient prior to surgery and CS is to reduce the induction dose of anaesthesia (Pascoe and Moon, 2001), reduce the minimum alveolar concentration of inhalation anaesthetics (Ryan and Wagner, 2006a), decrease maternal stress and anxiety (Ryan and Wagner, 2006b), provide analgesia and providing chemical restraint allowing preoperative preparation in cases where this is required. The use of alpha2-adrenergic agonists meet all these requirements as they are potent sedatives, may induce narcosis at high doses, act as analgesics (Kuusela et al., 2001), vastly reduce induction doses of propofol (Hammond and England, 1994) and reduces minimum alveolar concentration of isoflurane and sevoflurane (Aho et al., 1991; Aantaa et al., 1997; Lawrence and De Lange, 1997; Fragen and Fitzgerald, 1999).

Analgesics and non-steroidal anti-inflammatory agents in pregnant animals and humans are problematic for CS (Williams et al., 1999; Hoffmann et al., 1999; Landsbergen et al., 2001; Derrier and Mercatello, 1997; Luna et al., 2007; Poveda Roda et al., 2007; Gael et al., 2007; Risser et al., 2009; Mullins et al., 2012; Burdan et al., 2007; Chan et al., 2002; Koren et al., 2006; Ulinski et al., 2012; Goodger and Levy, 1973). Opioids provide analgesia but cross the

placenta and can cause significant central nervous system and respiratory depression in neonates (Goodger and Levy, 1973) which may take 2–6 d in canine neonates to eliminate (Mathews, 2008). Therefore some clinicians have historically avoided them (Mathews, 2008). Failing to administer analgesics for obstetric surgery has become unacceptable to the veterinary profession and its legislative bodies. However, there is consensus that a single intravenous administration of meloxicam at a dosage of 0.1 mg/kg immediately after delivery of the puppies is safe (Mathews, 2008). The analgesic properties of medetomidine intra-operatively and post-operatively have been demonstrated (Barnhart et al., 2000) and reviewed in the dog (Murrell and Hellebrekers, 2005) and demonstrated in the pregnant woman before and after CS without adverse neonatal effects (El-Tahan et al., 2012). However, reversal by atipamezole will also reverse the analgesic effects and for this reason the administration of other analgesics in the recovery and immediate post-operative period are indicated.

The low dose of 7 µg/kg may have minimized the cardiovascular effects of medetomidine in the current study as did a dose of 5 µg/kg in another (Pypendop and Verstegen, 1998). Although both maternal and neonatal cardiovascular depression associated with the use of medetomidine may have been present in the current study, our results show that it appeared not to have affected puppy survival rate and Apgar score or maternal survival rate. The route of administration of medetomidine is important as the iv route requires a much smaller dose to achieve the same effect as opposed to the im route (Sinclair, 2003).

In the absence of premedication the dose of propofol required to induce and intubate pregnant bitches approaches 6 mg/kg body weight (Doebeli et al., 2013). A significant induction dose sparing of propofol (reducing it to 1 mg/kg body weight) was recorded when medetomidine is used as premedicant at doses of 20–40 µg/kg (Hammond and England, 1994). Despite the use of a much lower dose of medetomidine in the current study, it was possible to intubate and surgically prepare all bitches never exceeding a total dose of 2 mg/kg of propofol.

The rate of stillbirth and neonatal death is known to be relatively high in dogs with mortality ranging from 17% to 30% within the first 8 weeks of life (Indrebo et al., 2007) and asphyxia being the leading cause of death (Andersen, 1957; Van der Beek et al., 1999). Stillbirths and deaths within the first week were responsible for most these losses. In a large study involving 10,810 litters, the perinatal mortality was present in 24.6% of litters. In the same study, 4.3% of puppies were stillborn and another 3.7% died within a week (Tønnessen et al., 2012).

Survival was 92% in the first week, and only another 1% dying from 8 days to 8 weeks of age (Tønnessen et al., 2012). The puppy survival rate in other studies varied from 92.4% to 88.1% (Linde-Forsberg and Forsberg, 1989; Linde-Forsberg and Forsberg, 1993; Gavrilovic et al., 2008; Böhm and Hoy, 1999). In a study by Moon et al. (1988), survival rates immediately, 2 hours and 7 d after delivery were 92%, 87%, and 80%, respectively, for puppies delivered by CS (n = 3,410) and 86%, 83%, and 75%, respectively, for 498 puppies born naturally. Moon et al. (1998) reported that in 76% of litters delivered by CS, all the puppies were born alive. The maternal mortality rate was 1% (Moon et al., 1998). Our results show that medetomidine appeared not to have affected puppy survival rate and maternal survival rate.

In a study involving 37 CSs performed on English Bulldogs, 14.9% of the puppies were stillborn, 8.2% of the puppies alive, were deformed (mainly palatoschisis and anasarca) and 10% of the puppies died before the age of weaning (Wydooghe et al., 2013). This is similar to the 6.9% birth defects, 15.91% 24 h mortality that Batista et al. (2014) reported for elective CS in English Bulldogs. The results from the current study in English Bulldogs were similar with respect to the number of deformed puppies (5.59%) but better for percent live birth (96.67%). In 193 puppies from 42 litters, 65 born by spontaneous delivery, 66 by assisted delivery and 62 by CS, the percentage of stillbirths were 14%, 20% and 8% respectively (Veronesi et al., 2009).

Puppy survival rates at birth and at 2 h after birth supposedly reflect the effects of perioperative conditions more specifically than at 7 d after delivery (Moon et al., 2000). This is because deaths within the first week are often associated with factors relating to maternal care, agalactia, undetected birth defects and infectious causes (Indrebo et al., 2007).

It is important to establish prior to a treatment or procedure whether all the foetuses are alive and well prior to the intervention (Fontbonne et al., 2009) to avoid a false overestimation of the effect of the intervention on puppy mortality. Pre-operative ultrasound enables one to account for at least some foetuses that died before and therefore those stillbirths may be considered independent of the intervention. The current study identified 45.6% (36/79) of stillborn puppies on ultrasound examination immediately before the CS was performed. Because the aim of the current study was to evaluate the effect of our anaesthetic protocol on puppy survival rates, puppy vigour and maternal survival rates, attempts were made to exclude confounders which may have affected outcome not related to anaesthetic protocol. These were foetuses detected dead on ultrasound examination prior to administering any

drugs included in the anaesthetic protocol and puppies euthanized because they were deformed. By eliminating puppy mortality unrelated to anaesthesia from the data, a true reflection of puppy survival rates was obtained in the current study and seems a more accurate way to compare anaesthetic protocols. This correction is particularly useful when comparing puppy survival rates of the English Bulldog breed to those of others. English Bulldog puppies not only have poorer survival rates at 2 h and at weaning age but also an increased risk of being stillborn or being born with defects requiring euthanasia (Wydooghe et al., 2013; Batista et al., 2014).

The maternal survival rate in this study was good as all but one of the 292 bitches anaesthetized were alive at 7 d post CS. A single Boerboel bitch died from gastric dilatation and volvulus two days after discharge. Gastric dilatation and volvulus is a well-recognized cause of sudden death in large breeds (Glickman et al., 2000). Therefore, our results support the finding by another worker that premedication with medetomidine was not identified as an increased risk factor for anaesthesia in the dog (Brodbelt et al., 2008b). In contrast, xylazine has safety concerns for both dam and neonate when used for CS in the bitch (Navarro and Friedman, 1975; Moon et al., 2000; Clarke and Hall, 1990; Dyson et al., 1998; Traas, 2008b). The demonstrated safety recorded in the current study may in part be explained by; improved alpha 1 : alpha 2 specificity exhibited by medetomidine compared to xylazine, greater awareness of the physiologic effects of alpha2-adrenergic agonists (Brodbelt et al., 2008b) and the use of a relative low dose (7 µg/kg) of medetomidine. Although dexmedetomidine and medetomidine induced similar clinical effects (Granholm et al., 2007), there are theoretical pharmacological advantages of the non-racemic alpha2-adrenergic agonist, dexmedetomidine, over medetomidine (Flaherty, 2013a).

Apgar scores have been shown to be influenced by anaesthetic agents. Alfaxalone (1–2 mg/kg) has been shown to be slightly superior to propofol (2–6 mg/kg) in this respect (Doebeli et al., 2013; Metcalfe et al., 2014). Overall, the Apgar scores achieved in the current study were higher than those reported in other studies irrespective of method of delivery or anaesthetic protocol used (Doebeli et al., 2013; Veronesi et al., 2009; Groppetti et al., 2010; Metcalfe et al., 2014). The time at which Apgar scores are measured after birth is important. Although in early studies in man, a score at one minute was used (Apgar, 1966), it was later demonstrated that the score at five minutes is more predictive of survival (Casey et al., 2001). Similarly, in puppies, evaluations performed at five minutes after birth were less predictive

than those done at 15 min and 60 min after delivery (Veronesi et al., 2009; Groppetti et al., 2010; Doebeli et al., 2013). This may be because more time was allowed to elapse for removal of depressive effects of the anaesthetic agents before an Apgar score was evaluated. In the current study the delay from delivery till Apgar score evaluation may have allowed for complete reversal of medetomidine in the puppy and for the concentration of propofol in the central nervous system to diminish by redistribution which is reported to take 15–20 min (Funkquist et al., 1997; Short and Bufalari, 1999). Furthermore, the low induction dose of propofol used and atipamezole administration, may also have favourably influenced Apgar scores.

In humans, the Apgar score is easily performed as there is only usually only one baby to evaluate. In polytocous species such as the bitch there may be a considerable time delay between the delivery of the first puppy and the last puppy, depending on the litter size. In the Boerboel- and English Bulldog litters combined, the average time it took to deliver an individual puppy was 42.6 s. The delivery times of foetuses increased with an increase in distance between where a foetus was situated and the incision line in the uterine body through which the foetuses were delivered. Foetuses suffering from *hydrops foetalis* also took longer to deliver than those unaffected.

In agreement with the studies by others (Doebeli et al., 2013; Veronesi et al., 2009), the current study shows that Apgar scores correlate with mortality, with lower scores being associated with higher mortality rates. Although another study has shown that English Bulldog puppies were at increased risk compared to other breeds for suffering from poor vigour after CS (Moon-Massat and Erb, 2002), our study showed only slightly reduced Apgar scores when compared to other purebreds and Boerboel puppies. The method of delivery may impact on puppy vigour. In contrast to findings by others, in the current study, we recorded higher Apgar scores for puppies from caesarean deliveries than those of the eutocic vaginally delivered puppies reported in the literature (Silva et al., 2015; Vassalo et al., 2015). These differences may reflect the different anaesthetic protocols used in the different studies and timing of obstetric intervention. Our results show that medetomidine appeared not to have affected the Apgar score.

Although, by comparing puppy survival rates, it can be concluded that the anaesthetic protocol in the current study was at least as safe as other published reports using other anaesthetic protocols, the effect of early intervention by CS prior to foetal compromise may

in part explain the good results obtained in our study. This early intervention eliminated or minimized the effects of obstetrical factors negatively impacting on puppy survival and vigour. This is in agreement with the literature reporting positive correlation between timeous intervention and puppy survival (Lennoz-Roland, 1998; Stolla et al., 1999; Moon et al., 2000; Polster et al., 2005; Bergström et al., 2006b; Gendler et al., 2007; Pretzer, 2008; Traas, 2008b; Michel and Reichler, 2008b; Munnich and Kuchenmeister, 2009).

4.5. Conclusions

This study shows that anaesthetic protocol using medetomidine hydrochloride at 7 µg/kg iv as premedicant combined with 1–2 mg/kg propofol as induction agent and 2% sevoflurane in oxygen as maintenance, is safe for CS in the bitch. The Apgar scores 15 min after delivery, as well as the puppy and maternal survival rates at delivery, 2 h, and 7 d compare favourably to those reported in publications using other anaesthetic protocols. The use of medetomidine as premedicant permits the use of less than ½ the induction dose of propofol usually required when no premedicant is used. Accurate comparison of anaesthetic protocols requires correction for both puppies dead before anaesthesia and puppies euthanized due to malformations.

4.6. Author contributions

J O Nöthling and K Joubert supervised and assisted in drafting the protocol and manuscript. K.G.M. De Cramer was the main person involved in experimental work and wrote the protocol and manuscript.

4.7. Conflict of interest statement

The authors have declared no conflicts of interest.

Chapter 5. Haematocrit changes in healthy periparturient bitches that underwent elective caesarean section

The content of this chapter has been published in a different format as "De Cramer, K.G.M., Joubert, K.E., Nöthling, J.O., 2016. Hematocrit changes in healthy periparturient bitches that underwent elective cesarean section. Theriogenology 86**,** 1333-1340."

Abstract

Haematocrits were measured before each of 406 CSs performed on 324 bitches at term and again following crystalloid fluid therapy administered at 35 ml/kg over 1½ to 2 h starting from induction. The mean haematocrit was 44.2% (95% CI 43.8–44.6%) before CS and 37.8% (95% CI 37.3–38.2%) following CS and fluid therapy, with a mean decrease of 6.4 percentage points (95% CI 6.1–6.7%) over all 406 CSs. These results provide the clinician with clear guidelines of the normal expected ranges of haematocrits in bitches before and after CS. Results of this study show that bitches have haematocrits at term that are at the lower end of the normal reference ranges for non-pregnant dogs and that there is no true anaemia of pregnancy. It is therefore suggested that if late term bitches present with anaemia, other causes besides pregnancy should be considered.

Keywords*:* Caesarean section, haematocrit, dog, blood loss, anaemia, blood transfusion trigger

5.1. Introduction

Poor oxygen delivery may negatively affect bitches undergoing a CS and their foetuses (Ryan and Wagner, 2006b). Various physiological changes during pregnancy affect oxygen delivery. Oxygen delivery depends on oxygen carrying capacity (haemoglobin), cardiovascular function and respiratory function (Ryan and Wagner, 2006b). As early as 1977, Concannon et al. referred to a physiological normocytic, normochromic anaemia in pregnant bitches. They reported that the increase in body weight of the bitches they observed during pregnancy was accompanied by a decrease in haematocrit and proposed that this may have been due to a large increase in plasma volume.

The haematocrit of normal healthy non-pregnant dogs may lie between 42% and 62%

(average 52%) (Moritz et al., 2004) or between 37% and 55% (average 50%) (Duncan and Prasse, 2011). Four studies have reported a lowering of haematocrit during gestation in bitches: In 1974, Hayashi reported a steady decrease in the haematocrit of 15 bitches that became statistically significant on Day 50 of pregnancy, when it also reached a nadir of 33.7% (SEM 1.8%) from when onwards it increased to 38.7% (SEM 1.8%) on Day 60. Hayashi also reported the mean postpartum haematocrit as 37%. In 1977 Concannon et al. reported that the haematocrit in 12 pregnant bitches was consistently lower than that of 12 non-pregnant bitches from Day 20 after the onset of oestrus onwards and continued to decline to reach a nadir of 30.6% (SD 0.8) by Day 60–62. In 1993, Kaneko et al. (1993) showed that the haematocrit of 23 beagles decreased from between 40% and 52% before pregnancy to between 28% and 42% at term and that the litter size had an influence on haematocrit. Finally, in 2013, Dimço et al. (2013) reported a mean haematocrit of 45.4% (SD 3.6%) in 16 non-pregnant bitches and a mean of 41% (SD 4.9%) in 16 bitches of similar body weight and age in the last third of gestation . Due to varying results, litter sizes and the small sample sizes of these studies, no definitive conclusions about haematocrits during pregnancy can be made.

The haematocrit of bitches that had been pregnant increased slowly after parturition but were still slightly lower by 145 d after the onset of oestrus than those of bitches that had had non-pregnant oestrous cycles (Concannon et al., 1977b). There is no absolute decrease in erythrocyte mass, and the haematocrit returns to normal within 8–12 weeks after parturition as the plasma volume returns to normal (Ryan and Wagner, 2006b).

Fluid therapy is recommended as a standard for CS in bitches (Ryan and Wagner, 2006a; Moon et al., 2000; Robertson and Moon, 2003; Traas, 2008b; Von Heimendahl and Cariou, 2009; Kushnir and Epstein, 2012; Smith, 2012). Fluids are given to correct any fluid and electrolyte deficits, acid-base balances, the hypotensive effects of anaesthesia and maintain cardiac output and uterine blood flow (Ryan and Wagner, 2006a). Fluid rates of 10–30 ml/kg/h with additional boluses have been suggested to maintain perfusion (Ryan and Wagner, 2006a; Robertson and Moon, 2003; Smith, 2012). Fluid therapy has the potential to cause additional change to the haematocrit.

Caesarean sections are associated with additional blood loss from surgery. There is no literature describing the haematocrits of bitches in late pregnancy before and after CSs. This is a stumbling block in the periparturient risk assessment of bitches that delivered by CS. Following CS, bitches and their puppies are generally not kept in veterinary hospitals for long

due to the risk of disease exposure and better nursing environments at home. Assessing risk prior to discharge following CS in bitches is particularly important because this may take place as soon as 2–3 h after surgery. This study comprises a retrospective analysis of data on haematocrits before and after CSs in healthy bitches undergoing elective CS to assess what changes can be expected.

5.2. Materials and Methods

The protocol was approved by the Animal Ethics Committee of the Faculty of Veterinary Science, University of Pretoria, (Onderstepoort, South Africa) (Project numbers v010-14, v048-14, v021-15 and v079-15). All experimental animals were housed and fed commercial dry pellets twice daily and with ad-lib water. This study included 406 CSs in 324 healthy, privately owned bitches presented to a private veterinary clinic for management of parturition during May 2012 to September 2015. Only healthy bitches destined for elective CS were included in the current study. No ovariohysterectomies were done and the placentas were removed with each puppy. A blood smear evaluation was performed before surgery. A clinical examination was performed before and after surgery which included assessment of: skin for turgor, mucous membranes for colour, moistness and capillary refill time, respiratory and heart rates, rectal temperature and habitus. The decision to perform a CS was based upon the first appearance of dilatation of the cervix on vaginoscopy performed every 6 h. The bitches where weighed immediately prior to surgery and anaesthetised using the standard anaesthetic protocol in the practice which included low dose alpha2-adrenergic agonist premedication (Medetomidine 7 µg/kg iv) (Zoetis Animal Health, Sandton, South Africa), propofol (1–2 mg/kg iv) (Fresenius Kabi, Midrand, South Africa) as induction agent and sevoflurane (1–2%) (Safeline Pharmaceuticals, Northcliff, South Africa) in oxygen for maintenance of anaesthesia. The CS was performed in standard fashion as described by Gilson (Gilson, 2003). The blood required for haematocrit assessment (approximately 1 ml) was collected by jugular venipuncture using a syringe and 23 G needle directly before anaesthetising the bitch for surgery and again 1½ to 2 h following induction for surgery and after the bitch had already received the set fluid volume (Ringer lactate, Fresenius Kabi, South Africa). No blood was collected from indwelling catheters used for fluid and drug administration as this would lead to potential errors in measuring the haematocrit. The blood was immediately transferred to a heparinised (sodium heparin 80 iu/ml) microhaematocrit capillary tube (Marienfield laboratory glassware, Germany) (74.5–75.5 mm in length and 1.1–1.2 mm internal diameter)

and centrifuged at 12000 revolutions per min for 10 min producing a relative centrifugal force of 14800 g and the haematocrit expressed as a percentage. This calculation was performed by measuring the red blood cell column in mm and dividing this value by the total length in mm of the blood column (plasma and packed cell column) and multiplying by 100 to obtain a percentage. If the serum appeared with reddish discolouration after centrifugation it was assumed that haemolysis had taken place during the blood collection or centrifugation processes and blood collection was then repeated.

The total volume of fluids administered was 35 ml/kg body weight to each bitch. The fluid was administered over 1½–2 h including surgery time, starting at time of induction, using simple fluid administration sets with the fluid rate ranging from 17.5 to 26.25 ml/kg/h. In order to standardise the effect of haemodilution on haematocrit in all the bitches, it was ensured that the bitch got the set fluid volume and approximate fluid rates. This was achieved by calculating the required amount of fluids the bitch should receive and removing it from the fluid bag. For instance, if the dog weighed 20 kg, the required amount is 700 ml and thus 300 ml would be removed from the one litre fluid bag in a sterile fashion. Because infusion pumps were not used, care was taken to ensure that the calculated fluid volume did not infuse in a time shorter than 1½ hour and not longer than 2 hours following induction for anaesthesia. The haematocrit after CS was not collected until the required fluid volume had been infused. For each bitch the following data were recorded: Date of CS, name of owner, name of bitch, breed, haematocrit before CS (Htbefore), haematocrit after CS (Htafter), body weight before CS, total number of puppies delivered (litter size)—irrespective of whether they were stillborn or delivered alive. Figure 5.1 shows the path diagram of possible effects of independent variables on factors affecting haematocrit before (Htbefore) and haematocrit after (Htafter) caesarean section in bitches.

5.2.1. Data analysis

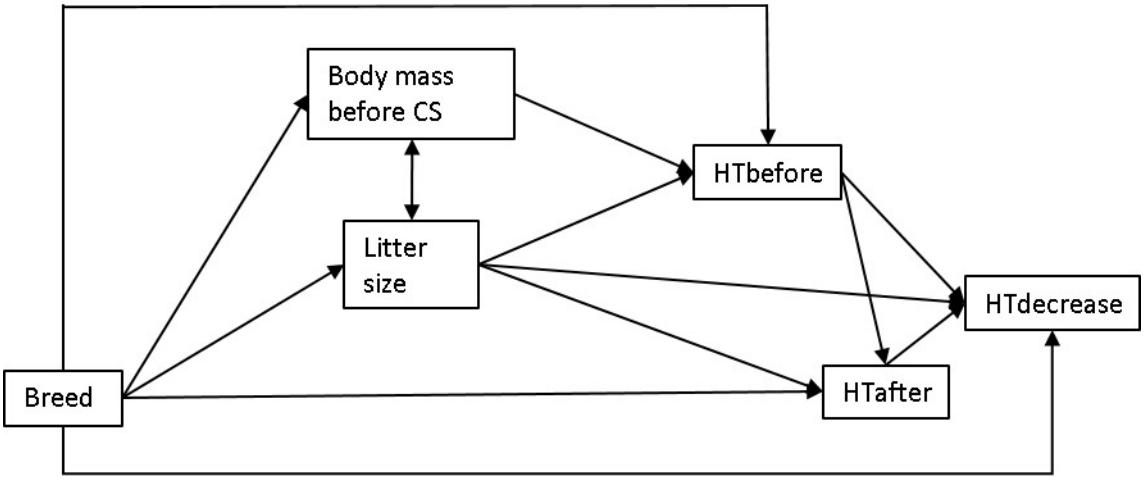


Figure 5.1
Path diagram of possible effects of independent variables on factors affecting haematocrit before (Htbefore) and haematocrit after (Htafter) caesarean section in bitches.

Linear regression was used to determine the effects of breed and litter size on Htbefore, and of Htbefore on Htafter. Each breed having 11 or more CSs in the data set was included in the regression models. These breeds were English Bulldogs (n = 119 CSs, labelled "Bulld"), Boerboels (n = 203 CSs, labelled "Boerb"), Bull Terriers (n = 21 CSs, labelled "Bull t"), German shepherds (n = 11 CSs, labelled "G s d") and Labrador Retrievers (n = 11 CSs, labelled "Labr"). For analyses that include breed as independent variable, the English Bulldog was used as the baseline category and each other breed was compared thereto. The English Bulldog was chosen as the baseline group because the group was large and, globally, English Bulldogs often requires CS.

Prior inspection revealed that litter size was significantly larger in Boerboels than in English Bulldogs, Bull Terriers, German shepherds and Labrador Retrievers, whereas it was the same among the latter four breeds. In all models where litter size was the independent variable of interest, breed was included to control its confounding effect on that of litter size (Dohoo et al., 2009).

Prior inspection of body weight revealed that it was distinctly bimodal because each Boerboel bitch was heavier than any other bitch included in the regression models. When the regression models were run with and without body weight, we found that the effect of body weight is spurious and it is perfectly included in the effect of breed. Bodyweight was then excluded

from all the regression models.

Although not included in the regression analyses, Chows (seven CSs on seven bitches), Staffordshire terriers (seven CSs on five bitches), 16 CSs on a group of 14 bitches belonging to eight breeds varying in size from Pomeranian to Rottweiler that occurred in low frequency in the data set (labelled "rare") and 11 CSs on a group of 11 bitches of which the breed was not recorded, varying in size from 3 kg to 72 kg (labelled "unknown"), were included in the summary statistics of the raw data.

Sixty-three bitches each underwent two or more CSs. Data were considered clustered in these bitches and a mixed-effects linear regression was used to determine the effect of repeated CS on Htbefore and Htafter, respectively. For this analysis, repeated CS was used as a categorical variable with values of 1, 2, 3, 4 or 5 for the first, second, third, fourth and fifth CSs, respectively. The haematocrits of the second to fifth CSs were each compared to those of the first.

For regressions of Htbefore and Htafter on independent variables, each CS, including the second to fifth CSs done on 63 bitches, was used as an independent observation.

Huber/White sandwich estimators of the standard errors were used for all regression analyses. No interactions were included in the analyses. Effects were considered significant if $P < 0.05$. Data analysis was done using Stata 14 (Stata Corp College Station, Texas, USA).

5.3. Results

Based on the clinical examinations, no bitch showed any illness or clinical evident dehydration before induction of anaesthesia or prior to discharge of the bitch and her puppies.

5.3.1. Summary of the data

Table 5.1 summarizes the haematocrits before and after CS, as well as the decrease in haematocrit during the CS in the various breeds.

Table 5.1

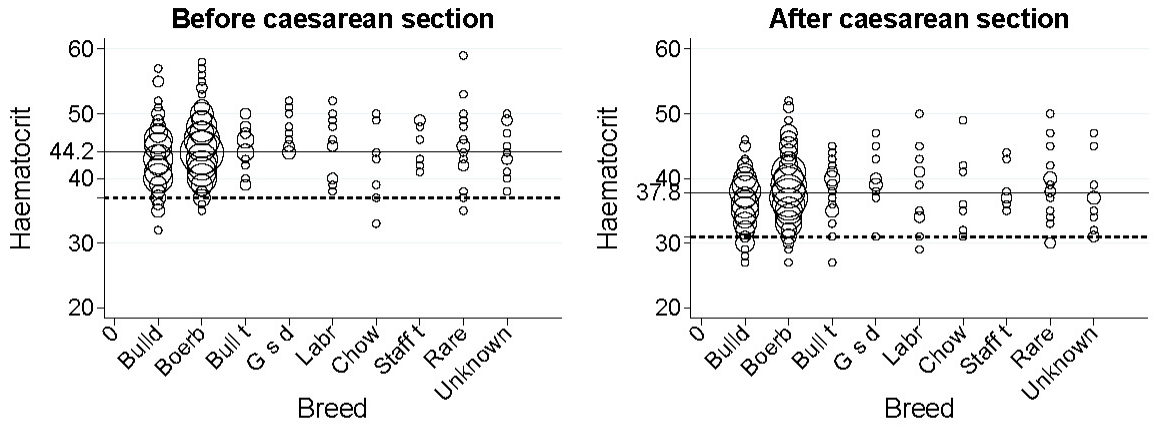
Haematocrit of healthy bitches undergoing elective caesarean section (CS) before and after CS, and the decrease during CS

	No. of CSs	No. of bitches	Before CS		After CS		Decrease during CS	
			Mean	SD	Mean	SD	Mean	SD
English Bulldog	119	89	43.15	4.25	36.40	3.65	6.75	2.93
Boerboel	203	162	44.46	3.93	38.33	4.26	6.13	2.78
Bull Terrier	21	16	44.90	3.13	38.14	4.41	6.76	3.37
German Shepherd Dog	11	11	46.91	2.95	39.82	4.24	7.09	3.56
Labrador Retriever	11	9	44.73	4.84	38.36	6.38	6.36	2.46
Chow Chow	7	7	42.14	6.23	38.00	6.38	4.14	2.73
Staffordshire Bull Terrier	7	5	45.43	3.41	38.57	3.51	6.86	2.61
Rare[a]	16	14	45.06	6.04	38.63	5.63	6.65	2.40
Unknown[b]	11	11	44.45	3.96	37.69	5.35	7.64	4.11
Over all	406	324	44.17	4.18	37.77	4.35	6.41	2.92

[a] This group consists of eight breeds occurring in low frequency in the data set; they varied in size from Pomeranian to Rottweiler.

[b] The breeds of these bitches were unknown or not recorded; they varied in body mass from 3 to 72 kg.

Figure 5.2 shows the distribution of the haematocrits before and after CS according to breed and Figure 5.3 shows the distribution of haematocrits before and after CS according to litter size.



Smallest circles indicate single values, larger circles indicate more frequent occurrences of haematocrits.
Solid lines indicate overall means.
Dashed lines indicate 5th percentiles (37.0 before CS, 31.0 after CS).

Figure 5.2
Distribution of haematocrits before and after 406 elective caesarean sections on healthy bitches according to breed.
(Bulld = Bulldog, Boerb = Boerboel, Bull t = Bull Terrier, G s d = German Shepherd Dog, Labr = Labrador Retriever, Chow = Chow, Staff t = Staffordshire Terrier, Rare = eight breeds occurring in low frequency in the data set, Unknown = 16 bitches for which the breed is unknown).

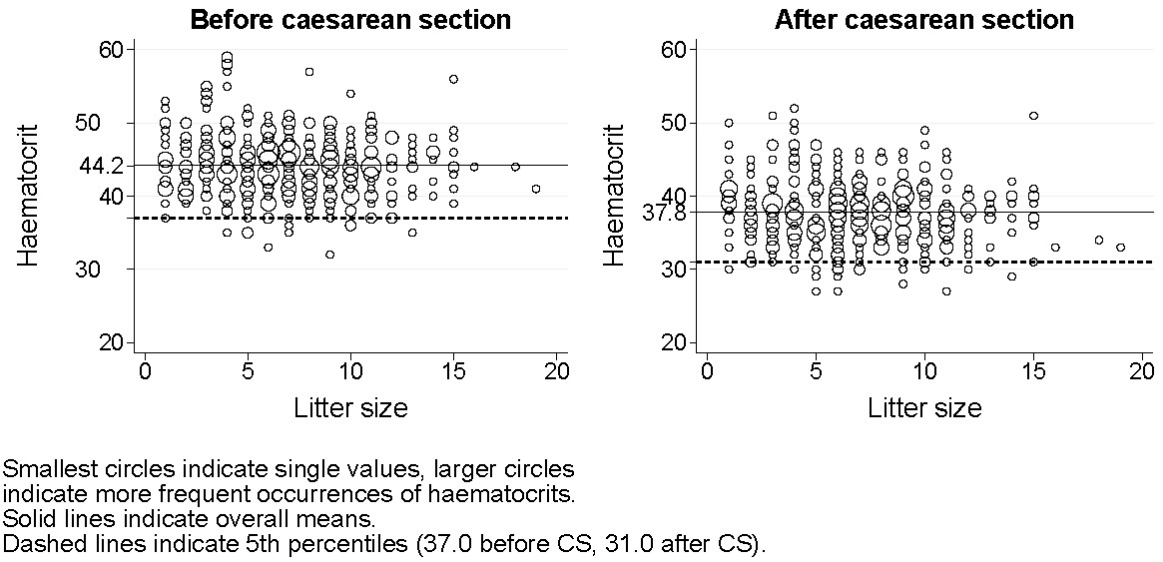


Figure 5.3

Distribution of haematocrits before and after 406 elective caesarean sections on healthy bitches according to litter size

The mean haematocrit before CS was 44.2% (95% CI 43.8–44.6%) and the 5th percentile of Htbefore over all 406 CSs 37.0% (95% CI 37–38.8%). Ten haematocrits were below 37% prior to CS (mean 34.8%, SD 1.32%, minimum 32%). The mean haematocrit after CS was 37.8% (95% CI 37.3–38.2%) and the 5th percentile over all 406 CSs was 31% (95% CI 30.0–31.8%). The mean decrease in haematocrit during CS and fluid therapy was 6.4 percentage points (95% CI 6.1–6.7 percentage points) over all 406 CSs.

The decrease in haematocrit was quite symmetrically distributed around the median of six percentage points (Figure 5.4), with 50% of CSs associated with decreases between five and eight percentage points and 95% with decreases between one and 13 percentage points. Five percent of CSs were associated with a decrease in haematocrit of 12 percentage points or more (12–18 percentage points).

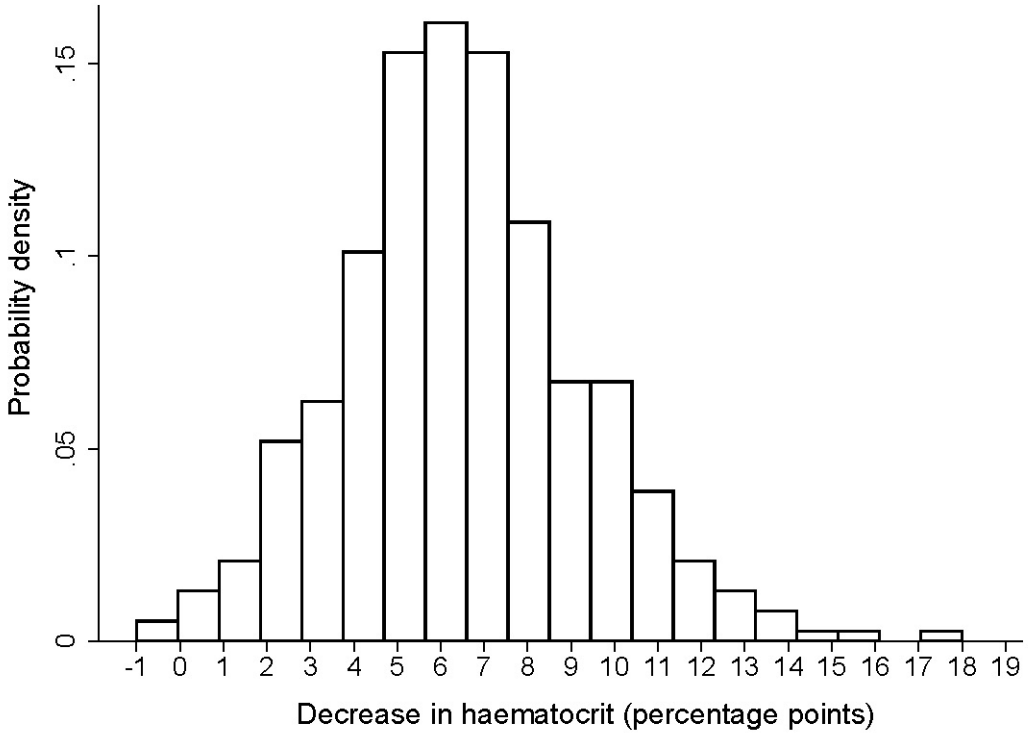


Figure 5.4

Histogram of the decrease in haematocrit during 406 caesarean sections in bitches.

Sixty-three bitches each had two to five CSs (Table 5.2). Htbefore and Htafter were independent of the number of CSs a bitch had ($P > 0.1$).

Table 5.2

For bitches that underwent more than one caesarean section (CS), haematocrit before and after CS was independent of the number of CSs a bitch had (P > 0.1)

Caesarean section	n[a]	Haematocrit			P[d]
		Mean	95% CI L[b]	95% CI U[c]	
Haematocrit before CS					
First of 2 or more	63	44.70	43.40	46.00	
Second of 2 or more	63	43.67	42.71	44.62	0.21
Third of 3 or more	13	44.85	42.75	46.94	0.91
Fourth of 4 or more	5	43.40	40.95	45.85	0.36
Fifth	1	44			0.29
Haematocrit after CS					
First of 2 or more	63	38.03	36.72	39.34	
Second of 2 or more	63	37.25	36.24	38.27	0.36
Third of 3 or more	13	39.85	37.77	41.92	0.15
Fourth of 4 or more	5	39.2	36.11	42.29	0.49
Fifth	1	37			0.12

[a] Number of caesarean sections in the category

[b,c] Lower and upper limit of the 95% confidence interval of the mean

[d] Within a time relative to CS (before or after), the mean haematocrits of the second to fifth CS were each compared to the mean of the first CS.

5.3.2. The effects of breed and litter size on Htbefore

Regressing Htbefore on breed shows that breed had a significant unconditional effect on Htbefore ($F(4,360) = 4.81$, $P < 0.001$). Apart from the G s d, which had a mean Htbefore that was 3.76 percentage points higher than that of the breed with the lowest Htbefore (Bulld), the difference among breeds were below two percentage points (Table 5.3).

Table 5.3

Linear prediction of the effect of breed on haematocrit before caesarean section

	Haematocrit (%)				
Breed	Mean	95% CI [La]	95% CI [Ub]	Effect size[c]	P[d]
Bulldog	43.15	42.38	43.92		
Boerboel	44.46	43.92	45.01	1.31	0.006
Bull Terrier	44.90	43.58	46.22	1.75	0.024
German Shepherd Dog	46.91	45.23	48.59	3.76	0.000
Labrador Retriever	44.72	41.97	47.48	1.58	0.279

[a, b] Lower and upper limits of the 95% confidence interval of the mean

[c, d] Magnitude (percentage points) and significance of the difference between the breed and the baseline breed (Bulldog)

Figure 5.5 graphically displays the effect of breed on Htbefore.

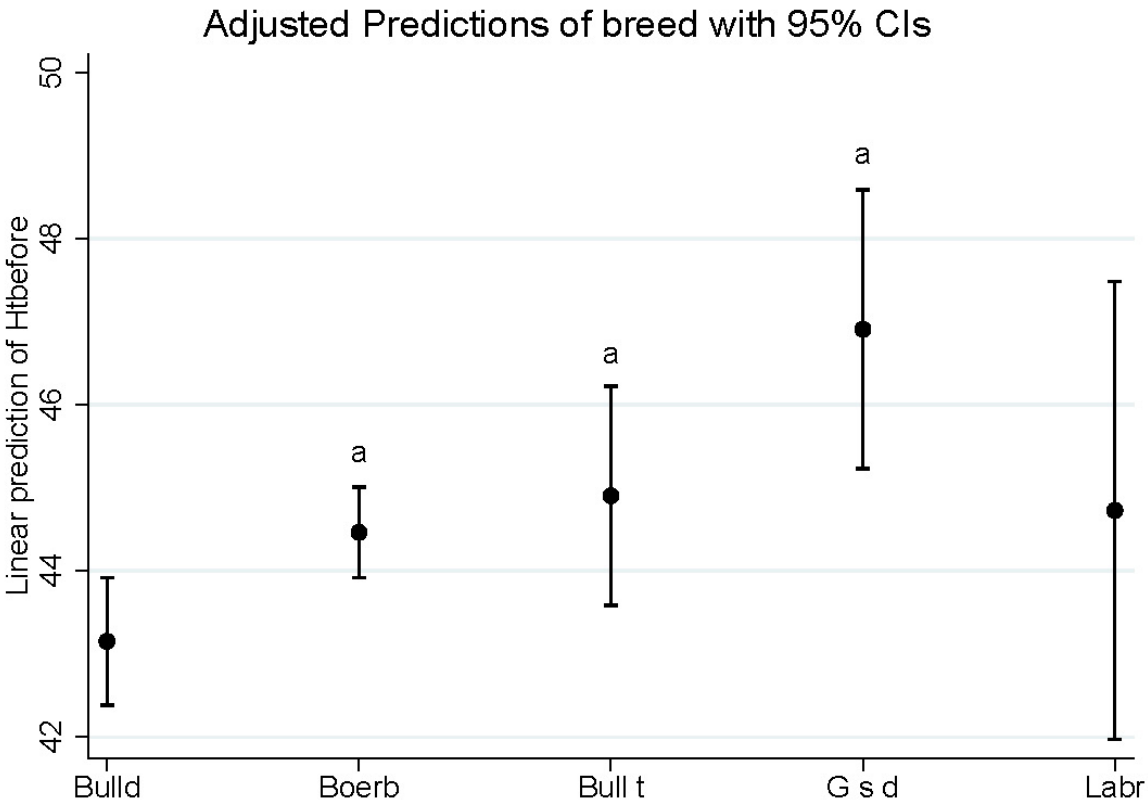


Figure 5.5

Unconditional effect of breed on haematocrit before CS (Htbefore). Predicted means marked "a" are significantly higher than that of Bulld ($P < 0.05$). (Bulld = Bulldog, Boerb = Boerboel, Bull t = Bull Terrier, G s d = German Shepherd Dog, Labr = Labrador Retriever).

Figure 5.6 suggests that, in English Bulldog-, Boerboel-, German shepherd- and Labrador Retriever bitches, there exists a variable negative linear relationship between Htbefore and litter size. Regression of Htbefore on litter size and breed showed that, controlling for breed, there was a significant ($P = 0.013$) but weak effect of litter size on breed. Htbefore is expected to decrease by 0.15 percentage points (95% CI 0.03–0.28 percentage points) for each unit increase in litter size.

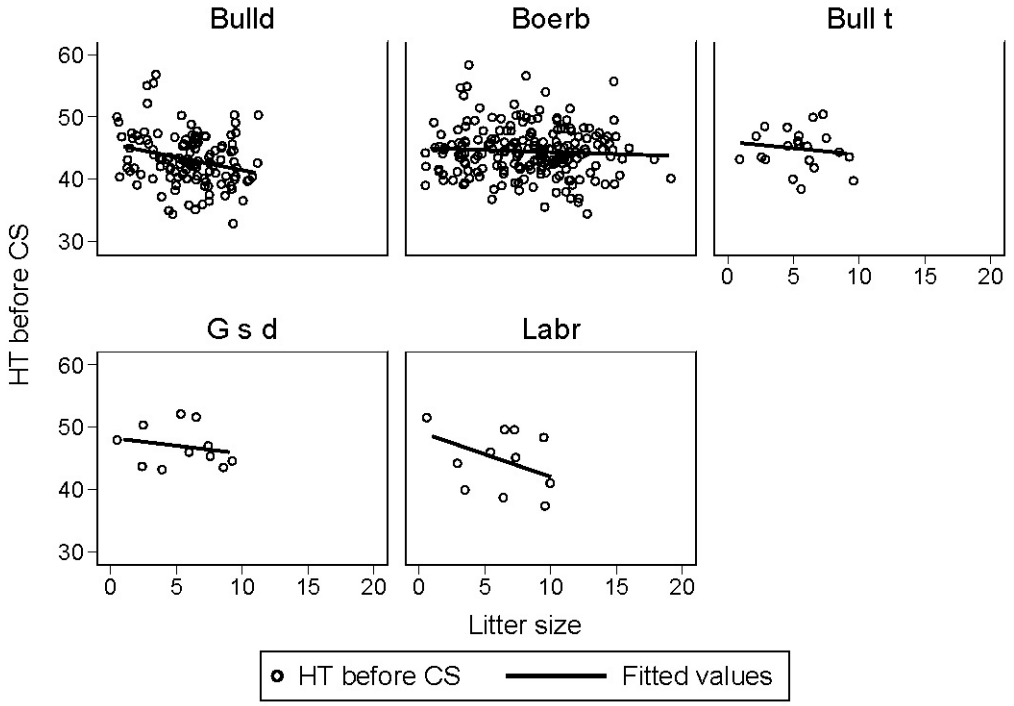


Figure 5.6

Haematocrit before caesarean section against litter size, with the best linear prediction in five breeds. (Bulld = Bulldog, Boerb = Boerboel, Bull t = Bull Terrier, G s d = German Shepherd Dog, Labr = Labrador).

5.3.3. The effect of Htbefore on Htafter

Figure 5.7 suggests that Htbefore has a strong positive, linear effect on Htafter in English Bulldog-, Boerboel-, German shepherd and Labrador Retriever bitches. Regressing Htafter on Htbefore with litter size and breed revealed that, controlling for litter size and breed, Htafter increases by 0.77 percentage points (95% CI 0.70–0.84 percentage points) for each percentage point increase in Htbefore ($P < 0.001$).

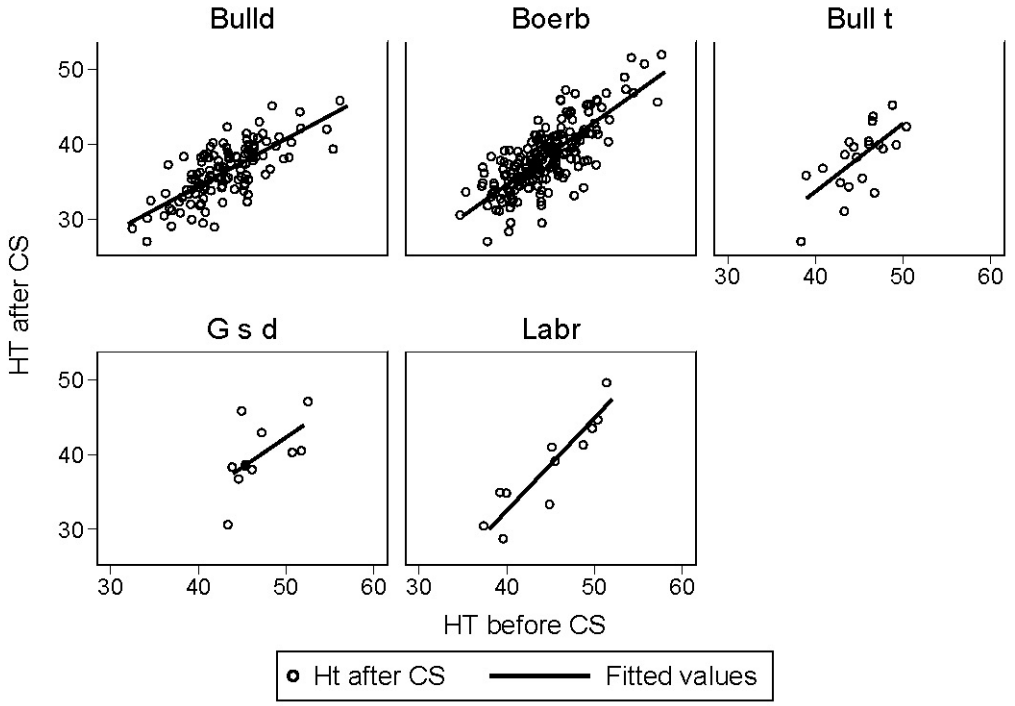


Figure 5.7

Haematocrit after caesarean section against haematocrit before caesarean section, with the best linear prediction in five breeds. (Bulld = Bulldog, Boerb = Boerboel, Bull t = Bull Terrier, G s d = German Shepherd Dog, Labr = Labrador).

5.4. Discussion

Anaemia may be expressed as haematocrit but haemoglobin concentration in the blood is the preferred way (Goonewardene et al., 2012). Haematocrit was chosen in the current study as it requires no laboratory assistance, is easily available to all veterinary practitioners and is rapidly performed in any private practice. Furthermore in humans, haematocrit change was reported as an objective indicator of blood loss and anaemia (Hidar et al., 2004; Stafford et al., 2010) and is well correlated to blood haemoglobin content (World Health Organization, 2001).

Concannon et al. (1977) reported that pregnant bitches develop a physiologic anaemia presumably due to plasma expansion, with mean haematocrits in close-to-term bitches of 30.6 (SEM 0.8%, n = 12). This was later termed anaemia of pregnancy (Concannon et al., 1989). In contrast, the current study shows that the expected haematocrit of healthy bitches of various breeds before CS is substantially higher at 44.2% (SD 4.2%), with a 95% confidence interval for the mean of 43.8–44.6%. Should the study be repeated, the mean haematocrit is expected with 95% certainty to fall within this narrow confidence interval. The Japanese have reported

a preterm haematocrit of 38.7% (SEM 1.8%) in 15 dogs (Hayashi, 1974). In contrast, Dimço et al. (2013) reported haematocrits of 41% (SD 4.9%, n = 16) and are in closer agreement to the results in the current study. In another study, all haematological parameters and serum biochemical profiles in pregnant bitches remained within normal ranges without adjustment to account for haemodilution and concluded that these parameters did not differ significantly from those found in normal adult dogs (Kimberely et al., 2006). Our results have an important clinical implication as pregnant bitches presenting with haematocrits below the normal reference range should be examined for other concurrent disease that may cause anaemia. This is particularly important if such bitches are close to term, giving natural birth or are to be subjected to CS and further potential blood loss. The reference intervals for normal healthy non-pregnant dogs' haematocrits at a laboratory at similar altitude (1500 m above sea-level) to the experimental animals were 37–55% (Personal communication, Scheepers 2014). In the current study, only 10 of the 406 CSs were associated with a haematocrit before CS below 37%. This may in part be explained because all the bitches in the experimental group were from well-managed breeding colonies where the nutrition and veterinary care was of a high standard.

Kaneko et al. (1993) studied the effects of litter size on the extent of anaemia of pregnancy in 23 beagle dogs and observed a correlation between litter size and a decrease in haematocrit, haemoglobin concentration and red blood cell count. They reported a 1.1% decrease in haematocrit for each puppy present. The current study shows that, controlling for breed, litter size has a significant but weak effect on Htbefore, with Htbefore on average decreasing by 0.14 percentage points for each increase of litter size by one puppy. Over the range of litter sizes seen in the current study this means that the mean litter size is expected to decrease by 2.5 percentage points from the smallest litter size of one to the largest of 19. The expected decrease in haematocrit before CS with an increase in litter size found in the current study is seven to eight times smaller than that found by Kaneko et al. (1993). The small sample size in the study by Kaneko et al. (1993) may have resulted in a less precise estimation of the relationship between haematocrit and litter size. Although the current study shows that increased litter size results in a slightly lower haematocrit, the difference is of minimal clinical significance.

The current study shows that breed affects the haematocrit of bitches before CS. Controlling for litter size, Boerboel and Bull Terrier bitches are expected to have 1.7% higher and German shepherd bitches 3.7% higher mean haematocrits before CS than the mean (43.2%) of English

Bulldog bitches. Breed specific changes in haematological variables have been reported and related to genetic phenotypes (Lawrence et al., 2013). Our data support this contention.

It seems unlikely that the effects of breed and litter size would be sufficient to explain the distinctly lower haematocrits before CS reported in other studies (Kaneko et al., 1993; Hayashi, 1974; Concannon et al., 1977b) compared to those reported in the current study.

The current study shows that the haematocrit of a bitch prior to CS is independent of the number of previous CSs that she has had.

The effects of altitude on haematocrit is not expected to have influenced results in the current study. This assumption is based on the finding that although exposure to hypoxia in dogs occurring at moderate to marked high altitude results in measurable cardiovascular changes, including increased heart rate, increased systemic and pulmonary artery pressure, and changes in systolic and diastolic cardiac function, it does not result in an increase in haematocrit (Glaus et al., 2003).

The haematocrit after CS in this study was decreased by both the surgery and the fluid therapy. Nevertheless, fluid therapy should be considered standard practice during surgery for CS section in the bitch (Ryan and Wagner, 2006b). In the current study, the hydration status of the bitches was assessed using assessment of skin turgor, mucous membranes and capillary refill time. Although imperfect, clinical assessment of hydration by looking for gross abnormalities during a clinical examination appears practical.

During normal parturition in the bitch a small amount of blood loss occurs and this has been shown in one study to reduce the mean haematocrit by 1.7% from 38.7% (SEM 1.8%) to 37.0% (SEM 0.8%) (Hayashi, 1974). No study prior to the current one has reported the effects of CS on haematocrit in dogs.

Changes in haematocrit have been used to estimate blood loss during obstetric surgery in women (Stafford et al., 2010) and allowed the prediction of bleeding even with ongoing fluid resuscitation (Thorson et al., 2013). Stafford et al. (Stafford et al., 2010), used a formula to reliably identify excessive blood loss during CS in women from the haematocrits before and after CS. Fluid redistribution following acute blood loss may take several hours but may be achieved in one hour when fluids are administered intravenously (Thorson et al., 2013). If not enough time is allowed for fluid redistribution following acute blood loss, haematocrit is likely

to be inaccurate and give false low estimates of blood lost and or false high haematocrits (Nicol et al., 1997). In the current study 1½ to 2 h was allowed for equilibration but this may not have been long enough. Despite the shortcomings of haematocrit, the American College of Obstetricians and Gynaecologists has used haematocrit in their official definition of postpartum haemorrhage as a drop of more than 10% in haematocrit during delivery ([ACOG] American College of Obstetricians and Gynaecologists, 1998).

In the current study the average haematocrit after CS was 37.8 (SD 4.4) and was largely dependent on haematocrit before CS. Controlling for litter size and breed, Htafter is expected to decrease by 0.77 for each decrease of one in haematocrit before CS. The average decrease in haematocrit following CS and fluid therapy was 6.4% (SD 2.9). It can be speculated that the extent of blood loss and, hence, the haematocrits may be different following CS when the placentas are left in situ or removed and whether an ovariohysterectomy was performed or not. In this study, no ovariohysterectomies were performed and the placentas were removed from the uterus with each puppy. The effect of blood loss associated with vaginal births and CS in humans is ameliorated for by autotransfusion of blood into the circulation from the contracting uterus if left in place (Ciliberto and Marx, 1998). It is fair to speculate that autotransfusion may also act as a compensatory mechanism to protect against effects of maternal blood loss during natural whelp and CS in dogs as has been reported in woman (Ciliberto and Marx, 1998). In the current study, the effect of autotransfusion on haematocrit was not studied.

In a report on CS in bitches it was suggested that blood loss during surgery should be compensated for at a level of 3 ml of crystalloid for every 1 ml of blood lost (Smith, 2012). The author did not explain how the amount of blood lost was estimated or determined. Change in haematocrit before and after CS in the bitch seems an objective parameter to mitigate this shortcoming.

All our bitches made an uneventful recovery and survived beyond 7 d and nursed litters successfully, yet five percent of bitches showed a decrease in haematocrit of 12–18 percentage points. It should however not be assumed that a decrease in haematocrit by 12–18 percentage points is never a reason for concern. A study in woman suggested that a change in haematocrit of more than 10 percentage points may indicate a need for blood transfusion (Stafford et al., 2010). The significance of such a decrease should be based on basic veterinary clinical principles. In humans both a minimum haematocrit as well as a maximum change in haematocrit before and after surgery are used as trigger for blood transfusion (Van Woerkens

et al., 1992; Freitag et al., 2002; Mannucci and Levi, 2007). Further research is required to better define transfusion triggers in the periparturient bitch undergoing CS.

5.5. Conclusions

Results of this study show that bitches have haematocrits at term that are at the lower end of the normal reference ranges for non-pregnant dogs and that there is no true anaemia of pregnancy. In contrast to current recommendations (Linde-Forsberg and Eneroth, 2000), pregnant bitches near term with haematocrits below normal reference ranges for non-pregnant dogs should be evaluated for disease that may affect haematocrit. Also, our results show that there is a small yet significant negative correlation between litter size and haematocrit at term in bitches but that this has no clinical significance. On average the haematocrit decreased by 6.4% during CS, while bitches were receiving 35 ml/kg of crystalloids over 1½ to 2 h. Further research is required to better define transfusion triggers in the periparturient bitch undergoing CS.

5.6. Author contributions

J.O. Nöthling and K. Joubert were the supervisors of the scientific protocol and assisted in drafting the protocol and manuscript up to the final drafts. J.O. Nöthling performed the statistical analyses. K.G.M. De Cramer was the main person involved in experimental work and wrote the protocol and manuscript.

5.7. Conflicts of interest

Conflicts of interest: none.

Chapter 6. Is the biparietal diameter of foetuses in late gestation too variable to predict readiness for caesarean section in dogs?

The content of this chapter has been submitted for publication in a different format and is currently under review for consideration as an article by K G M De Cramer and J O Nöthling and under the title "Is the biparietal diameter of foetuses in late gestation too variable to predict readiness for CS in dogs?"

Abstract

Correct assessment of readiness for CS is essential for timing elective CS during late pregnancy in the bitch. In humans, biparietal diameter (BPD) is sufficiently precise and accurate and used in a clinical setting daily. The objectives of this study were to determine whether foetal BPD in late gestation in the dog could be used to predict readiness for CS by having reached a minimum cut-off value and to correlate the BPD to birth weight. The BPD of 208 puppies in 34 litters from 31 English Bulldog bitches and 660 puppies in 78 litters from 70 Boerboel bitches were measured immediately after delivery by CS, performed at full term, using digital calipers. At the same time the birth weight of the same 208 English Bulldog puppies and 494 of the same Boerboel puppies in 59 litters from 54 bitches was measured by means of an electronic scale. With a CS, all the puppies in a litter are delivered simultaneously and readiness for CS must be determined for a litter. Litters and not puppies were therefore considered experimental units. Over all puppies, ignoring the fact that the data were clustered in litters, the BPD varied from 28.8 to 49.8 mm (mean 38.63 mm, SD 3.20 mm) in the 208 English Bulldog puppies and from 27.8 to 54.3 mm (mean 42.04, SD 3.60) in the 660 Boerboel puppies. The range in BPD among puppies of the same litter varied from 0 to 12 mm (mean 4.99 mm, SD 2.78 mm) in the 34 English Bulldog litters and from 0 to 17.99 mm (mean 7.63 mm, SD 3.61 mm) in the 78 Boerboel litters.The minimum, median and maximum BPD varied from 21.1 to 47.8, 32.9 to 50.0 and 34.2 to 58.2 mm, respectively, among English Bulldog litters and from 18.4 to 48.7, 35.5 to 49.7 and 39.8 to 54.3 mm among Boerboel litters. This large variation suggests that BPD is too variable within and among litters to be useful as a means of determining readiness for CS.

Keywords: Caesarean section, bitch, biparietal diameter, birth weight, gestational age, parturition date

6.1. Introduction

Correct assessment of readiness for CS is essential for timing elective CS during late pregnancy in the bitch and requires more precision compared to merely predicting an approximate parturition date to limit the number of days wherein parturition may be expected to occur. Readiness for CS may precede the time of spontaneous parturition in the bitch by an unknown time interval. For many bitches presented to veterinary clinicians for management of parturition, there is no information available of events during the peri-oestrous period or mid-pregnancy to help estimate gestational age. Today, in contrast to the dog, ultrasound measurement of foetal dimensions (foetal biometric measurements) are widely used for estimating gestational age in pregnant women (Henrihsen et al., 1995). The most common foetal biometric measurements in women taken are; biparietal diameter (BPD), crown rump length, head circumference, abdominal circumference and femur length (Davis et al., 1993; Verburg et al., 2008). In women, the accuracy of BPD to predict the spontaneous onset of parturition within 14 d was 89.4% ($n = 1788$, $p < 0.001$) when measured in the second trimester (Waldenström et al., 1990) and in a large meta-analysis, the discrepancy was approximately 21 d (Kurtz et al., 1980). In women, term is a well-defined safe period of four weeks (37 to 41 weeks of gestation), wherein a foetus may be delivered without having increased risk of complications compared to foetuses delivered at the time of spontaneous parturition (Fleischman et al., 2010a). This period may be described as the safe period of intervention by CS. Planned term CSs are only possible in a species when the safe period of intervention is known and a method exists to precisely determine that the gestational age of the foetuses has advanced to within that critical period. There is no literature on this safe period of intervention by CS in the bitch and we do not know how long this period is. In dogs, ultrasonographic measurement of the inner diameter of the inner chorionic cavity proved an accurate method to evaluate gestational age and to predict the day of parturition when the bitch is examined for pregnancy diagnosis during early gestation (England et al., 1990; Luvoni and Grioni, 2000; Kim and Son, 2008; Socha et al., 2008) but different equations, derived from growth curves from the various breed sizes were required (Luvoni and Grioni, 2000; Son et al., 2001; Yeager et al., 1992) or correction factors were required for giant breeds (Kutzler et al., 2003b). In late pregnancy, it was concluded that the BPD of puppies was most accurate in predicting gestational age and that the crown rump length may be difficult to measure because of foetal flexion and foetal lengths that exceeds the size of the ultrasound image (Kutzler et al., 2003b). A study on English Bulldogs suggested that CSs can be scheduled safely once the foetal BPD

has reached 29.5 mm or above but the author did not put the theory to test (Batista et al., 2014). Although numerous studies reported on gestational age estimation in dogs by ultrasonographic assessment of foetal biometric measurements, none explores the safety of using such estimated dates to time CSs (England et al., 1990; Luvoni and Grioni, 2000; Kim and Son, 2008; Socha et al., 2008; Yeager et al., 1992; Batista et al., 2014; Kutzler et al., 2003b; Son et al., 2001; Lenard et al., 2007).

Evans and Sack (1973) showed that the growth of dog foetuses is logarithmic, reaching a steady plateau during late gestation. From this, we assumed that the pattern of variation among BPD and birth weight of neonates would be similar to the pattern occurring in foetuses in late gestation. Given this assumption, the aim of the present study was to determine whether the variability in BPD and birth weight of newborn puppies in two breeds are sufficiently small to allow veterinary obstetricians to potentially plan CSs based on these measurements having reached a minimum value as suggested by Batista et al. (2014).

6.2. Materials and Methods

The protocol was approved by the Animal Ethics Committee of the Faculty of Veterinary Science, University of Pretoria, (Onderstepoort, South Africa) (Project numbers v010-14 amend 1 and v079-15). All experimental animals were housed and fed commercial dry pellets twice daily and had access to ad-lib water. All the bitches were selected from the general obstetric population because of increased obstetric risk. High-risk pregnancies were those in English Bulldogs (Wydooghe et al., 2013) and those in Boerboels with a history of dystocia (Stolla et al., 1999) or a history of having had a prior CS or those known to carry very large litters (Bennett, 1974). A trial of labour (attempt at spontaneous unassisted parturition) was declined by all the owners of the bitches in the current study. Caesarean section was performed upon the first appearance of any degree of dilatation of the cervix. The bitches where anaesthetized using an anaesthetic protocol which included the alpha2-adrenergic agonist medetomidine hydrochloride (Domitor®, Zoetis Animal Health, Sandton, South Africa) at 7 µg/kg iv as premedicant, followed one minute later by propofol (Fresenius propoven®1%, Fresenius Kabi, Midrand, South Africa), (1–2 mg/kg iv) as induction agent and sevoflurane (Safeline Pharmaceuticals, Northcliff) in oxygen (1%–2%) for maintenance of anaesthesia. The CS was performed in standard fashion as described by Gilson (2003).

Starting 15 min after delivery, the puppies were weighed on an electronic scale and the BPD

was measured in a transverse plane, with the base of the Vernier calipers dorsal to the skull, making certain that the blades contact the cranium at its widest diameter.

In order to assess the precision of measurement, 28 puppies of three litters (two English Bulldog and one Boerboel) were each weighed two times and the BPD of each measured two times. The puppies of each of these three litters were presented for the second measurement of BPD in a different order than was the case for the first. The second measurement of BPD on each puppy was done in a way that the operator could not see the value of the first measurement. All BPD measurements were done by the same operator.

The survival rates at delivery, 2 h and 7 d were recorded for all the puppies delivered.

In 31 English Bulldog bitches, 231 puppies were delivered during 36 CSs. Of these puppies, 23 from eight litters were excluded from the study and 208 puppies from 34 CSs from 31 bitches were retained in the study. BPD and birth weight were measured in each of these 208 puppies. In 70 Boerboel bitches, 673 puppies were delivered during 79 CSs. Of these puppies, 13 puppies from nine litters were excluded from the study whereas 660 puppies from 78 CSs in 70 bitches were retained in the study. BPD were measured in each of these 660 puppies and birth weight in 494 puppies from 59 litters in 54 bitches. The reason for exclusion of puppies in both breeds included *hydrops foetalis*, dysmaturity, mummified puppies and hydrocephalus.

6.3. Data analysis

For the 28 puppies that were measured two times each, the difference between the two measurements was determined by subtracting the smaller measurement from the larger. The relative difference was obtained by expressing the difference as a percentage of the smaller measurement.

With a CS in a polytocous species such as the bitch, all puppies in a litter are delivered simultaneously and readiness for CS must be determined for a litter as a whole. Therefore, litters and not puppies (foetuses in the clinical setting) were the experimental units**.** Mixed-effects regression was used to assess the effect of birth weight and litter size on BPD in the 208 puppies from 34 English Bulldog litters and 494 puppies from 59 Boerboel litters in which both, BPD and birth weight were recorded. Data were clustered in litters. The data of the two breeds were analysed separately.

Data on BPD were summarized by litter, graphically displayed and assessed by direct visual inspection. We assumed that, if a few foetal BPDs from a large litter are measured during antenatal ultrasound, the probability that the largest measured BPD will be higher than the median is high. (Basic probability theory suggests that the probability is 50%, 75%, 88% and 94% if one, two, three or four BPDs from the litter are measured). We further assumed that ultrasound during late gestation would enable one to measure foetal BPD to the nearest millimetre. Given these assumptions, we determined the frequency with which the median BPD was at least one millimetre higher than the maximum of another.

6.4. Results

The mean birth weight and BPD of the 28 puppies that were weighed two times were 359.8 g (SD 54.0) and 38.2 mm (SD 3.80). The mean difference between the two measurements in each puppy was 1.92 g (SD 1.27) for birth weight and 0.53 mm (SD 0.30) for BPD, with mean relative differences of 0.54% (SD 0.348) for birth weight and 1.42% (SD 0.866) for BPD.

In both breeds, BPD depends on the birth weight of puppies ($P < 0.001$) but not on litter size ($p = 0.18$ and $P = 0.93$, for English Bulldogs and Boerboels, respectively). Controlling for litter size, the value of BPD increased by 0.038 mm (95% CI 0.034–0.043) for each one-gram increase in the value of birth weight of English Bulldog puppies and by 0.024 mm (95% CI 0.023–0.026) in Boerboel puppies. Figure 6.1 shows the positive relationship between BPD and birth weight in both breeds.

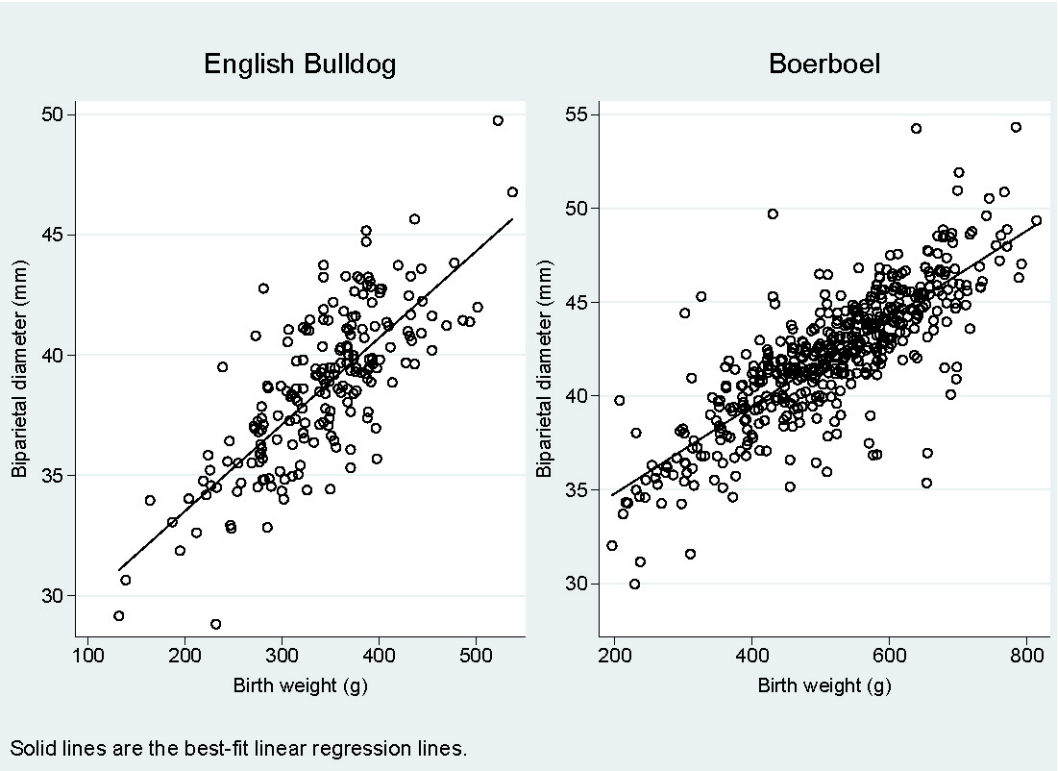


Figure 6.1

Scatterplot showing that the value of biparietal diameter of English Bulldog- and Boerboel puppies increases with the value of birth weight

Figure 6.2 shows the BPD of all puppies within their litters. Over all puppies, ignoring the fact that the data were clustered in litters, the BPD varied from 28.8 to 49.8 mm (mean 38.63 mm, SD 3.20 mm) in the 208 English Bulldog puppies and from 27.8 to 54.3 mm (mean 42.04, SD 3.60) in the 660 Boerboel puppies. The range in BPD among puppies of the same litter varied from 0 to 12 mm (mean 4.99 mm, SD 2.78 mm) in the 34 English Bulldog litters and from 0 to 17.99 mm (mean 7.63 mm, SD 3.61 mm) in the 78 Boerboel litters.

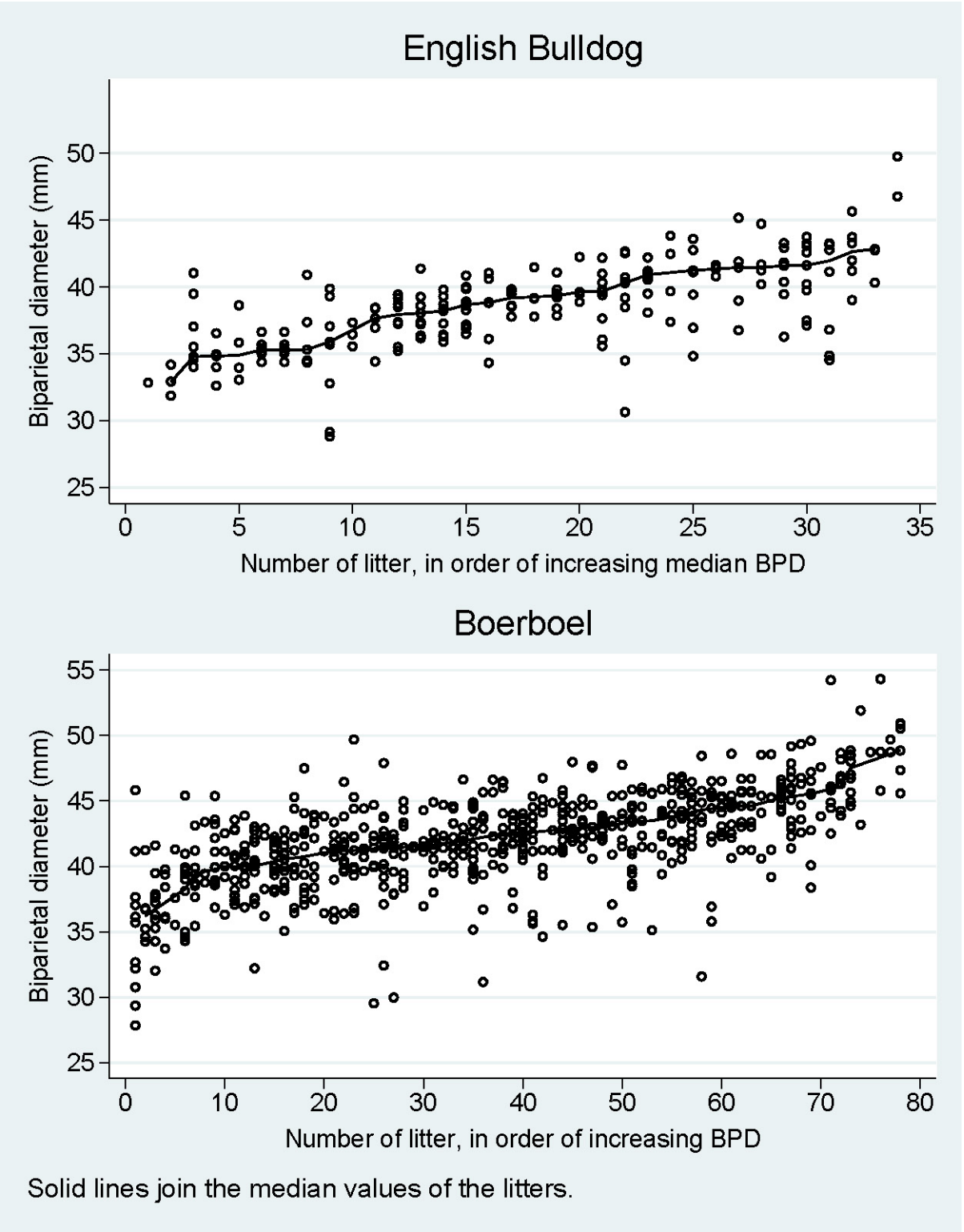


Figure 6.2

Biparietal diameter of the puppies of 34 English Bulldog litters and 78 Boerboel litters

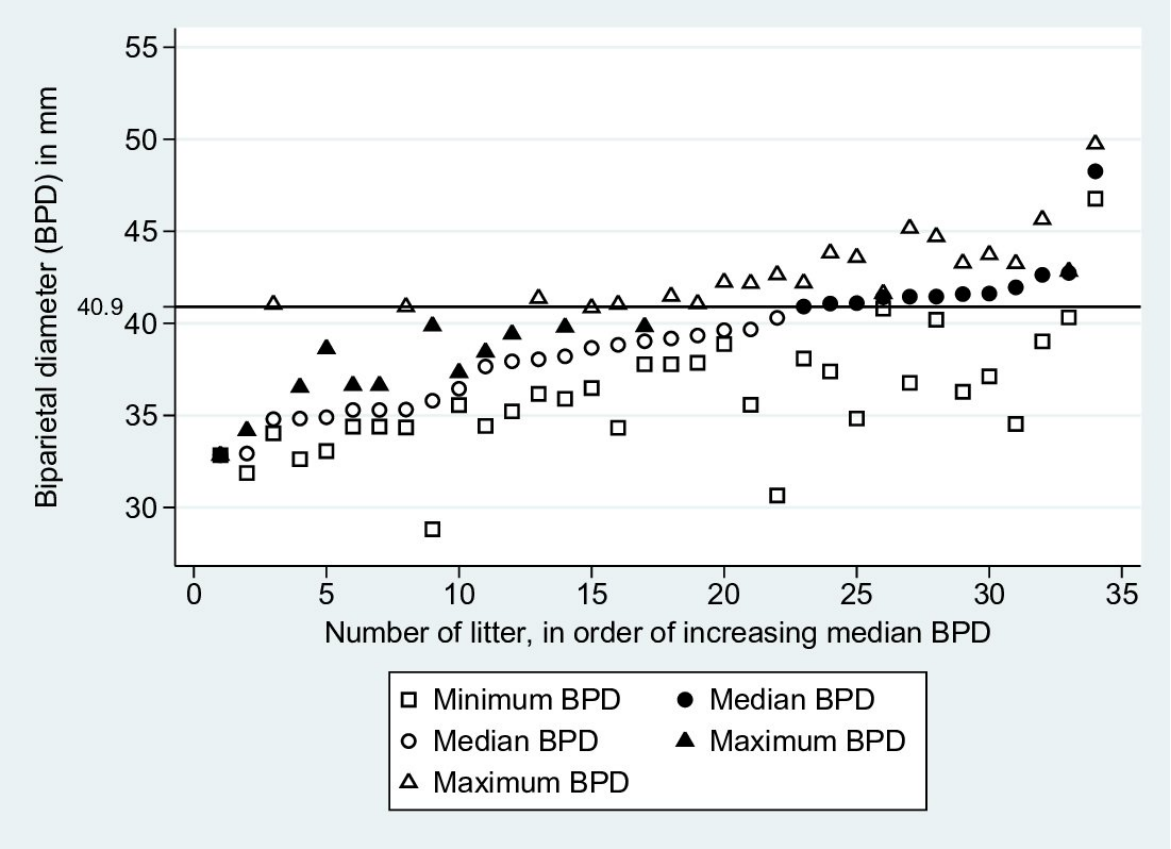


Figure 6.3

The minimum, median and maximum biparietal diameters of 34 English Bulldog litters

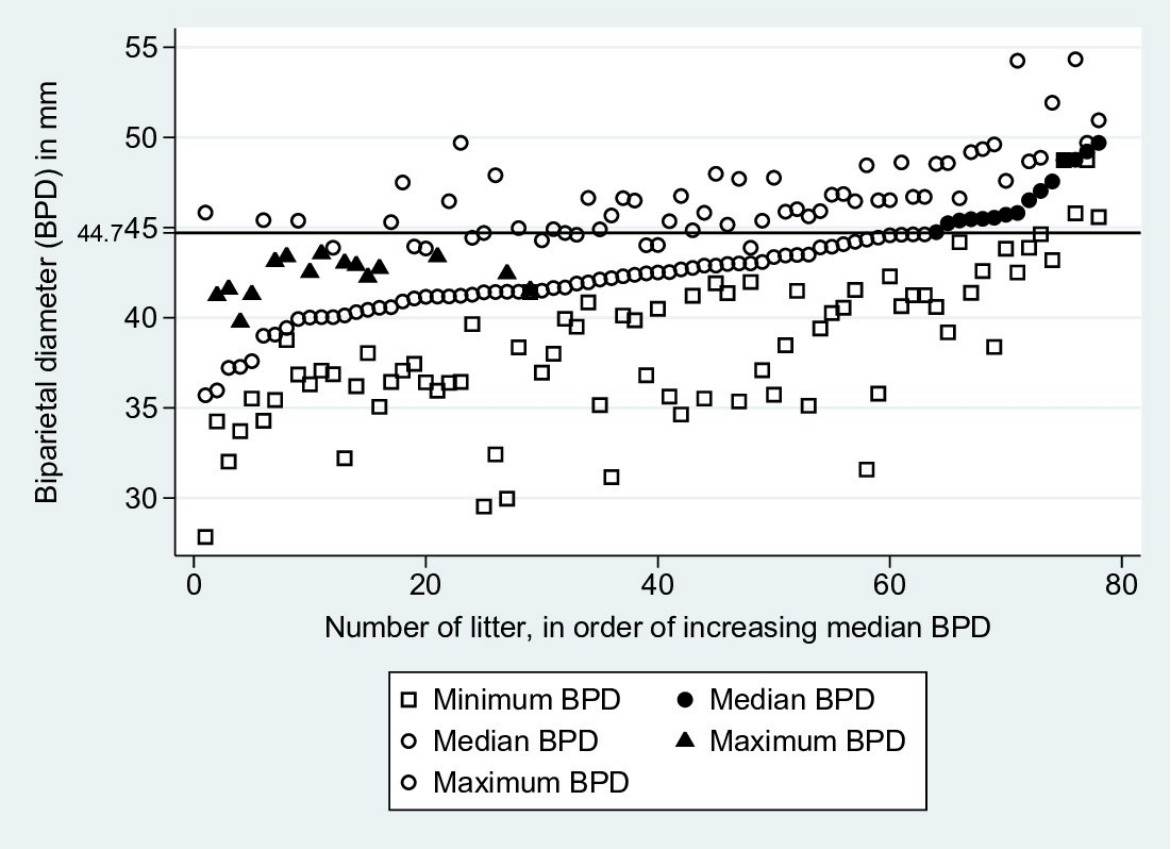


Figure 6.4
The minimum, median and maximum biparietal diameters of 78 Boerboel litters

The median BPD of 12 English Bulldog litters (Figure 6.3, solid circles) was 40.9 mm or higher, which is at least 1 mm larger than the maximum BPD of 12 other English Bulldog litters (Figure 6.3, solid triangles). The median BPD of 15 Boerboel litters (Figure 6.4, solid circles) was 44.7 mm or higher, which is at least 1 mm larger than the maximum BPD of 15 other Boerboel litters (Figure 6.4, solid triangles).

Thirty nine of the 231 English Bulldog puppies from 18 litters died before the age of 7 d, resulting in an overall survival ratio of 0.82. Among the 673 Boerboel puppies, 72 from 43 litters died, resulting in an overall survival ratio of 0.89.

6.5. Discussion

The precision of measurement of both the BPD and birth weight was high. Because, in the current study, there was a low mean difference between the two measurements of the BPD 0.53 mm (SD 0.30, n = 28) and because there was a large range and SD for the BPD, 28.8 mm to 49.8 mm (mean 38.63 mm, SD 3.20, n = 208) and 27.8 mm to 54.3 mm (mean 42.04, SD

3.60, n = 660) in the English Bulldog and Boerboel puppies respectively, it is unlikely that lack of precision of the method of measurement may have influenced our results.

The current study established the normal ranges for BPD for two breeds 15 minutes after delivery by CS at full term. The growth curve of canine foetuses is logarithmic with a steady plateau towards the end (Evans and Sack, 1973). For the purposes of the current study, only the last few days of gestation are of interest. Given the findings of Evans and Sack, we assumed that the variation in BPD measured by Vernier caliper within minutes after birth reflects the variation in BPD of foetuses measured by ultrasonography during late gestation.

The current study showed large variability in BPD among litters and among litter mates in both breeds. The ranges of the BPD for English Bulldog puppies (28.8 mm to 49.8 mm) and for Boerboel puppies (27.8 mm to 54.3 mm), found minutes after birth were markedly larger than the narrow range found by Batista et al. (2014) mean value (±SEM) of BPD 30.8 ± 0.1 mm (range 29.5–31.8 mm, n = 126). The median BPD of 12 English Bulldog litters was at least one millimetre larger than the maximum BPD of 12 other English Bulldog litters from the 34 litters studied. Similarly, the median BPD of 15 Boerboel litters was at least one millimetre larger than the maximum BPD of 15 other Boerboel litters from the 78 litters studied. Assuming that ultrasound enables one to measure foetal BPD to the nearest millimetre and that the variation in neonatal BPD is similar to the variation in foetal BPD during late gestation, this large variation should also be evident upon ultrasound examination of foetuses during late gestation.

The range in days whereby a method predicts the date of parturition is of clinical importance when such method is intended to establish readiness for CS in the bitch. Based on current knowledge it may be assumed that a foetus can be delivered and remain viable without assistance if it is delivered 48 h or less prior to the onset of spontaneous parturition (Baan et al., 2005; Levy et al., 2009; Vannucchi et al., 2012). Therefore, when determining readiness for CS, the method used to predict the date of parturition should be sufficiently accurate and precise to ensure that the pregnancy has advanced to within this critical 48 h period. Beccaglia and Luvoni (2006) showed that foetal BPD predicted parturition date to within ± 1 d in 75% and 63% and within ± 2 days in 88% and 81% of small and medium size bitches, with the precision not affected by litter size or sex ratio (Beccaglia and Luvoni, 2006).

The lowest median BPD of an English Bulldog litter was 32.8 mm and the highest 48.3 mm, constituting a difference in median BPD of 15.4 mm or 47% of the lowest median. Although

we did not measure how long it takes for the BPD of English Bulldog foetuses to grow from 32.8 to 48.3 mm, using Evans and Sack's notion of the nature of the growth curve of dog foetuses, we postulate that it would be more than two days. Given this and assuming for now that all BPDs in a litter are equal—which they are not—we would have a problem in deciding on a cut-off BPD by which to determine when an English Bulldog litter is sufficiently mature for delivery by CS: Proposing to use the smallest median BPD (32.8 mm) as the cut-off would most likely have caused us to declare the litter that eventually had a median BPD of 48.3 mm ready for delivery more than two days before cervical dilatation. Using any value above 32.8 mm as cut-off would have caused us to never declare litters with median BPDs that were below the cut-off at full term as being ready for delivery by CS. To further complicate matters we must acknowledge that BPD varies among newborn puppies—and therefore also full-term foetuses—in a litter. Using any cut-off may cause one to declare a litter ready for CS or not yet ready depending on which BPDs of a litter one measures. This problem also presents itself for the Boerboel and perhaps for various if not all other breeds.

England et al., (1990) and Lenard et al., (2007) failed to accurately determine litter size in pregnant bitches by means of ultrasonography, exemplifying the limitation of using ultrasonography to observe each foetus in a litter, and observing it once only. Being impossible to accurately count the number of foetuses during mid-pregnancy, suggests that it may be more difficult to make foetal biometric measurements of all the foetuses in each litter in late pregnancy. This would be so because foetuses—especially their calcified skeletons—and a gas-filled intestinal tract in the near ultrasonographic field may both obscure foetuses in the distant field. The model used in the current study overcame these limitations of ultrasound by allowing us to obtain measurements of all the puppies in all the litters. This enabled us to accurately assess variability of the BPD and birth weight among puppies of the same breed and within the same litter. Another limitation that was overcome in the current study was the precision attained in individual measurements of the BPD. This was because the BPD in all puppies could be measured across identical planes. In contrast, the accuracy of ultrasonographically obtained foetal biometric measurements depends on the spatial arrangements of the foetuses within the uterus and ultrasonographic planes (Luvoni and Grioni, 2000) and may further be influenced by intra- and inter observer variability (Dudley, 2005).

The results of the current study show that the BPD of both breeds were positively correlated to the birth weight, which also showed large variability. This suggests that the variability of any

foetal biometric measurement should be critically evaluated before it may be considered for determining readiness for CS.

The observation that litter size did not influence BPD and birth weight suggests that the size of the foetus is likely influenced by factors other than litter size, which may include the genotype of the foetuses.

The puppy survival rates at 7 d in the current study compare favourably to the puppies alive at 2 h after CS in non-brachiocephalic breeds 87% (Moon et al., 1998) and puppies alive at 2 hours post CS in brachiocephalic breeds 79% (Wydooghe et al., 2013). The high puppy survival rate obtained in the current study not only confirms good timing of the CS and a safe anaesthetic protocol, but also that the BPD and birth weight values obtained are those of mature puppies of generally normal viability.

We assumed that foetuses suffering from *hydrops foetalis* and those that are unusually small or dysmature would be evident on ultrasonography. Not knowing whether their BPDs are representative of the remainder of the litter, they would most likely have been ignored in the clinical setting when estimating whether a bitch is ready for CS or not. It was therefore just to exclude the 23 English Bulldog puppies and 13 Boerboel puppies from the current study.

6.6. Conclusions

Results of this study show that even with the most reliable measurement of BPD or volumetric measurement to estimate birth weight, their variability among littermates and within litters close to the time of spontaneous parturition is so large that they are not suitable to predict readiness for CS of dog foetuses.

6.7. Author contributions

J O Nöthling was the supervisor of the scientific protocol and assisted in drafting the protocol and manuscript and performed the statistics. K.G.M. De Cramer was the main person involved in experimental work and wrote the protocol and manuscript.

6.8. Conflicts of interest

The authors have declared no conflicts of interest.

Chapter 7. Clinical impact in bitches of measuring progesterone with the Immulite® 1000 lkpw1 assay compared to the coat-a-count® radioimmunoassay

The content of this chapter will be submitted for publication in a different format as an article by J O Nöthling and K G M De Cramer under the title "Clinical impact in bitches of measuring progesterone with the Immulite® 1000 LKPW1 assay compared to the Coat-A-Count® radioimmunoassay".

Abstract

The Coat-A-Count® radioimmunoassay has been long and widely used to determine the concentration of progesterone in serum or plasma of bitches (progRIA) but was discontinued in 2014. The Immulite® 1000 LKPG1 chemiluminescence immunoassay gained prominence since 2003 to determine the concentration of progesterone in serum of bitches but the assay changed in 2012 (Immulite® 1000 LKPW1). This study assessed the agreement between progRIA and the concentration of progesterone in serum of bitches measured with LKPW1 (progImm), determined the ability of progImm to estimate various time points during oestrus and compared progRIA and progImm 2 and 3 d after the first or only day of the LH surge (LH1). On average, the value of progImm was 85% of that of progRIA (95% CI 58% to 112%, n = 110). ProgImm exceeded 5.1 nmol/L or 13.6 nmol/L on the same day that progRIA first exceeded 6 nmol/L or 16 nmol/L or the day before in 90% (n = 31) and 97% (n = 35) of oestrous periods. ProgImm first exceeded 5.4 nmol/L on LH1 or the day before in 95% (n = 25) of oestrous periods. The mean of progRIA was 1.5 and 1.2 nmol/L higher than the 10.1 and 16.7 nmol/L of progImm 2 and 3 d after LH1 ($P \leq 0.001$). In conclusion, progImm underestimated progRIA, and the days on which progImm first exceeded 5.1 nmol/L, 13.6 nmol/L and 5.4 nmol/L were effective in estimating the days on which progRIA reached 6 nmol/L or 16 nmol/L or LH1.

Key words: progesterone, bitch, radioimmunoassay, Coat-A-Count, Immulite, LH

7.1. Introduction

The concentration of progesterone in blood plasma (plasma) or serum of bitches is determined

and used for various clinical decisions. One such decision is deciding whether a bitch is in anoestrus: concentration ≤1.3 nmol/L (Jeffcoate, 1992) or ≤1.6 nmol/L (Concannon, 2011). Another is identifying the first day of the oestrous cycle on which the concentration exceeds 6 nmol/L, as that day allows one to predict the day on which the highest fertility should be expected with the use of frozen-thawed spermatozoa (Steckler et al., 2013). Another is identifying the day on which the LH surge is estimated to start: 3 nmol/L (Concannon, 2011). Yet another is identifying the day on which the LH peak is estimated to occur: 5.1 nmol/L (Concannon et al., 1975) or 8.3 nmol/L (Concannon et al., 1977a) or 9.8 nmol/L (Bergeron et al., 2013). Another is to estimate the day of ovulation: 26 of 39 bitches ovulated once the concentration of progesterone in plasma reached 15.9 nmol/L and 55 of 59 within 1 d thereof, whereas the mean concentration at the time of ovulation was 19.4 nmol/L (SD 3.72) (Groppetti et al., 2015). Ovulation in the bitch occurs 2–3 d after the LH surge (Concannon et al., 1977a) and the time of ovulation has been estimated indirectly from the concentration of progesterone in serum or plasma 2 d after the LH peak (Bouchard et al., 1991). Finally, PC of 16 nmol/L or lower would indicate that near-term bitches are within 36 h (Concannon et al., 1978) or 30 h (Baan et al., 2005) from whelping.

Following ovulation, the concentration of progesterone not only increases rapidly but its value at comparable times across bitches vary progressively more as the time since ovulation increases. So, for example, the concentration of progesterone in plasma varied from 29 to 83 nmol/L at the time of fertilisation and from 48 to 102 nmol/L at the onset of cytological dioestrum (Badinand et al., 1993)and from 47 to 254 nmol/L at the peak during early dioestrum (Concannon, 2011).

From the above follows that it is more important for an assay used to measure the concentration of progesterone in serum or plasma to be precise and accurate at concentrations associated with ovulation or lower. The Coat-A-Count ^{125}I radioimmunoassay was more suitable than a qualitative ELISA kit to determine the optimal mating time of bitches (Van Klaveren et al., 2001). A radioimmunoassay used to measure the concentration of progesterone in the plasma of bitches had better assay performance than the qualitative and quantitative ELISA assays it was compared to (Moxon et al., 2010). The production of the Coat-A-Count ^{125}I radioimmunoassay for progesterone (Siemens)—which has been used to determine the concentration of progesterone in the serum or plasma of bitches for decades (Reimers et al., 1991; Gerstenberg and Nöthling, 1995; Okkens et al., 2001; Kutzler et al., 2003a; Luz et al.,

2006; Steckler et al., 2013)—was due to stop during the last quarter of 2014.

Reports since 2003 show that the chemiluminescent immunoassay Immulite has become popular for measuring the concentration of progesterone in the serum of bitches (Kutzler et al., 2003a; Volkmann, 2006; Chapwanya et al., 2008; Rota et al., 2015; Schmicke et al., 2016).

The Immulite chemiluminescence assay has been compared to the Coat-A-Count ^{125}I radioimmunoassay (Kutzler et al., 2003a; Volkmann, 2006). In addition, Chapwanya et al. (2009) compared it to another radioimmunoassay that was described by Hoffmann et al. (1973). In these studies serum samples were used that contained progesterone at concentrations of 1.56–71 nmol/L (Kutzler et al., 2003a), near zero to 95 nmol/L as estimated from Figure 2 in Volkmann (2006) and 0.7–102 nmol/L (Chapwanya et al., 2008). These concentrations extended well above those expected at ovulation or earlier during the oestrous cycle, or those expected near parturition. These studies reported strong, positive, linear correlations between the concentrations determined by Immulite compared to those determined in the same serum samples by radioimmunoassay. What appears as strong linearity over such a large range in PC may mask weak linearity among concentrations measured with radioimmunoassay and Immulite lying near the low, clinically important end of the range included in the correlation analysis (Fisher L.D. and Van Belle G., 1993).

A linear regression showed that the concentration of progesterone measured by Immulite in the serum of bitches was expected to be equal to 95.1% of the concentration measured with the Coat-A-Count ^{125}I radioimmunoassay in nmol/L, minus 0.51 nmol/L (Kutzler et al., 2003a). This regression not only suggests that the concentration of progesterone measured with Immulite is expected to be lower than that measured by radioimmunoassay, but that the difference will be larger at low concentrations of progesterone. In another study, Volkmann (2006) reported that the concentration of progesterone in the serum of bitches, as measured with Immulite was about two-thirds of the concentration measured in serum with the Coat-A-Count ^{125}I radioimmunoassay, which is even lower than what had been reported in Kutzler et al. (2003). In contrast, Chapwanya et al. (2008) reported that Immulite showed higher concentrations than the radioimmunoassay that they used.

Since the studies by Kutzler et al. (2003a) and Volkmann (2006) and Chapwanya et al. (2008), the Immulite assay (Catalogue number LKPG1), which was a 2-cycle competitive immunoassay that used a bead coated with monoclonal mouse anti-progesterone antibodies,

changed to a one-cycle competitive immunoassay using a bead coated with polyclonal rabbit anti-progesterone antibodies, referred to by catalogue number LKPW1 (Ludewig et al., 2012; Schmicke et al., 2016). Schmicke et al. (2016) reported that LKPW1 showed distinctly lower concentrations of progesterone than LKPG1. Immulite LKPW1 used at the time of ovulation in 7 bitches showed serum PC from 6.4 to 14.3 nmol/L with a mean of 10.8 nmol/L (SD 2.86), which is distinctly lower than the widely-accepted concentrations of 16–25.4 nmol/L.

Using plasma or serum had no effect on the concentration of progesterone measured with a chemiluminescence assay (Tahir et al., 2013). In contrast, serum of bitches yielded higher concentrations of progesterone than plasma when an ELISA was used (Thuróczy et al., 2003). The manufacturers of the Immulite 1000 assay prescribes the use of serum. Kutzler et al. (2003a) and Volkmann (2006) used serum to compare the concentrations of progesterone measured with the Coat-A-Count radioimmunoassay to those measured with the Immulite 1000 progesterone assay. The Coat-A-Count radioimmunoassay has, however, also been used on the plasma of bitches (Gerstenberg and Nöthling, 1995; Okkens et al., 2001; Van Klaveren et al., 2001; Luz et al., 2006; Steckler et al., 2013). In the assay brochure, the suppliers of the Coat-A-Count radioimmunoassay state that, in humans, the mean concentration of progesterone in heparinized plasma was almost identical to the mean in serum. They also state that the concentration of progesterone measured with Immulite is approximately 90% of the mean obtained with the radioimmunoassay.

Given the differences in the results obtained by Kutzler et al. (2003a) and Volkmann (2006), the different effects of plasma and serum reported by Thuróczy et al. (2003) and Tahir et al. (2013), the wide-spread use of the Coat-A-Count radioimmunoassay and the Immulite 1000 progesterone assay in practice and research, as well as the effect of the change in the Immulite assay in 2012 (Schmicke et al., 2016), there was a need to compare the concentrations of progesterone measured with the Coat-A-Count radioimmunoassay in plasma of bitches with those measured in serum with the Immulite 1000 LKPW1 assay before Coat-A-Count progesterone assay was removed from the market.

The aim of Phase 1 of this study was to compare the concentrations of progesterone in the plasma of dogs as determined with the Coat-A-Count radioimmunoassay (abbreviated as RIA) and the Immulite 1000 LKPW1 Progesterone assay (referred to as Immulite) for concentrations of progesterone at or below those typically associated with ovulation.

Should RIA and Immulite yield different concentrations of progesterone in the same bitch at the same time, it is important to know the clinical impact of such differences. The aim of Phase 2 was to determine the clinical impact of using Immulite instead of RIA in bitches to (1) identify the date on which the concentration of progesterone in the plasma or serum first exceeds 6 nmol/L, (2) identify the date on which the concentration of progesterone in the plasma or serum first exceeds 16 nmol/L, (3) to identify the concentrations of progesterone in the plasma or serum on the first or only day of the LH surge in the serum, (4) to determine whether the number of days between the first or only date on which the LH surge and the date on which it is expected to occur based on the concentration of progesterone in plasma or serum differs for RIA and Immulite and (5) to compare the concentrations of progesterone found with RIA and Immulite 2 and 3 d after the first or only day of the LH surge.

7.2. Materials and methods

The project was approved by the Animal Ethics Committee of the Faculty of Veterinary Science of the University of Pretoria (Project V071-13 and Project V010-14) .

7.2.1. Phase 1

Two vials of blood, one in a 10-ml red-stoppered glass vial (BD Vacutainer ® (Plain), BD Plymouth, UK) and another in a 10-ml green-stoppered heparinized glass vial (BD Vacutainer ® (170 iu lithium heparin), BD Plymouth, UK) were collected at the same time, once every 24 or 48 h on 401 occasions from 62 bitches during pro-oestrus and oestrus and four times per day from 24 bitches during the last few days before parturition. The heparinized blood was centrifuged within 30 min after collection, following which the plasma was collected. The red-stopped vials were left standing at room temperature for approximately 2 h for the clot to form and contract, following which the serum was collected. In this way 401 contemporaneous pairs of plasma and serum were obtained. Plasma and serum were stored in cryotubes labelled with unique 4-digit random numbers only and frozen at -18°C until evaluation.

All determinations of the concentration of progesterone in plasma were done with RIA (Coat-A-Count® radioimmunoassay; Siemens Health Care Diagnostics Inc. Los Angeles, CA 90045 USA) and in serum with Immulite (Immulite® 1000 Catalogue number LKPW1) Progesterone assay; Siemens Medical Solutions Diagnostics, 5210 Pacific Concourse Drive Los Angeles, CA 90045-6900 USA). All determinations were done in duplicate (two replicates simultaneously done in the same assay). The mean of the two replicate concentrations of

progesterone determined with RIA and Immulite are respectively referred to as progRIA (progRIAs in the plural) or progImm (progImms in the plural).

Using a 100-well RIA kit, the concentration of progesterone was determined in an initial selection of 41 plasma samples from bitches estimated to have been in late pro-oestrus or in early oestrus by means of vaginal cytology and vaginal speculum examination. The 7 standards supplied with the kit ranged in concentration from zero to 127.2 nmol/L and were included in the assay. This process was repeated until 285 progRIAs were determined from which 89 could be selected that were as near as possible to uniformly distributed between 0.5 and 25 nmol/L, which is the range of interest. To further improve the uniform distribution of progRIAs within this range, 21 plasma samples with suitable progRIAs were selected from those of the preparturient bitches. Using three 100-tube Immulite assays, progImm was also determined in each of the 110 serum samples that had been collected at the same time as these 110 selected plasma samples.

Six of the 110 plasma samples—two with progRIAs near the low end of the range (2 nmol/L and 4 nmol/L), two near the middle of the range (13 nmol/L and 14 nmol/L) and two near the upper end thereof (22 nmol/L and 23 nmol/L)—were each analysed with RIA on six or seven occasions, using a different assay on each occasion. The six serum samples that were collected from the same bitches at the same time as the six plasma samples just described were each analysed on three occasions, using three different Immulite assays.

These 110 progRIAs and progImms, which came from 40 bitches (18 English Bulldogs, 22 Boerboels), were used to compare the agreement between the Coat-A-Count® and Immulite® 1000 LKPW1 progesterone assays.

7.2.2. Phase 2

In addition to the 89 of which progImm had already been determined in Phase 1, progImm was determined in the serum of another 105 of the 285 sample pairs for which progRIA had been determined in Phase 1 to yield 194 contemporaneous pairs of progRIA and progImm from 43 bitches (18 English Bulldogs and 25 Boerboels).

In an attempt to identify the LH surge we used the Witness® LH test (Synbiotics Europe, Lyon, France) on 203 of the serum samples collected in Phase 1. Of these, 174 were members of the 285 contemporaneous pairs for which progRIAs had been determined as described in Phase 1

and 159 were members of the 194 pairs for which both, progRIA and progImm had been determined.

Following the above, progRIA and progImm were both available from 32 bitches on the day that progRIA first exceeded 6 nmol/L as well as the day before, 35 on the day on which progRIA first exceeded 16 nmol/L as well as the day before, 27 on the first or only day that the concentration of LH in the serum was elevated, 27 two days after and 25 three days after the first or only day of the LH surge. Each bitch was represented during one oestrous period only.

7.3. Data analysis

7.3.1. Phase 1

a) Comparing the precision of RIA and Immulite

The intra assay CV for RIA and Immulite were respectively determined for each of the 110 plasma- or serum samples, using the PC of the two replicates that were simultaneously done on each sample. The mean of the 110 CVs for RIA and Immulite are reported as their intra-assay CVs.

The progRIAs of the six plasma samples that were each analysed in six or seven assays were used to calculate the interassay CV for RIA. The progImms of the six serum samples that were each analysed in three assays were used to determine the interassay CV for Immulite.

The percentage difference between the maximum and minimum progesterone concentration of two replicates done on the same plasma sample (RIA) or serum sample (Immulite) in the same assay were compared using Wilcoxon's rank sum test.

b) Assessing the agreement between RIA and Immulite

To remove heteroskedasticity, the square root of progImm was regressed on the square root of progRIA. Squaring both sides of the derived regression equation provided a formula by which to predict progImm from progRIA.

ProgImm was then expressed as a percentage of progRIA and the percentages regressed on progRIA. Two of the percentages were above 150. They occurred at low values of progRIA (1.18 nmol/L and 0.52 nmol/L) and were 3.7 and 6.5 standard deviations above the mean percentage. These two outliers were excluded from the regression.

7.3.2. Phase 2

Having shown in Phase 1 that progImm underestimated progRIA, we determined whether progRIA at each of five clinically important time points were higher than progImm at the same times. The time points were (1) the day on which progRIA first exceeded 6 nmol/L, (2) the day on which progRIA first exceeded 16 nmol/L, (3) the first or only day of the LH surge, (4) 2 d after the first day of the LH surge and (5) 3 d after the first day of the LH surge. A one-tailed paired t-test was used for these comparisons or, where the data did not meet the requirements therefore, Wilcoxon's signed rank test or the sign test was used.

The number of days from progRIA having first exceeded 6 nmol/L until progImm did so was determined by direct inspection of the dataset. The same was done to determine the number of days from progRIA having first exceeded 6 nmol/L until progImm first exceeded 5.1 nmol/L, which is the expected value of progImm when progRIA was 6 nmol/L ($5.1 = 0.85 \times 6$). The proportion of bitches where progImm first exceeded 6 nmol/L on the same day as progRIA did was compared to the proportion of bitches where progImm first exceeded 5.1 nmol/L on the same day that progRIA first exceeded 6 nmol/L. The proportion of bitches in which progImm first exceeded 6 nmol/L during a 2-day period that included the day on which progRIA first exceeded 6 nmol/L was compared to the proportion of bitches in which the day on which progImm first exceeded 5.1 nmol/L did the same.

The above was repeated with respect to the interval between progRIA having first exceeded 16 nmol/L until progImm first exceeded 16 nmol/L or 13.6 nmol/L, which is the expected progImm when progRIA was 16 nmol/L ($13.6 = 0.85 \times 16$).

The number of days between the first or only date on which the LH surge occurred and the date on which it was expected to occur based on progRIA or progImm was determined by direct inspection of the data.

The proportion of bitches in which the first or only day of the LH surge occurred on the expected day based on progRIA was compared to the proportion in which it occurred on the expected day based on progImm. The proportion of bitches in which the first or only day of the LH surge occurred during a 2-day period that included the expected day based on progRIA was compared to the proportion of bitches in which it occurred during a 2-day period that included the expected day based on progImm.

Fisher's exact test was used to compare the proportions.

Data analysis was done using Stata 14 (Stata Corp College Station, Texas, USA.)

7.4. Results

7.4.1. Phase 1

a) The precision of RIA and Immulite

The intra- and interassay coefficients of variation were 5.85% and 8.45% for RIA and 6.70% and 9.16% for Immulite.

Table 7.1 shows that the distributions of the percentage difference between the maximum and minimum progesterone concentration of two replicates done on the same plasma sample (RIA) or serum sample (Immulite) in the same assay were similar. The table also shows that in 25% of samples the difference in progesterone concentration between two replicates fell between 11% and 31% for RIA and between 13% and 31% for Immulite.

Table 7.1
The percentage difference between the maximum and minimum concentrations of progesterone in two replicates simultaneously done on the same plasma or serum sample in the same Coat-A-Count® RIA or Immulite® 1000 LKPW1 assay were similar (P = 0.64)

	RIA	Immulite
Minimum	0	0
25th Percentile	3.37	2.67
Median	6.54	7.79
75th Percentile	11.41	12.54
Maximum	31.05	30.95
Number of pairs of replicates	110	110

b) The agreement between Immulite and RIA

Figure 7.1 shows that, for all but 13 of 110 blood samples progImm was lower than progRIA.

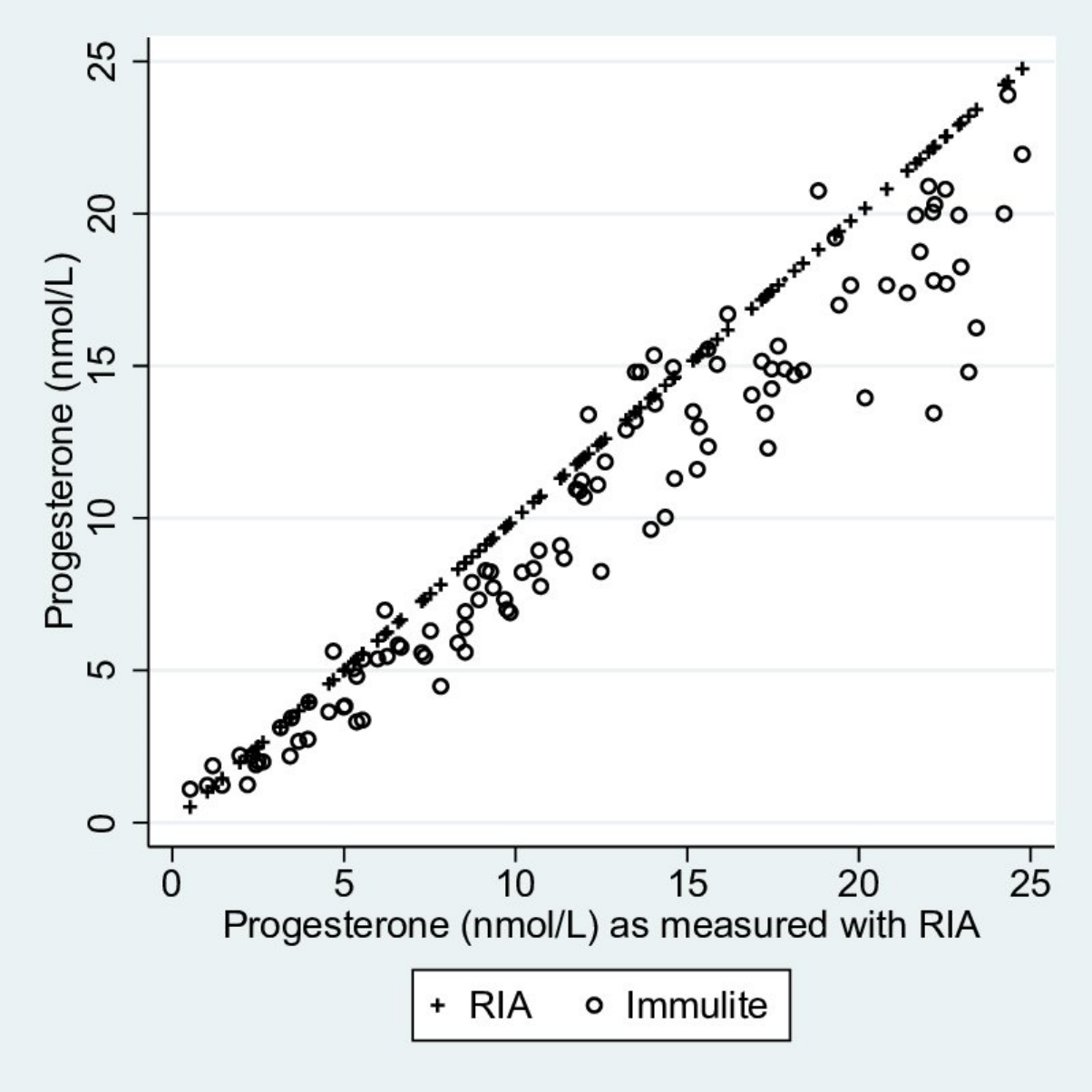


Figure 7.1

The concentrations of progesterone measured with Immulite® 1000 LKPW1 in 110 serum samples and with Coat-A-Count® radioimmunoassay in 110 plasma samples—with each pair of serum and plasma samples drawn from the same bitch at the same time—plotted against the concentrations in the 110 plasma samples

Figure 7.2 shows the strong linear relationship between the square roots of progImm against the square roots of progRIA. The linear regression of the square root of progImm on the square root of progRIA showed that the square root of progImm is highly dependent on the square root of progRIA (the F-statistic with one and 108 degrees of freedom was 1872, $P < 0.0001$).

The R-squared for this regression was 0.945, indicating that 94.5% of the variance of the square

root of progImm can be predicted from the square root of the concentration of progRIA. The coefficients for this regression are shown in Table 7.2.

Table 7.2

Coefficients of the linear regression of the square root of the concentration of progesterone in serum as measured with Immulite® 1000 LKPW1 on the square root of the concentration in plasma as measured with Coat-A-Count® RIA (progRIA, n = 110)

	Coefficient	Standard error	95% Confidence interval	P
Square root of progRIA	0.91162	0.02107	0.86985–0.95339	0.0005
Constant	0.03785	0.07375	-0.10834–0.18403	0.61

The model pertaining to the regression in Table 7.2 was as follows:

$progImm^{0.5} = 0.03785 + 0.91162 \times progRIA^{0.5} + error$, where *progImm* is the concentration of progesterone expected with Immulite, *progRIA* is the concentration of progesterone measured with RIA and *error* has the expected value of zero.

Squaring on both sides of the above equation yielded the following equation by which *progImm* may be estimated from *progRIA*:

$ProgImm = 0.001433 + 0.06901 \times progRIA^{0.5} + 0.831051 \times progRIA$

Using this equation to estimate progImm from progRIA yielded estimated values for progImm from 87% to 90% of progRIA for progRIAs of 3 nmol/L or below, and 85% to 86% for progRIAs above 3 nmol/L.

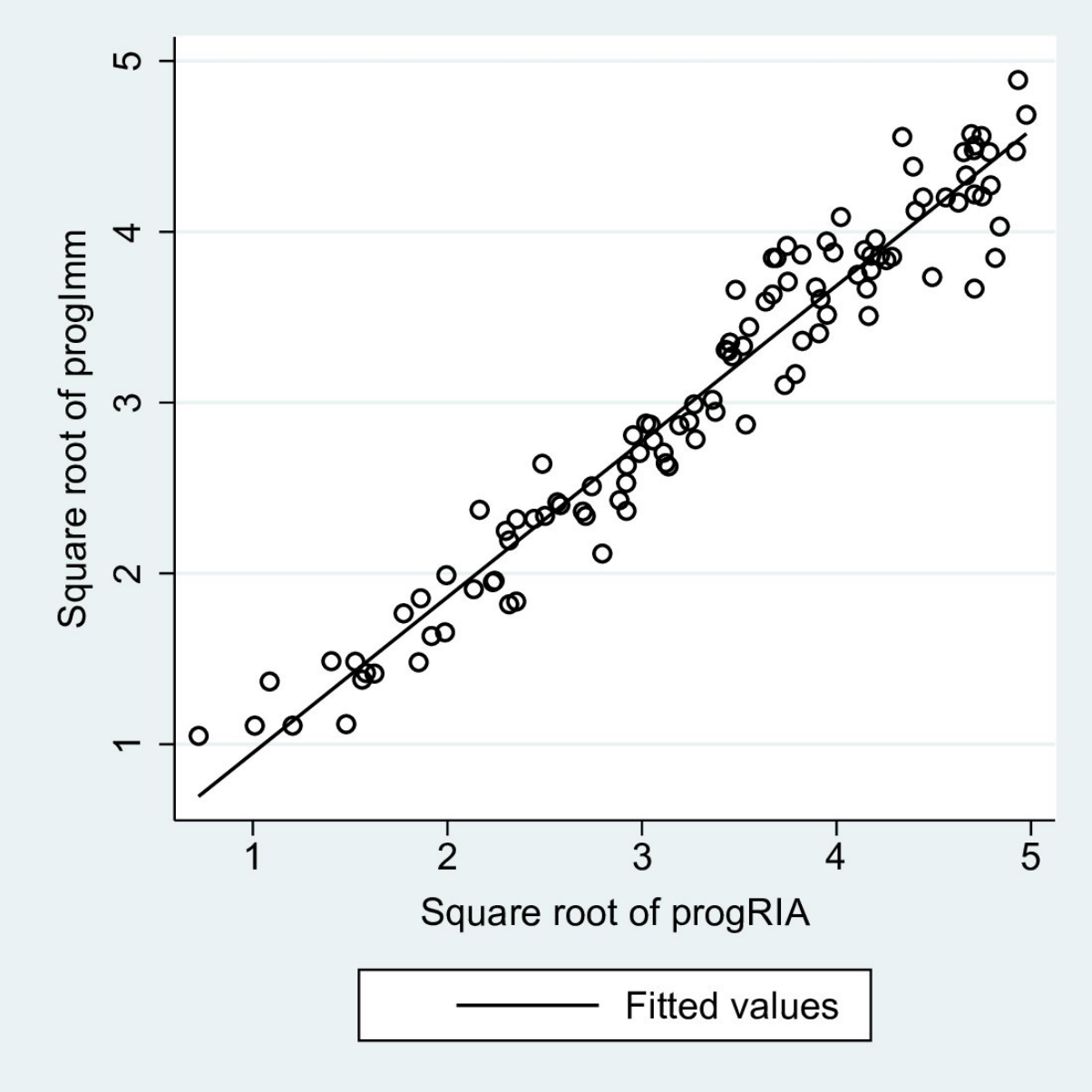


Figure 7.2

Scatterplot of the square roots of the concentrations of progesterone in serum measured with Immulite® 1000 LKPW1 (progImm) against the square roots of the concentrations of progesterone measured in plasma with Coat-A-Count® radioimmunoassay (progRIA), and the regression line (n = 110 pairs)

The linear regression of progImm—expressed as a percentage of progRIA—on progRIA showed that progImm expressed as a percentage of progRIA was independent of progRIA (the F-statistic with one and 108 degrees of freedom was 0.00, P = 0.96). The R-squared was 0.000, showing that less than 0.1% of the variance in progImm expressed as a percentage of progRIA was explained by progRIA. Table 7.3 shows the regression coefficients of this regression. The

95% confidence interval of the slope of the regression line (the coefficient for progRIA) was symmetrically arranged zero (P = 0.96), while the y-intercept (the constant) had a 95% confidence interval that was narrowly spread around the mean of 85.2% (P < 0.001).

Table 7.3

Coefficients of the linear regression of the concentration of progesterone measured with Immulite® 1000 LKPW1 in serum as a percentage of the concentration measured with Coat-A-Count® RIA in plasma on the concentration measured with the RIA (n = 108)

	Coefficient	Standard error	95% Confidence interval	P
Progesterone measured with RIA	0.0096	0.1950	-0.3770–0.3963	0.96
Constant	85.172	2.753	79.714–90.630	0.0005

In line with the regression in Table 7.3, Figure 7.3 shows that, across the range in progRIA, the predicted value of progImm is, on average, equal to 85% of progRIA. There is, however, a large scatter around the mean of 85%, as shown by the 95% confidence interval for an individual forecast lying between 58% and 112% (Figure 7.3). The mean prediction is similar to the percentages estimated with the regression equation derived from the regression of the square root of progImm on progRIA, as shown in Table 7.3.

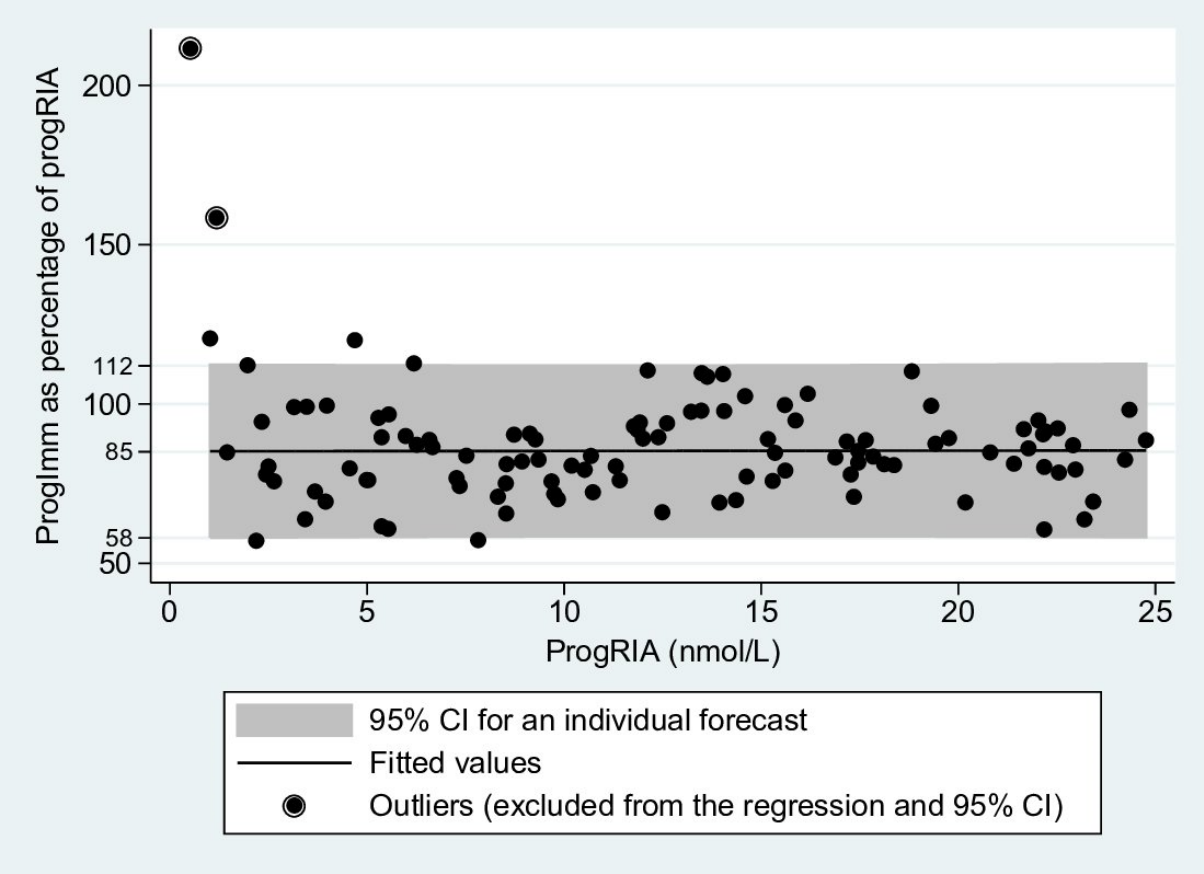


Figure 7.3
Scatterplot of the concentrations of progesterone measured with Immulite® 1000 LKPW1 (ProgImm) in serum of bitches, expressed as a percentage of the concentration measured with Coat-A-Count® radioimmunoassay (RIA) in plasma (progRIA), against progRIA (n = 110 pairs). The linear regression line with the 95% CI (mean ± 1.96 SD) for an individual forecast are also shown.

7.4.2. Phase 2

a) The impact of using Immulite to identify the day on which the concentration of progesterone measured with RIA first exceeded 6 nmol/L

Table 7.4 shows that progImm was lower than progRIA on the day that progRIA first exceeded 6 nmol/L (P = 0.035).

Figure 7.4 shows that progImm first exceeded 6 nmol/L on the same day that progRIA did in 21 (66%) of 32 bitches (95% CI 47% to 81%), which was similar to the 23 (74%) of 31 bitches (95% CI 55% to 88%) in which progImm first exceeded 5.1 nmol/L on the same day that progRIA first exceeded 6 nmol/L (P = 0.58). ProgImm first exceeded 5.1 nmol/L within 2 d of

progRIA having first exceeded 6 nmol/L, compared to a variation of 4 d in the interval between progImm reaching 6 nmol/L and progRIA having done so (Figure 7.4).

Figure 7.4 shows that the day on which progImm first exceeded 6 nmol/L coincided with the day on which progRIA first exceeded 6 nmol/L or the day following that in 26 (82%) of 32 bitches (95% CI 64% to 93%). This proportion was similar to the 28 (90%) of 31 of bitches (95% CI 74% to 98%) in which progImm first exceeded 5.1 nmol/L on the day on which progRIA first exceeded 6 nmol/L or the day preceding that (P = 0.48).

Table 7.4

Concentrations of progesterone measured with Coat-A-Count® RIA and Immulite® 1000 LKPW1 at important times during oestrus in bitches

	Minimum	25th[a]	Median	75th[b]	Maximum	Mean	SD[c]	n	P[d]
On the day that RIA exceeded 6 nmol/L									
RIA	6.2	7.0	8.1	9.3	10.5	8.11	1.38	32	0.0354
Immulite	4.1	6.2	7.1	8.3	14.2	7.65	2.09	32	
On the day that RIA exceeded 16 nmol/L									
RIA	16.2	20.2	23.0	28.3	52.6	24.67	7.43	35	< 0.001
Immulite	13.5	15.7	20.0	24.8	50.9	21.43	7.87	35	
On the first or only day of the LH surge									
RIA	1.5	4.9	6.2	7.4	10.0	6.06	2.09	27	0.007
Immulite	1.1	4.0	5.6	7.0	9.1	5.41	1.96	27	
Two days after the first or only day of the LH surge									
RIA	5.0	9.5	10.7	12.5	22.2	11.58	3.71	27	0.001
Immulite	3.8	8.3	9.5	11.7	18.8	10.08	2.96	27	
Three days after the first or only day of the LH surge									
RIA	9.8	15.2	17.8	23.0	28.5	18.95	5.30	25	< 0.001
Immulite	9.9	13.5	15.1	20.2	28.8	16.73	4.68	25	

[a] Twenty fifth percentile

[b] Seventy fifth percentile

[c] Standard deviation

[d] One-tailed test, testing whether the concentrations of progesterone measured with Immulite are lower than those measured with RIA

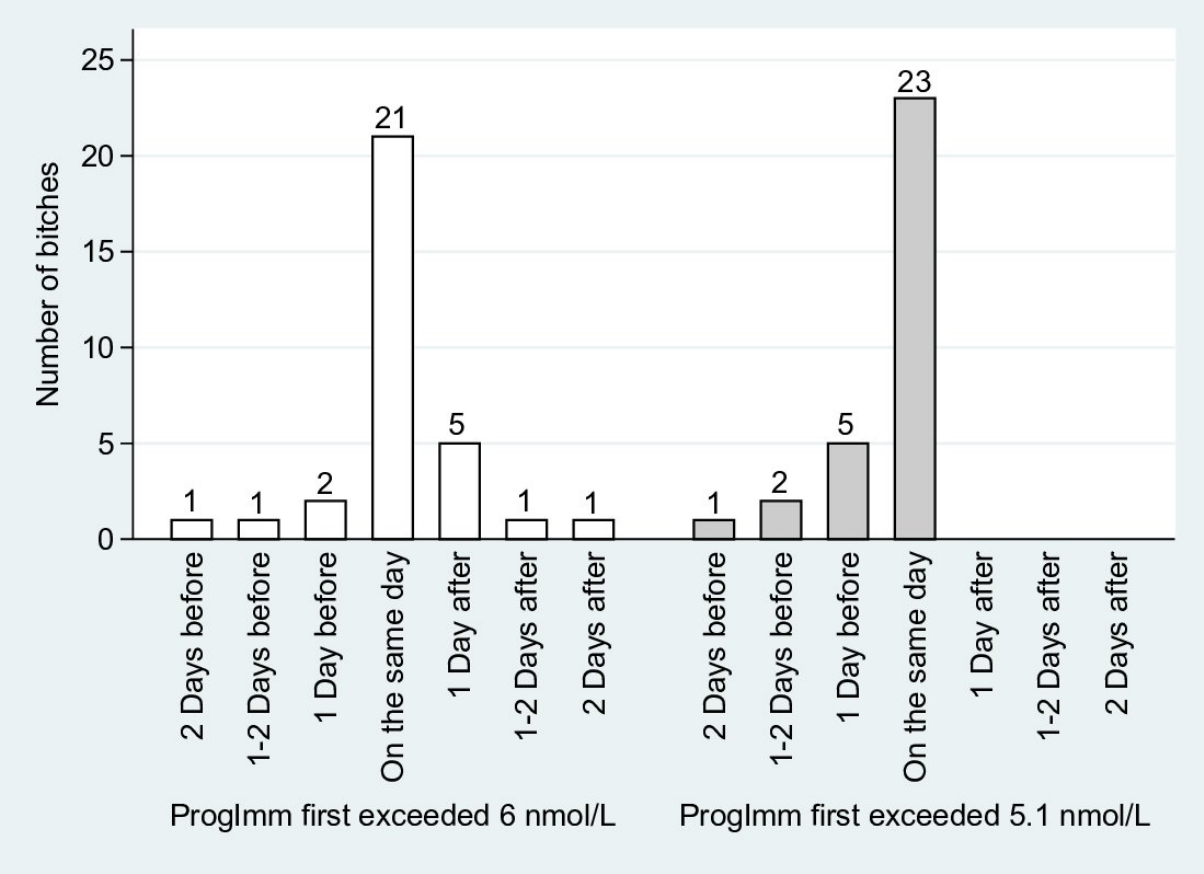


Figure 7.4

Days—relative to the day on which the concentration of progesterone measured with Coat-A-Count® radioimmunoassay in plasma of bitches first exceeded 6 nmol/L—on which the concentration measured in their serum with Immulite® 1000 LKPW1 (ProgImm) first exceeded 6 nmol/L or its predicted value of 5.1 nmol/L

b) The impact of using Immulite to identify the day on which the concentration of progesterone measured with RIA first exceeded 16 nmol/L

On the day that progRIA first exceeded 16 nmol/L progImm was lower than progRIA ($P < 0.001$, Table 7.4).

Figure 7.5 shows that progImm first exceeded 16 nmol/L on the same day that progRIA did in 25 (69%) of 36 bitches (95% CI 52% to 84%), which was similar to the 28 (80%) of 35 bitches (95% CI 63% to 92%) in which progImm first exceeded 13.6 nmol/L on the same day that progRIA first exceeded 16 nmol/L ($P = 0.42$). The range in interval between progRIA having first exceeded 16 nmol/L and progImm having first exceeded 16 nmol/L or 13.6 nmol/L was 2 d in each case (Figure 7.5).

Figure 7.5 shows that the day on which progImm first exceeded 16 nmol/L coincided with the day on which progRIA first exceeded 16 nmol/L or the day following that in 34 (94%) of 36 bitches (95% CI 81% to 99%). This proportion was similar to the 34 (97%) of 35 bitches (95% CI 85% to 100%) in which progImm first exceeded 13.6 nmol/L on the day on which progRIA first exceeded 16 nmol/L or the day before (P = 1.0).

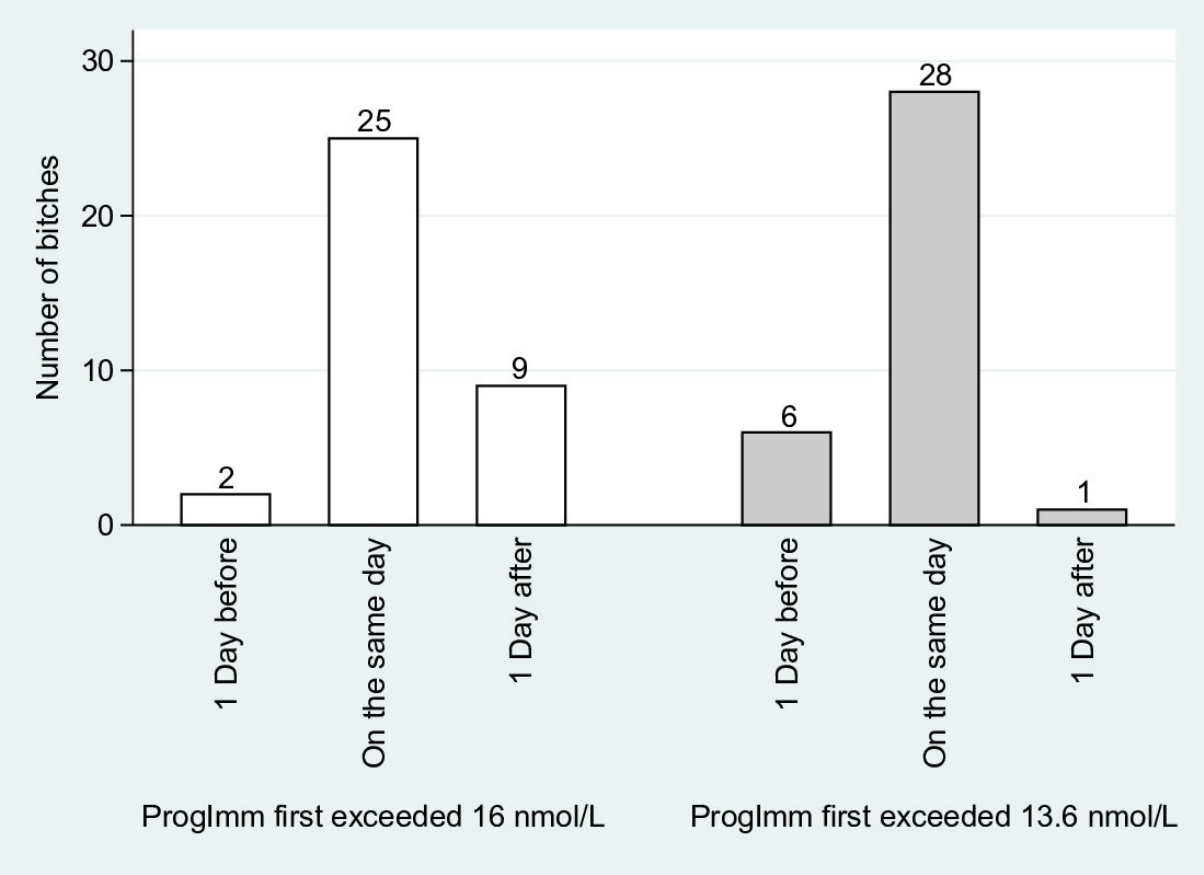


Figure 7.5
Days—relative to the day on which the concentration of progesterone measured with Coat-A-Count® radioimmunoassay in plasma of bitches first exceeded 16 nmol/L—on which the concentration measured in their serum with Immulite® 1000 LKPW1 (ProgImm) first exceeded 16 nmol/L or its predicted value of 13.6 nmol/L

c) The ability to identify the day of the LH surge using the concentration of progesterone

The LH surge was detected in 32 bitches and its duration determined in 25 by having at least one day without an elevated concentration of LH in the serum before and another after the days with elevated concentrations. The first day of the LH surge was identified in 27 bitches by having at least one day without an elevated concentration preceding a day with an elevated

concentration. The LH surge lasted one day in 18 bitches, 2 d in 5 d, 3 d in two, at least 1 d in three, at least 2 d in three and at least 4 d in one.

The mean progImm on the first or only day of the LH surge was 5.41 nmol/L, which was lower than the 6.06 nmol/L for progRIA (P = 0.007, Table 7.4).

Figure 7.6 shows that the first or only day of the LH surge occurred on the same day that progRIA first exceeded 6.06 nmol/L in 14 (52%) of 27 bitches (95% CI 32% to 71%). This proportion is similar to the 15 (60%) of 25 bitches (95% CI 39% to 79%) in which the first or only day of the LH surge occurred on the same day that progImm first exceeded 5.41 nmol/L (P = 0.59).

Figure 7.6 also shows that in 24 (89%) of 27 bitches (95% CI 71% to 98%) the first or only day of the LH surge occurred on the same day that progRIA first exceeded 6.06 nmol/L or on the preceding day. This proportion is similar to the 24 (96%) of 25 bitches (95% CI 80% to 100%) in which the first or only day of the LH surge occurred on the day that progImm first exceeded 5.41 nmol/L or on the preceding day (P = 0.61).

The range in interval between the first or only day of the LH surge and progRIA first exceeding 6.06 nmol/L was 3 d and that between the LH surge and progImm first exceeding 5.41 nmol/L 2 d.

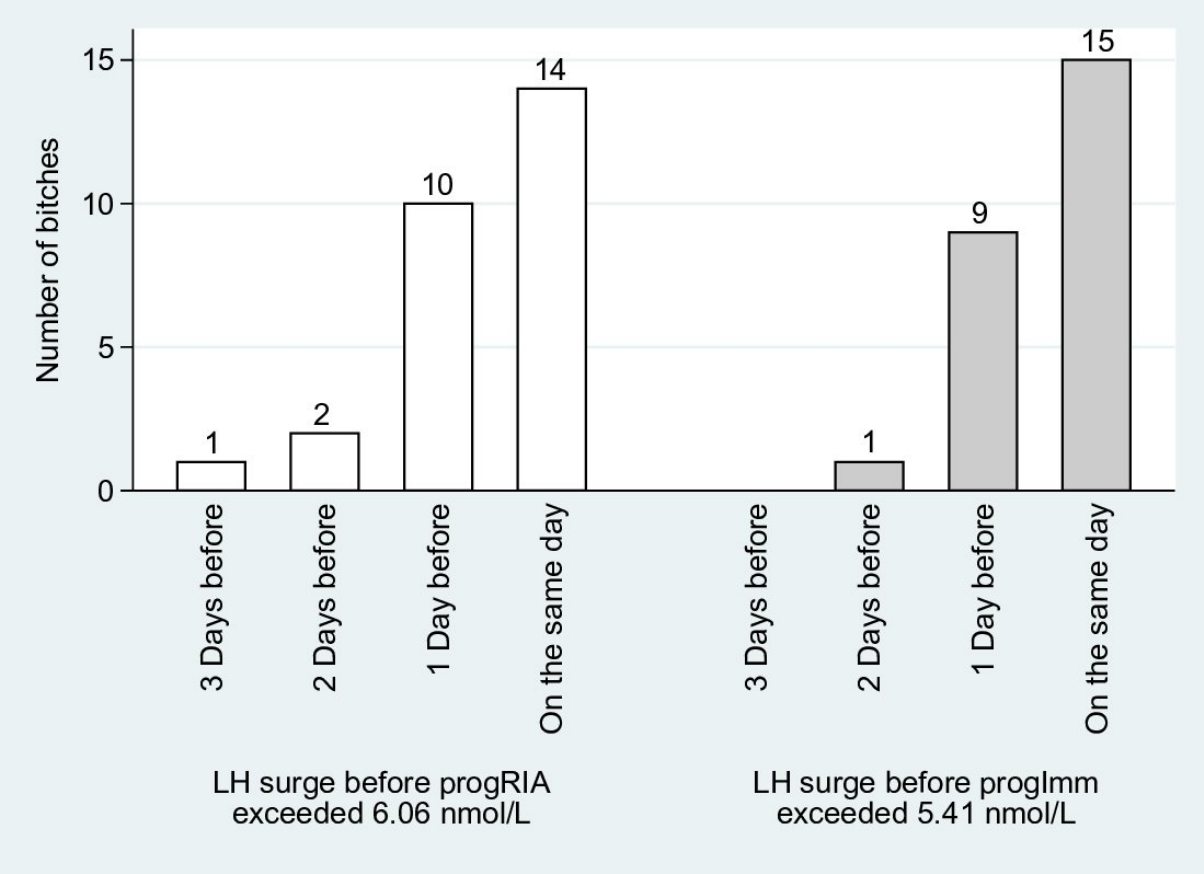


Figure 7.6

Days, relative to the first or only day of the LH surge, on which the concentration of progesterone measured with Coat-A-Count® radioimmunoassay in the plasma of 27 bitches (progRIA) or with Immulite® 1000 LKPW1 in the serum of 25 (progImm) first exceeded their respective mean concentrations of 6.06 nmol/L and 5.41 nmol/L as they were on the day of the LH surge

d) ProgRIA and progImm 2 and 3 d after the first or only day of the LH surge

Although significantly lower, Table 7.4 shows that progImm was numerically close to progRIA 2 and 3 d after the first or only day of the LH surge.

7.5. Discussion

7.5.1. The precision of the assays

The intra-assay coefficients of variation found in the current study are similar to the 6% Okkens et al. (2001) found for the same RIA assay and the 11% that Kutzler et al. (2003) reported for the Immulite 1000 LKPG1 assay and the 7.1% that Schmicke et al. (2016) reported for the same Immulite 1000 (LKPW1) assay that was used in the current study. The interassay

coefficients of variation found in the current study are similar to the 10.8% Okkens et al. (2001) found for the same RIA assay and the 6.7% and 11.9% that Kutzler et al. (2003) found with Immulite LKPG1 and the 8.9% that Schmicke et al. (2016) found with the Immulite 1000 LKPW1 assay.

Clinically important decisions in the bitch depend on precise measurements of the concentration of progesterone in plasma or serum. With the Coat-A-Count RIA and Immulite 1000 LKPW1 assays, differences as large as 11% to 30% occur in 25% of replicates in the same assay. This suggests that samples should be analysed in duplicate to identify large differences between replicate measurements and the assay repeated if a more precise assessment is required.

7.5.2. The agreement between progImm and progRIA

The current study shows that, given PC between 0.5 and 25 nmol/L when measured in plasma with the Coat-A-Count RIA assay, the Immulite 1000 LKPW1 assay will find lower concentrations in 88% of samples. On average, progImm will be 15% lower than progRIA throughout the range from 0.5 to 25 nmol/L in progRIA. As is the case with the Immulite 1000 LKPW1 assay used in the current study, the Immulite 1000 LKPG1 assay also yielded lower concentrations of progesterone than did the Coat-A-Count RIA as shown by Kutzler et al. (2003) and Volkmann (2006).

In contrast to the current study, Volkmann (2006) found that the average concentration of progesterone found in serum with Immulite 1000 LKPG1 was about one-third lower than that found with the RIA, which seems a larger difference than that found in the current study. Although the current study shows that progImm is on average 85% of progRIA, there is a wide scatter around this mean, with the 95% CI as wide as 28% percentage points above and 28% percentage points below the mean. This variability suggests that a clinician should not see a progesterone concentration obtained in isolation but in the light of other historic, clinical and laboratory findings pertaining to the bitch.

These results suggest that care be taken when applying what was found using one assay to a situation where another assay is being used and suggests careful comparison among assays.

7.5.3. Using progImm to estimate the day on which progRIA would first have exceeded 6 nmol/L

Steckler et al. (2013) showed that the fertility of frozen-thawed spermatozoa is higher when bitches are inseminated 6 d after progRIA first exceeded 6 nmol/L than 5 d or 7 d thereafter. The low expected probability and its wide 95% CI found in the current study show that progImm is not effective in estimating the day that progRIA would first exceed 6 nmol/L. The high probability of a progImm of 5.1 nmol/L correctly estimating the day on which progRIA would first exceed 6 nmol/L or the day before renders it useful to predict the optimal time to inseminate bitches with frozen-thawed spermatozoa: There is a 90% probability (95% CI 74% to 98%) that a bitch receiving her first insemination 6 d after progImm has first exceeded 5.1 nmol/L will be inseminated 5 d or 6 d after progRIA would first have exceeded 6 nmol/L. Inseminating her again 24 h later would, again with a 90% probability of success, ensure that the second insemination occurs either 6 or 7 d after progRIA first exceeded 6 nmol/L. Such an insemination schedule will ensure insemination on the most fertile day together with one day before or after, which have been shown to result in more conceptuses in a bitch than would be the case if she is only inseminated on the most fertile day (Steckler et al., 2013).

7.5.4. Using progImm to estimate the day on which progRIA would first have exceeded 16 nmol/L

The day on which the concentration of progesterone in plasma reaches 16 nmol/L provides a good estimate of the time of ovulation (Fontbonne, 2008). The optimal time for insemination has been determined relative to the day on which the concentration of progesterone measured with the Coat-A-Count RIA first exceeds 16 nmol/L (Okkens et al., 2001). The current study shows that the day on which progImm first exceeds 16 nmol/L or 13.6 nmol/L is ineffective in estimating the day on which progRIA would first exceed 16 nmol/L. The day on which progImm first exceeds 13.6 nmol/L provides a very good estimate of the day on which progRIA would first exceed 16 nmol/L or the day before, with a 97% probability of being correct and a 95% CI of 85% to 100%. The day on which progImm first exceeds 13.6 nmol/L therefore provides a suitable means of estimating the time of ovulation (Fontbonne, 2008) and the time of breeding (Okkens et al., 2001).

7.5.5. Using progImm to estimate the first or only day of the LH surge

Nishiyama et al. (1999) showed that the day of the LH surge identified with the LH assay kit

now known as Witness, corresponds well with the day identified by measuring the concentration of LH with a radioimmunoassay (Nishiyama et al., 1999). Tsumagari et al. (2003) inseminated bitches 5 d and 7 d after the LH surge as determined using the same LH kit (Tsumagari et al., 2003). Among studies in which progesterone in plasma or serum was assayed once a day, the mean progRIA of 6.06 nmol/L (SD 2.09) on the first or only day of the LH surge in the current study is similar ($P > 0.05$) to the 5.088 nmol/L (SE 0.64) and the 7.0 nmol/L (SE 1.69) that Concannon et al. (1975) and Fontbonne (2008) reported on the day when the concentration of LH in the plasma was highest but differs ($P < 0.05$) from the 9.76 nmol/L (SD 3.37) reported by Bergeron et al. (2013). The mean concentration of progesterone on the first or only day of the LH surge in the current study differs from the 8.14 nmol/L (SE 0.95) at the time of the LH peak in a study where plasma was collected 8-hourly (Concannon et al., 1977a). In the current study 18 of 32 LH surges lasted 1 d only and the remainder 2 d or more. This finding supports that of Concannon et al. (1975) that showed that 10 of 20 LH surges lasted 1 d and the remainder 2 d or more.

The current study shows that the day on which progRIA first exceeded 6.06 nmol/L and the day on which progImm first exceeded 5.41 nmol/L are ineffective in estimating the first or only day of the LH surge. Both are effective in estimating the first or only day of the LH surge or the day preceding that, with 89% and 96% probabilities of being correct. The latter probability has a 95% CI of 80% to 100%, suggesting that the day on which progImm first exceeds 5.41 nmol/L is expected to correctly indicate the first or only day of the LH surge or the day preceding it in at least 80% of oestrous periods.

7.5.6. The agreement between progImm and progRIA 2d and 3 d after the first or only day of the LH surge

Ovulation occurs 2–3 d after the LH peak (Fontbonne, 2008; Phemister et al., 1973; Concannon et al., 1977a). Although significantly lower than progRIA, various summary statistics of progImm were numerically close to those of progRIA 2 and 3 d after the first or only day of the LH surge. The mean progImm of 10.1 nmol/L (SD 2.96) 2 d after the LH surge was close to the 10.8 nmol/L (SD 2.86) that Schmicke et al. (2016) found at the time of ovulation using the same assay as the one used in the current study.

7.6. Final conclusion

On average, the concentration of progesterone in serum as measured with Immulite® 1000

LKPW1 is equal to 85% of the concentration of progesterone in plasma collected from the same bitch at the same time and measured with the Coat-A-Count® radioimmunoassay over a range in concentrations from 0.5 to 25 nmol/L. For individual blood samples, the concentration of progesterone measured with Immulite relative to that measured with the radioimmunoassay vary widely, with a 95% confidence interval from 59% to 112%. In spite of this variability, progImm serves as a useful predictor of three important clinical times during the oestrous period of bitches: They are (1) the day on which progImm first exceeds 5.1 nmol/L is expected to coincide with the day on which progRIA would first exceed 6 nmol/L or the preceding day in at least 74% of oestrous periods, (2) the day on which progImm first exceeds 13.6 nmol/L is expected to coincide with the day on which progRIA would first exceed 16 nmol/L or the preceding day in at least 85% of oestrous periods and (3) the day on which progImm first exceeds 5.4 nmol/L is expected to occur on the first or only day of the LH surge or on the preceding day in at least 80% of oestrous periods.

7.7. Acknowledgements

Funding: The National Research Foundation funded the Coat-A-Count radioimmunoassay kits, some of the Immulite kits, as well as the laboratory fees for the determination of concentrations of progesterone. Zoetis donated the Witness LH kits and Siemens donated some of the Immulite kits.

Chapter 8. The precision of peri-oestrous predictors of the date of onset of parturition in the bitch

The content of this chapter has been submitted for publication in a different format as an article by J.O. Nöthling and K.G.M. De Cramer under the title "The precision of peri-oestrous predictors of the date of onset of parturition in the bitch" and is currently under review

Abstract

Precise prediction of the date of onset of parturition in the bitch is clinically important. The current study compared the precision with which four peri-oestrous predictors predict the date of onset of parturition (taken as the date of onset of cervical dilatation, measured to the nearest 6 h). The predictors evaluated in 24 bitches were; the date of the first or only day of the LH surge, the date on which the concentration of progesterone in the blood plasma first exceeded 6 nmol/L, the date on which the concentration of progesterone in the blood plasma first exceeded 16 nmol/L and the date of onset of cytological dioestrus. Among the 24 bitches, the date of onset of cytological dioestrus predicted the date of onset of parturition with greater precision than the other three predictors. Following the evaluation of another 218 intervals between the onset of cytological dioestrus and the date of onset of parturition, it showed that the onset of cytological dioestrus predicted the date of onset parturition with a precision of ± 1 d, ± 2 d and ± 3 d in 88%, 99% and 100% of the 242 pregnancies. This study concludes that the first day of cytological dioestrus is a useful predictor of the date of onset of parturition.

Keywords*:* cytological dioestrus, parturition prediction, gestation duration, bitch

8.1. Introduction

Being able to predict the day on which spontaneous parturition would occur in the bitch is clinically important. The more precise one's ability is to predict the day of parturition, the smaller would be the range in days wherein parturition is expected to occur. The onset of parturition (the first stage of parturition) is characterized by the dilatation of the cervix which also signals readiness for CS (Smith, 2007). Therefore, it would be helpful if there were a method to predict the day of cervical dilatation (DCD), which corresponds to the day on which the onset of the first stage of spontaneous parturition occurs. Because the behavioural signs associated with early stage 1 of parturition may be variable (Linde-Forsberg and Eneroth,

2000), cervical dilatation offers a reliable and objective indicator of onset of the first stage of parturition. Following the first stage are the second and third stages of parturition, during which the foetuses and the placentas are delivered. A precise method of predicting the DCD will limit the time spent performing parturition observation. Precise prediction of DCD becomes more critical in case of timing CS (Kutzler et al., 2003a) and even more so when the intent is to perform a preparturient CS in cases where this is justified due to increased obstetric risk. Various variables during the peri-oestrous period may be of use to predict DCD. These variables include the dates on which the concentration of progesterone in the blood plasma or –serum first exceeds selected relatively low threshold values in the order of 5.1 nmol/L (Concannon et al., 1975), 6.36 nmol/L (Tsutsui et al., 2006; Steckler et al., 2013) or 8.3 nmol/L (Concannon et al., 1977a), or the higher threshold of 16 nmol/L (Fontbonne, 2008). The date or dates on which the concentration of LH in the blood plasma or –serum is elevated (Cohen et al., 2009) and the date of onset of cytological dioestrus (abbreviated as D0) (Eilts et al., 2005) may also be useful as predictors of DCD. Comparing the precision with which these variables predict DCD will reveal the best among them for routine in-practice use.

The aim of this study was to establish which of four peri-oestrous predictor of the day of onset of parturition (DCD), is the most precise. The peri-oestrous predictors were (1) the first or only day of the LH surge, (2) the first day on which PC exceeded 6 nmol/L (PC6), (3) the first day that PC exceeded 16 nmol/L (PC16) and (4) the day of onset of cytological dioestrus (D0).

8.2. Materials and Methods

The protocol was approved by the Animal Ethics Committee of the Faculty of Veterinary Science, University of Pretoria, (Onderstepoort, South Africa) (Project numbers v071-13, v010-14 and v048-15).

8.2.1. Bitches

The experimental animals were English Bulldog and Boerboel bitches presented to a private veterinary clinic for routine oestrus observation, artificial insemination and elective CS. The decision to plan an elective CS was based on breed in case of English Bulldogs and on the history of previous dystocia, puppy losses and CS in Boerboels. The owners of the bitches declined a trial of labour in any of their bitches. All bitches weighed more than 20 kg. The study involved two groups of bitches. Group A included 29 bitches (13 English Bulldog and 16 Boerboel) in which the peri-oestrous predictors of parturition were assessed. Group B

consisted of another 189 bitches (70 English Bulldog and 119 Boerboel) in which the interval from D0 to the onset of parturition were measured in order to further investigate the usefulness of D0 as a peri-oestrous predictor of parturition. All experimental animals were housed and fed commercial dry pellets twice daily with ad-lib water.

8.2.2. Collection of specimens and data

a) Group A bitches

During pro-oestrus and oestrus, vaginal speculum examinations were performed daily. Speculum examinations guided the investigators when to start and stop collecting blood to ensure collection of blood before, during and after the first or only day of the LH surge and the days on which PC first exceeded 6 nmol/L and 16 nmol/L. Vaginal speculum examinations also guided the time of artificial insemination of the bitches (Jeffcoate and Lindsay, 1989). Vaginal smears were made once daily as previously described by Olson et al. (1984) to establish D0, using the criteria set out by Holst and Phemister (1974). The bitch was considered to be in cytological dioestrus when her vaginal smear showed an abrupt change in the relative numbers of vaginal epithelial cells from the previous day, when nearly 100% of epithelial cells were of the superficial type. These changes were a decrease by at least 20% in the percentage of epithelial cells that were of the superficial type, irrespective of whether they were nucleated or anuclear, and an increase of at least 10% in cells from the deeper layers of the vaginal epithelium, such as early intermediate cells or parabasal cells (Holst and Phemister, 1974). In group A, two vials of blood was collected once daily between 7 and 9 a.m. from each bitch during pro-oestrus and oestrus; one in a ten-millilitre red-stoppered glass vial (BD Vacutainer ® (Plain), BD Plymouth, UK) and another in a ten-millilitre green-stoppered heparinized glass vial (BD Vacutainer ® (170 iu lithium heparin), BD Plymouth, UK). Blood was mostly collected by jugular venipuncture but if this failed in the shorter-necked English Bulldogs, the cephalic vein was used. All blood samples collected in the heparinized tubes was centrifuged within 30 min after collection and all blood samples collected in the serum vacuum tube after it had been left standing at room temperature for 2 h. After centrifugation, the plasma and serum was transferred to 1.8 ml cryo vials (Catalogue number 750273, PlastPro Scientific, Edenvale South Africa), labelled and frozen at -20°C until analysed.

Coat-A-Count® radioimmunoassay (Siemens Health Care Diagnostics Inc. Los Angeles, CA 90045 USA) was used to determine PC in the blood plasma. This assay has also been used on

dog blood plasma or serum by others (Gerstenberg and Nöthling, 1995; Kutzler et al., 2003a; Luz et al., 2006; Okkens et al., 2001; Reimers et al., 1991; Steckler et al., 2013; Van Klaveren et al., 2001). All determinations of PC were done in duplicate (2 replicates simultaneously done in the same assay).

The LH test was performed on an aliquot of the serum using a test kit (Witness® LH test, Synbiotics Europe, Lyon France).

Around D28 (28 days following D0), an ultrasound examination was performed to confirm pregnancy and identify possible singleton pregnancies. At D54, radiographs were taken of bitches suspected of carrying singleton pregnancies to confirm singleton status. Starting at D54, each Group A bitch was admitted for parturition management. A sterile Perspex tubular speculum (22 mm outer diameter, 17 mm inner diameter, length 280 mm) and cold light source was used to perform the vaginal speculum examinations every 6 h at 6:00, 12:00, 18:00 and 24:00 and more often approaching impending parturition, to determine whether the cervix had started to dilate. A bitch was considered ready for immediate CS upon first finding any degree of cervical dilatation (Smith, 2007). The day on which this occurred (DCD) was considered the day of onset of spontaneous parturition.

b) Group B bitches

Group B bitches were treated the same as Group A, but no blood was collected from them and, hence, no hormone analyses were done. In addition to the 24 intervals between D0 and DCD of the Group A bitches, another 218 intervals were measured in Group B (85 in the 70 English Bulldogs and 133 in the 119 Boerboels), bringing the total to 242 intervals.

8.3. Data analysis

Data analyses was performed on 24 bitches (11 English Bulldog and 13 Boerboel) for which all the intervals were available that carried more than one foetus.

8.3.1. The precision of the progesterone assay

The PCs of the two replicates of each sample from the 24 bitches were used to determine the intra-assay coefficient of variation.

Six plasma samples—two with PCs between 2 and 4 nmol/L, two with PCs between 13 and 14 nmol/L and two with PCs between 22 and 23 nmol/L—were each analysed in six or seven

different assays and the interassay coefficient of variation calculated.

8.3.2. The precision of peri-oestrous predictors of date of cervical dilatation

Four peri-oestrous predictors of DCD were used. They were (1) the first or only date of the LH surge, (2) the date on which the PC in blood first exceeded 6 nmol/L, (3) the date on which PC first exceeded 16 nmol/L and (4) the date on which a dioestrus vaginal smear first occurred (D0). Subtracting each predictor from DCD yielded four interval variables were created, namely (1) the interval between the first or only date of the LH surge and DCD, (2) the interval between PC first exceeding 6 nmol/L and DCD, (3) the interval between PC first exceeding 16 nmol/L and DCD and (4) the interval between D0 and DCD.

First, simple linear regression was used to determine whether breed affected the intervals between the first or only date of the LH surge and DCD, PC first exceeding 6 nmol/L and DCD, PC first exceeding 16 nmol/L and DCD and D0 and DCD. Following these regression analyses, the data of the two breeds were pooled to yield variables with n = 24.

Subtracting the minimum value for a particular interval variable from the value of the interval variable for each bitch yielded a set of 24 deviations for that particular interval variable. The same was done for each of the four interval variables. For each interval variable, the values of its set of 24 deviations varied from zero to the range of the interval variable.

The data of breeds were pooled and Friedman's test used to determine whether the four sets of 24 deviations (one set per interval variable) differ. In the Friedman's test the four deviations of each bitch were considered matched observations. Thereafter the six pairwise comparisons were done by means of Wilcoxon's sign rank test (with Bonferroni's correction) to determine which set of deviations was the smallest and, thereby, which interval variable was the least variable and which predictor allowed DCD to be predicted with the greatest precision.

8.3.3. The interval between the first day of cytological dioestrus and the day of cervical dilatation in 242 oestrous cycles

Using all 242 intervals between D0 and DCD, multiple linear regression analysis was used to determine whether breed, litter size and group (A or B) had significant effects ($P < 0.05$) on the interval between D0 and DCD. The interaction between breed and litter size was first included in the model and then removed.

The Friedman test was done with IBM SPSS Statistics Version 23, USA. All other analyses were done with STATA version 14 (StataCorp, 4905 Lakeway Drive, College Station, Texas 77845 USA).

8.4. Results

Only 24 out of the initial 29 bitches in Group A could be used as 2 bitches had incomplete data and another three bitches had singleton pregnancies.

8.4.1. Precision of the progesterone assay

The intra-assay coefficients of variation of the progesterone assay were 9.9% at or below 6 nmol/L (n = 57), 5.0% at or below 16 nmol/L but above 6 nmol/L (n = 70) and 4.0% above 16 nmol/L (n = 66). The interassay coefficient of variation was 8.5%.

8.4.2. The precision of peri-oestrous predictors in predicting the date of cervical dilatation in the 24 Group A bitches

The intervals between the first or only date of the LH surge and DCD, PC first exceeding 6 nmol/L and DCD, PC first exceeding 16 nmol/L and DCD and D0 and DCD were similar for English Bulldogs and Boerboels ($P > 0.05$).

Figure 8.1 shows the distribution of the interval between each peri-oestrous predictor and DCD. Table 8.1 shows that the day of onset of cytological dioestrus (D0) more precisely predicted DCD than each of the other three peri-oestrous predictors. Table 8.1 also shows that the day on which PC first exceeded 16 nmol/L more precisely predicted DCD than the day on which PC first exceeded 6 nmol/L.

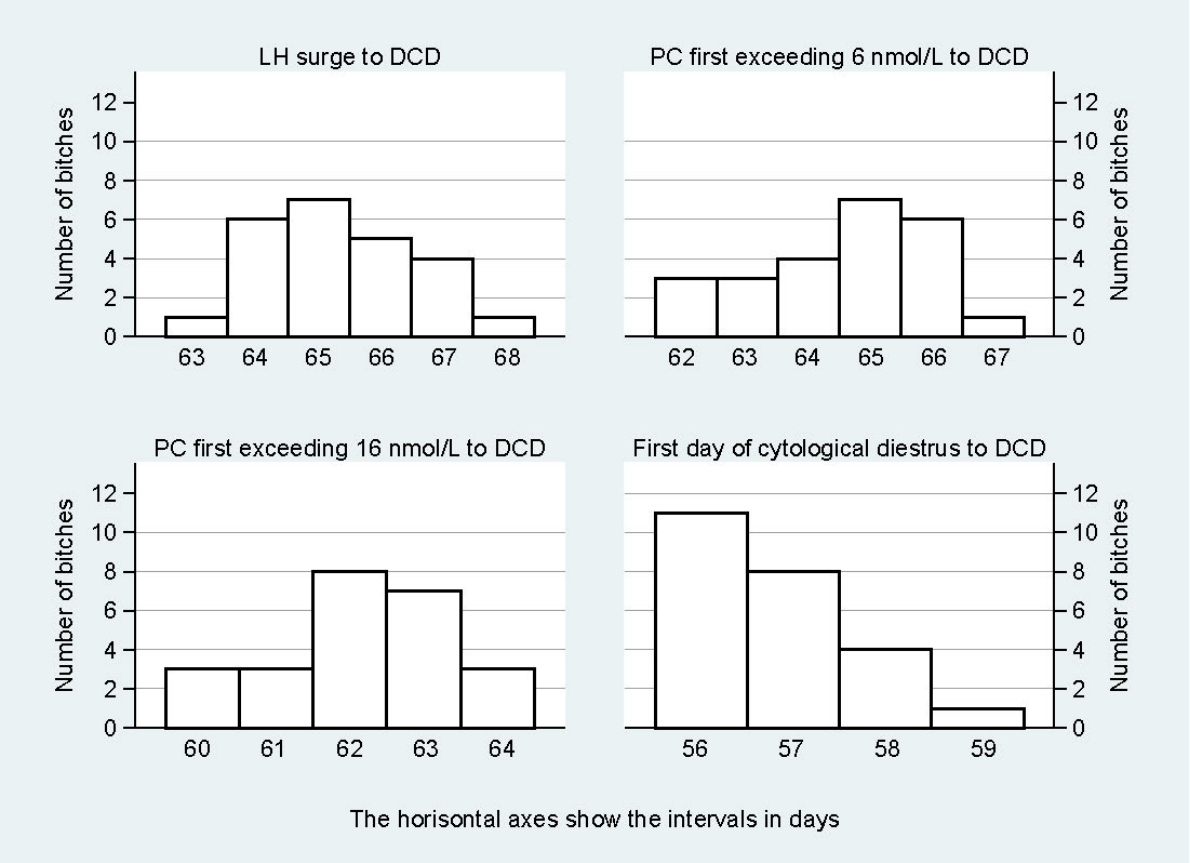


Figure 8.1

Intervals between each of four peri-oestrous predictors of the day of cervical dilatation (DCD) in 24 bitches

Table 8.1

Summary of the deviations of the interval in each of 24 bitches from the shortest interval among them for each of four interval variables between a peri-oestrous event and the day on which cervical dilatation started (DCD)

	Deviations from the minimum of each interval variable in days						
Interval variable	Minimum	Lower quartile	Median	Upper quartile	Maximum	n	Deviations differ
LH surge to DCD	0	1	2	3	5	24	a
PC reaching 6 nmol/L to DCD	0	1.5	3	4	5	24	a, c
PC reaching16 nmol/L to DCD	0	1.5	2	3	4	24	a, d
D0 to DCD	0	0	1	1	3	24	b

a, b Deviations of groups marked “a” differ from those of the group marked “b” ($P < 0.001$)

c, d Deviations of the group marked “c” differ from those of the group marked “d” ($P < 0.05$)

8.4.3. The interval between the first day of cytological dioestrus and the day of cervical dilatation in 242 pregnancies

Breed and litter size did not interact in their effects on the interval between D0 and DCD (P = 0.18) and the interaction term was removed from the model. The interval between D0 and DCD depended on breed (P = 0.04) and litter size (P = 0.01) but not on group (P = 0.68). On average, the interval between D0 and DCD was 0.278 d (95% CI 0.017–0.538) longer in Boerboel bitches than in English Bulldog bitches. The interval between D0 and DCD decreased by 0.047 d (95% CI 0.011–0.082) for each increase by one in litter size. The mean of the 24 Group A intervals between D0 and DCD was 56.74 d (95% CI 56.61 – 56.86), which was similar to the mean of 56.82 d (95% CI 56.44–57.20) of the 218 Group B intervals.

Figure 8.2 shows the frequency of the intervals between D0 and DCD in 242 pregnancies. The intervals were normally distributed with a mean of 56.74 d (SD 0.96 d). Accordingly, 95% of intervals between D0 and DCD were between 54.86 d and 58.63 d.

Figure 8.2 shows that 214 (87.6%), 239 (98.8%) and 242 (100%) of the intervals were respectively within one, two or three d of the expected interval of 57 d (the mean of 56.74 d rounded to 57 d).

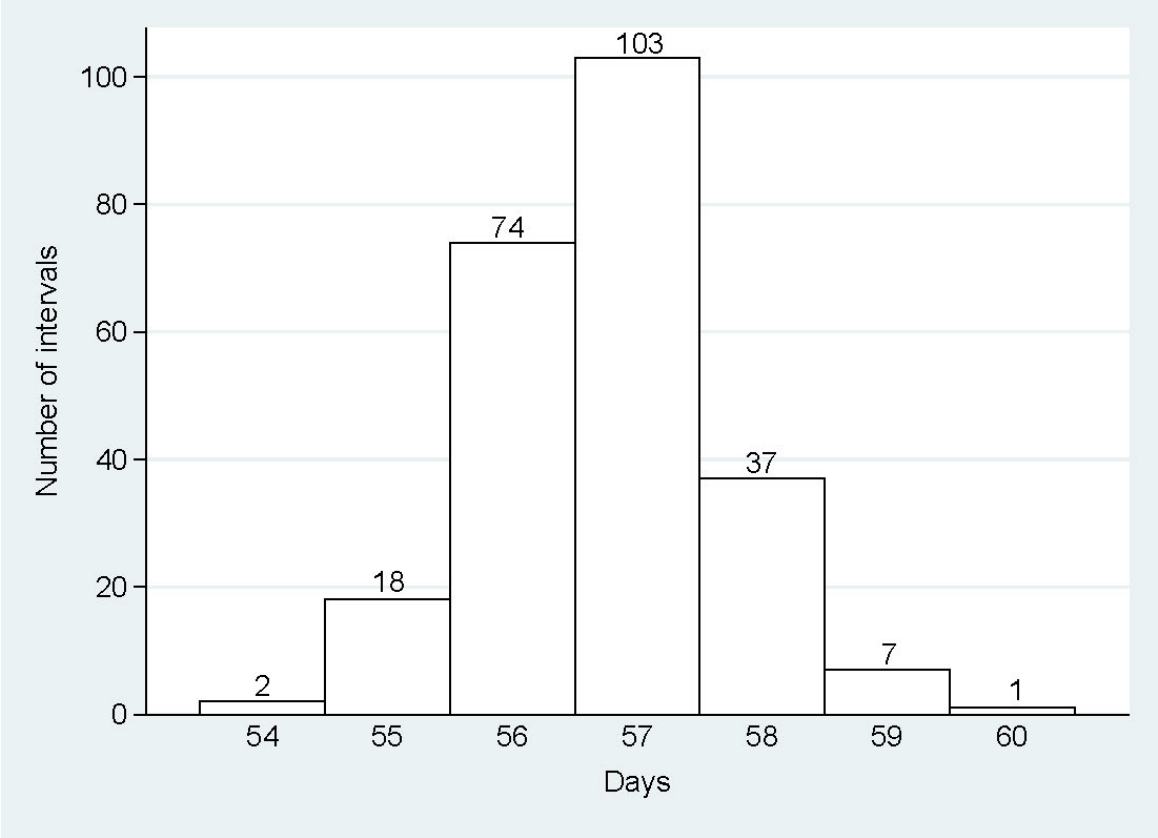


Figure 8.2
Histogram showing the intervals between the day of onset of cytological dioestrus and the day of cervical dilatation of 242 pregnancies in 213 bitches

8.5. Discussion

This study shows that D0 predicted DCD more precisely than the first or only day of the LH surge or the days on which the concentration of progesterone first exceeds 6 nmol/L or 16 nmol/L.

In the current study, D0 predicted DCD with a precision of ± 1 d, ± 2 d and ± 3 d in 88%, 99% and 100% of the 242 pregnancies. This precision agrees closely with the 82% and 100% with which the same LH test kit as the one used in the current study predicted the date of parturition within ± 1 d and ± 2 d (Cohen et al., 2009). The precision of D0 in the current study also compares favourably with the 67%, 90% and 100% with which the date on which PC first exceeded 5.7 nmol/L predicted the day of parturition within ± 1 d, ± 2 d and ± 3 d, respectively (Kutzler et al., 2003). In addition to its precision, D0 has the advantage that it is cheap and quick to obtain in practice.

Eilts et al. (2005) and Holst and Phemister (1974) respectively reported standard deviations of 2.8 d and 1.61 d for 152 and 93 intervals between D0 and parturition, which were higher than the 0.96 d for the 242 intervals in the current study ($P < 0.001$). The difference may be because the current study having evaluated the interval between D0 and cervical dilatation (first stage of parturition) and the other two the interval between D0 and the delivery of the first foetus. The interval between the onset of cervical dilatation and the delivery of the first foetus is unknown and requires further investigation.

Many singleton pregnancies fail to progress normally (King, 1978; Lopate, 2008; Smith, 2007; Johnson, 2008a; Wykes and Olson, 2003; Munnich and Kuchenmeister, 2009) and have increased gestation length (Holst and Phemister, 1974; Okkens et al., 1993). Therefore, it was justified to exclude singleton pregnancies form the current study.

Irrespective of the effect of singleton pregnancies, the effect of litter size on the duration of gestation is controversial. Linde-Forsberg et al. (1999), Tsutsui et al. (2006) and Seki et al. (2010) concluded that litter size did not affect the duration of gestation in their studies. In contrast, other workers found that small litters are carried significantly longer than larger litters (Okkens et al., 1993; Eilts et al., 2005; Gavrilovic et al., 2008; Mir et al., 2011). Having considered litters larger than one, the current study shows a significant, although small, decline by 0.047 d in the interval between D0 and DCD for each increase by one in litter size, which supports the latter three studies. This effect of litter size is unlikely to be of clinical significance.

The current study shows a small but significant difference of 0.28 d in the interval between D0 and DCD of English Bulldogs and Boerboels, which is unlikely to be of clinical significance. However, it cannot concluded that the differences between all breeds are the same and therefore a more general breed effect of clinical importance cannot be ruled out by the comparison of only two breeds.

8.6. Conclusions

This study shows that day of onset of cytological dioestrus (D0) predicts the day of cervical dilatation (DCD) more precisely than the first or only day of the LH surge or the days on which the concentration of progesterone in the blood plasma first exceeds 6 or 16 nmol/L. Although significant, the effects of breed (Boerboel vs English Bulldog) and litter size on the interval between D0 and DCD is unlikely to be clinically important.

8.7. Author contributions

J O Nöthling was the supervisor of the scientific protocol, performed the statistical analyses and assisted in drafting the protocol and manuscript. K.G.M. De Cramer was the main person involved in experimental work and wrote the protocol and manuscript.

8.8. Conflicts of interest

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of this manuscript.

Acknowledgements

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Chapter 9. The precision of predicting the time of onset of parturition in the bitch using the concentrations of progesterone and cortisol in the preparturient period

The content of this chapter will be submitted for publication in a different format as an article by K G M De Cramer and J O Nöthling.

Abstract

Precise prediction of the time of onset of parturition in the bitch is of clinical importance. Many parturition management cases in clinical practice are presented in the last third of gestation and are ones for which no estimate of the parturition date are available. The aims of this study were to compare the precision of the ability of PC and cortisol concentration in the blood of preparturient bitches to predict the time of onset of parturition. The temporal relationship between the respective decrease in progesterone and increase in cortisol concentrations and the time of cervical dilatation, which correlates to the onset of stage 1 of parturition, were evaluated in 25 bitches in the preparturient period. Our study showed that if the preparturient PC is above 15.8 nmol/L, there is a 99% probability that the bitch will not enter spontaneous parturition within the following 12 h and if above 8.7 nmol/L, a 98% probability of not entering spontaneous parturition within the following 12 h. Conversely, if the PC is below 15.8 nmol/L, there is a 99% probability of parturition within 96 h, if below 3.18 nmol/L a 100% probability of parturition within the next 24 h. Equally, for a PC of below 8.7 nmol/L, the probability is 99% of spontaneous parturition within the next 48 h and 86% of spontaneous parturition within 24 h. These results allow the veterinary obstetrician to make prompt decisions in the management of parturition. Cortisol concentrations in this study were too erratic to be of value in predicting the onset of parturition.

Keywords: Progesterone, cortisol, cytological diestrus, parturition, bitch

9.1. Introduction

Predicting the time of onset of spontaneous parturition in the bitch is clinically important. The onset of parturition (the first stage of parturition) is characterized by the dilatation of the cervix which also signals readiness for CS (Smith, 2007). Because the behavioural signs

associated with early stage 1 of parturition may be variable (Linde-Forsberg and Eneroth, 2000), cervical dilatation offers a reliable and objective indicator of onset of the first stage of parturition. Following the first stage are the second and third stages, during which the foetuses and the placentas are delivered, all forming part of parturition.

The majority of parturition management cases in practice are presented in the last third of gestation and are ones for which only mating dates are available. Mating dates are of no help in predicting parturition dates (Concannon et al., 1983; Holst and Phemister, 1974; Tsutsui et al., 2006; Shimatsu et al., 2007). In dogs, ultrasonographic measurement of the inner diameter of the inner chorionic cavity proved an accurate method to evaluate gestational age and to predict the day of parturition when the bitch is examined for pregnancy diagnosis during early gestation (England et al., 1990; Luvoni and Grioni, 2000; Kim and Son, 2008; Socha et al., 2008) but different equations, derived from growth curves from the various breed sizes were required (Luvoni and Grioni, 2000; Son et al., 2001; Yeager et al., 1992) or correction factors were required for giant breeds (Kutzler et al., 2003b). In late pregnancy it was concluded that the biparietal diameter was most accurate in predicting gestational age and that the crown rump length may be difficult to measure because of foetal flexion and foetal lengths that exceeds the size of the ultrasound image (Son et al., 2001; Luvoni and Beccaglia, 2006; Kutzler et al., 2003b). A study involving English Bulldogs suggested that (CSs can be scheduled safely when the foetal biparietal diameter having reached a value of 29.5 mm or above (Batista et al., 2014), but this theory was not put to test. Although these studies involve gestational age estimation in dogs by ultrasonographic assessment of foetal biometric measurements, it is not known whether it is safe using such estimated dates to time CS.

Progesterone appears thermogenic in the dog (Concannon et al., 1977b) but the effect may be variable (Tsutsui and Murata, 1982; Veronesi et al., 2002; Baan et al., 2005; Corrada et al., 2005; Long et al., 1978; Linde-Forsberg and Eneroth, 1998) and some bitches may not demonstrate a detectable preparturient drop in rectal temperature even when monitored three times daily (Johnston et al., 2001a). It may therefore be concluded that the preparturient temperature drop can give important clues but that it is not accurate or reliable enough to use as sole predictor of the time of parturition or indicating foetal maturity and safety for intervention by CS.

It is safe to perform a CS once the cervix has dilated (Smith, 2007). The time of onset of

cervical dilatation (TCD), corresponds to the time of onset of the first stage of spontaneous parturition and therefore it would be helpful if there were a method to predict the TCD in the preparturient period. It has been established that the PC decreases prior to parturition (Concannon et al., 1975; England and Verstegen, 1996a; Onclin and Verstegen, 1997) and that cortisol concentrations increase during the 24 to 30 h preceding the onset of spontaneous parturition (Concannon et al., 1978; Baan et al., 2008). However, their usefulness in predicting the TCD requires further investigation.

The aim of this study was to establish how precise the concentrations of progesterone and cortisol having respectively declined below a threshold or having reached a threshold, would be in predicting the TCD.

9.2. Materials and Methods

The protocol was approved by the Animal Ethics Committee of the Faculty of Veterinary Science, University of Pretoria, (Onderstepoort, South Africa) (Project numbers v071-13, v010-14, v010-14 amend 1, v048-14, v048 amend 1 and v021-15).

9.2.1. Bitches

The experimental animals were all English Bulldog and Boerboel bitches presented to a private veterinary clinic for routine oestrus observation, artificial insemination and elective CS. The decision to plan an elective CS was based on breed in the case of English Bulldogs and on the history of previous dystocia, puppy losses and CS in Boerboels. All bitches were in excess of 20 kg. All owners declined an attempt to let the bitches undergo spontaneous unassisted parturition. The current study included 28 bitches (12 English Bulldog and 16 Boerboel) in which the preparturient predictors of parturition were assessed. Bitches, other than these 28, that were nervous or resisted restraint were identified early in the experiment during oestrus monitoring and were excluded from the experiment. This was important as the experimental bitches were destined for frequent blood collections during the preparturient period and because we wanted to minimize the effect of stress and associated possible cortisol release, as confounder. All experimental animals were housed and fed commercial dry pellets twice daily with ad-lib water.

9.2.2. Collection of specimens and data

During pro-oestrus and oestrus vaginal speculum examinations and vaginal smears were

made once daily to establish D0, using the criteria set out by Holst and Phemister (1974). An ultrasound examination was performed around D26 to D35 to confirm pregnancy and to identify bitches carrying a singleton. In the preparturient period, starting at D54 (54 d after the onset of D0), each bitch was admitted for parturition management and if a small litter was suspected, a radiograph was taken to confirm litter size and exclude singleton pregnancies from the study. On the day of admission, a central venous catheter (18 gauge, 20 cm in length, Catalogue Number 04218, Arrow International, Pennsylvania, USA) was instilled in their jugular vein and heparinized blood was collected every 6 h at 6:00, 12:00, 18:00 and 24:00. Special care was taken using sterile technique to avoid catheter site infections and to ensure that the blood collected through the indwelling catheters was not diluted with any fluid other than the bitch's own contemporaneously circulating blood, such as ringer's lactate or heparinized saline. Immediately before each blood collection, a Perspex tubular speculum (22 mm outer diameter, 17 mm inner diameter, length 280 mm) was passed into the vagina to determine whether the cervix had dilated. A bitch was considered ready for immediate CS upon first finding any degree of cervical dilatation. The day and time on which this occurred was considered both the day and time of onset of spontaneous parturition.

The blood was immediately transferred to a 10-ml green-stoppered heparinized glass vial (BD Vacutainer ® (170 IU lithium heparin), BD Plymouth, UK) and was centrifuged within 30 min after collection, following which it was transferred to 1.8 ml cryo vials (Catalogue number 750273, PlastPro Scientific, Edenvale South Africa), labelled and frozen at -20 °C until analysed.

All determinations of the concentration of progesterone and cortisol in plasma were done with RIA (Coat-A-Count® radioimmunoassay; Siemens Health Care Diagnostics Inc. Los Angeles, CA 90045 USA). This assay has been commonly used in veterinary endocrine laboratories and validation data for this assay in dogs have appeared in several publications for cortisol assays (Kemppainen et al., 1983; Watson et al., 1993; Russell et al., 2007) and for progesterone assays (Srikandakumar et al., 1986). In addition the Coat-A-Count ^{125}I radioimmunoassay for progesterone (Siemens), has been used to determine the concentration of progesterone in the serum or plasma of bitches for decades (Reimers et al., 1991; Gerstenberg and Nöthling, 1995; Okkens et al., 2001; Kutzler et al., 2003a; Luz et al., 2006; Steckler et al., 2013).

All determinations of the concentration of progesterone and cortisol were done in duplicate (two replicates simultaneously done in the same assay). The intra-assay coefficient of variation was determined for the concentrations of the two replicates of each sample.

Six plasma samples from peri-oestrus bitches—two of which had concentrations of progesterone between 2 nmol/L and 4 nmol/L, two had concentrations between 13 nmol/L and 14 nmol/L and two had concentrations between 22 nmol/L and 23 nmol/L—were each analysed in six or seven different assays and the interassay coefficient of variation calculated.

The concentrations of cortisol of 19 plasma samples were each determined in two assays and the mean concentrations of each sample in each assay used to determine the interassay coefficient of variation.

9.3. Data analysis

Data of bitches carrying more than one foetus and on which a CS was performed once cervical dilatation was first noticed were used for analysis.

9.3.1. General investigation of the pattern of change, and variability of cortisol and progesterone concentrations during the preparturient period

First, a set of line graphs were compiled showing cortisol at each of the six-hourly measurements prior to TCD in each bitch. This was done to assess the pattern of change in individual bitches and the variability among bitches.

Thereafter a scatterplot was generated using all cortisols during each 24-hour period prior to TCD in order to assess the feasibility of predicting the number of days before TCD from a single cortisol or PC, taken at a random interval before TCD.

In the same way as described above for cortisol, a set of line graphs and a scatterplot reflecting the real time before TCD was generated for PC.

9.3.2. Selecting the progesterone concentration that would best predict the time to cervical dilatation

a) Identifying possible crosspoints

A scatterplot of PC against Time, where Time represents the time until cervical dilatation

was first noted (also referred to as TCD), which was denoted time zero. Progesterone concentration was plotted on the y-axis, with PC increasing with height on the axis. Time was plotted on the x-axis, with Time zero on its right extreme and the time most distantly preceding Time zero on its left. Vertical lines on the graph are referred to as timelines and horizontal lines as PClines. The point where a timeline and a PCline cross is called a crosspoint. All PCs from each of the 25 bitches were plotted on the scatterplot.

The scatterplot (Figure 9.4) was visually inspected and subjectively appraised for a pattern of change of PC over time. Given the downward trend in PC as time zero approached, a search for possible crosspoints was done.

Either of two criteria defined useful crosspoints. The first was if the top right quadrant (the zone above the PC line through the crosspoint and to the right of the timeline through it) has as few as possible PCs relative to the numbers in the other quadrants. (The lower the probability of bitches falling in the top right quadrant of a crosspoint relative to its other quadrants, the lower the probability that a specific bitch will undergo cervical dilatation sooner than suggested by the PCline through the crosspoint, in spite of her having a PC above the threshold suggested by the PCline through the crosspoint.) The second criterion was if the bottom left quadrant has as few as possible PCs relative to the numbers in the other quadrants. (Bitches with PCs in the bottom left quadrant would be clinically managed by follow-up obstetric examination and -intervention or premature CS if that is appropriate and they are closer to time zero than the maximum interval by which gestation may be shortened by a premature CS.)

With the above criteria in mind, the scatterplot was inspected at clinically important timelines to identify that PCline at each that would either have the top right quadrant or the bottom left quadrant or both sparsely populated or without PCs. The clinically important timelines were 12, 24, 48 and 96 h before TCD.

b) Defining the priorities by which to search for the best crosspoint

The suitability of various parameters was assessed for their ability to discriminate among crosspoints. These parameters were the sensitivity (SE), specificity (SP), false negative fraction (FNF), false positive fraction (FPF), positive predictive value (PPV) and negative predictive value (NPV) (Dohoo et al., 2009). Those parameters that would be most suitable to quantitatively characterise a crosspoint were short-listed and used to compare crosspoints.

Referring to Figure 9.4, the following symbols were used to facilitate description of a crosspoint: T_R = number of PCs in the top right quadrant, B_R = number of PCs in the bottom right quadrant, T_L = number of PCs in the top left quadrant and B_L = number of PCs in the bottom left quadrant.

c) Parameters pertaining to the top right quadrant

$FNF = T_R/(T_R + B_R)$. The fewer PCs in the top right quadrant relative to the number in the bottom right quadrant the lower the FNF will be. A low FNF is preferred.

$SE = B_R/(T_R + B_R)$. The fewer PCs in the top right quadrant relative to the number in the bottom right quadrant the higher the SE will be. A high SE is preferred.

$NPV = T_L/(T_L + T_R)$. The more PCs in the top left quadrant the fewer will be in the top left quadrant. A high NPV is preferred.

The FNF, SE and NPV all relate to the top right quadrant. In terms of the priorities, the optimal crosspoint with respect to the top right quarter will be the one for which the score consisting of the sum (1 - FNF) + SE + NPV is closest to the maximum of 3.

d) Parameters pertaining to the bottom left quadrant

$FPF = B_L/(B_L + T_L)$. The fewer PCs in the bottom left quadrant relative to the number in the top left quadrant, the lower the FPF will be. A low FPF is preferred.

$SP = T_L/(T_L + B_L)$. The fewer PCs in the bottom left quadrant relative to the number in the top left quadrant, the higher the SP will be. A high SP is preferred.

$PPV = B_R/(B_R + B_L)$. The fewer PCs in the bottom left quadrant relative to the number in the bottom right quadrant, the higher the PPV will be. A high PPV is preferred.

The FPF, SP and PPV all relate to the bottom left quadrant. In terms of the priorities, the optimal crosspoint with respect to the bottom left quarter will be the one for which the score consisting of the sum (1 - FPF) + SP + PPV is closest to the maximum of 3.

e) Timelines that were considered

The data allowed for the evaluation of a timeline within 96 h to 6 h of TCD.

Given the general decline in PCs as TCD approaches, timelines closer to TCD are more likely to identify optimal crosspoints of which the top right quadrant is devoid or sparsely

populated with PCs than timelines preceding TCD by longer intervals. Such crosspoints allow clinicians to classify bitches as unlikely to reach TCD within the interval signified by the timeline through the crosspoint, provided that her PC is above the PC line through the crosspoint. The 12-hours and 24-hours timelines were selected for investigation with such benefit in mind.

Given the general decline in PCs as TCD approaches suggests that timelines further ahead of TCD (closer to 96 h before TCD) are more likely to identify optimal crosspoints with higher PC lines of which the bottom left quadrants are devoid or sparsely populated with PCs. Such crosspoints will have the value of allowing clinicians to suitably schedule observation and follow-up examination of the preparturient bitch. The 96-hours, 72-hours and 48-hours timelines were selected for investigation with such benefit in mind. The 48-hours timeline was deemed important because Baan et al. (2005), Levy et al. (2009) and Vannucchi et al. (2012) have shown that foetuses are viable when delivered within 48 hours before the onset of spontaneous parturition.

Timelines closer to TCD may also identify crosspoints with low PC lines of which the bottom left quadrant is sparsely populated or free of PCs. Such crosspoints may allow clinicians to identify bitches in which TCD is due soon. If the timeline through the crosspoint is sufficiently close to TCD it may allow the clinician to safely perform a preparturient CS or to schedule it to follow shortly. The 12-hours and 24-hours timelines were selected for investigation with such benefit in mind.

Effects were considered significant if $P < 0.05$. All analyses were done with STATA version 14 (StataCorp, 4905 Lakeway Drive, College Station, Texas 77845 USA) or with Excel.

9.4. Results

One English Bulldog and two Boerboels were excluded because they had litters of one and because, in two of them, the CS was done while the cervix was still closed, leaving 25 bitches (11 English Bulldogs and 14 Boerboels) in the study.

9.4.1. Precision of the assays

a) Cortisol

The standard curve for the concentration of cortisol ranged from zero to 2000 nmol/L. The

intra-assay coefficients of variation of the concentration of cortisol in plasma samples from preparturient bitches with mean concentrations below 20 nmol/L (mean 13.8 nmol/L), between 40 nmol/L and 60 nmol//L (mean 49.9 nmol/L), and above 100 nmol/L (mean 143 nmol/L) were respectively 11.39% (n = 49), 6.26% (n = 91) and 5.42% (n = 33).

The mean interassay coefficient of variation for the concentration of cortisol in 19 plasma samples with concentrations from 11.8 to 96.61 nmol/L (mean 36.29 nmol/L) was 8.91%.

b) Progesterone

The standard curve for the concentration of progesterone ranged from zero to 127.2 nmol/L. The intra-assay coefficients of variation of progesterone in plasma samples from preparturient bitches with mean concentrations below 2 nmol/L, between 5.5 nmol/L and 6.5 nmol//L, between 16 nmol/L and 16.9 nmol/L and between 30 nmol/L and 38.7 nmol/L were respectively 9.21% (n = 20), 3.56% (n = 12), 5.83% (n = 16) and 2.96% (n = 16).

The mean interassay coefficient of variation was 12.45% for the two plasma samples that contained 2 nmol and 4 nmol of progesterone per litre, 7.21% for the two tubes that contained 13 nmol and 14 nmol of progesterone per litre and 5.69% for the two tubes that contained 22 nmol and 23 nmol of progesterone per litre.

9.4.2. Preparturient predictors of TCD

a) Cortisol as a possible predictor of TCD and general investigation of the pattern of change, and variability of cortisol during the preparturient period

Visual appraisal of Figure 9.1 shows that concentrations of cortisol above 100 nmol/L only occurred during the two days preceding TCD, although the concentration never exceeded 100 nmol/L in some bitches. Throughout the 108 h leading to TCD most concentrations of cortisol fluctuated widely below 100 nmol/L. Nevertheless, Figure 9.2 shows a general trend of rising concentrations of cortisol during the 36 h preceding TCD.

No pattern in either the concentration of cortisol in the blood plasma or the change thereof over time occurred that was sufficiently consistent among bitches to warrant further appraisal of cortisol as a predictor of TCD.

b) General pattern of change of progesterone concentration during the preparturient

period

Figure 9.3 shows the change in PC over time during the last few days before TCD in 25 individual bitches. Generally, there was a decline in PC that started 48 h or less prior to TCD. Both, the pattern of decline and the concentrations of PC varied substantially among bitches. In each of the 25 bitches except Shakira, PC decreased to below 6.4 nmol/L although in two (Amie and Lady) it increased again to above 6.4 nmol/L at TCD and in another (Zandrie) it increased from 3.15 nmol/L at TCD to 12.84 nmol/L one hour later, immediately before the CS was performed on her. The two bitches with PC that rapidly increased during the 6 h before TCD to reach values above 8 nmol/L at TCD (Amie and Zandrie) were interesting. These two bitches also showed sharp and severe rises in cortisol to above 240 nmol/L during the same period.

For the 25 bitches, the median and interquartile range in PC at TCD were 3.43 (1.59–6.1) nmol/L, with values ranging from 0.95 to 12.84 nmol/L.

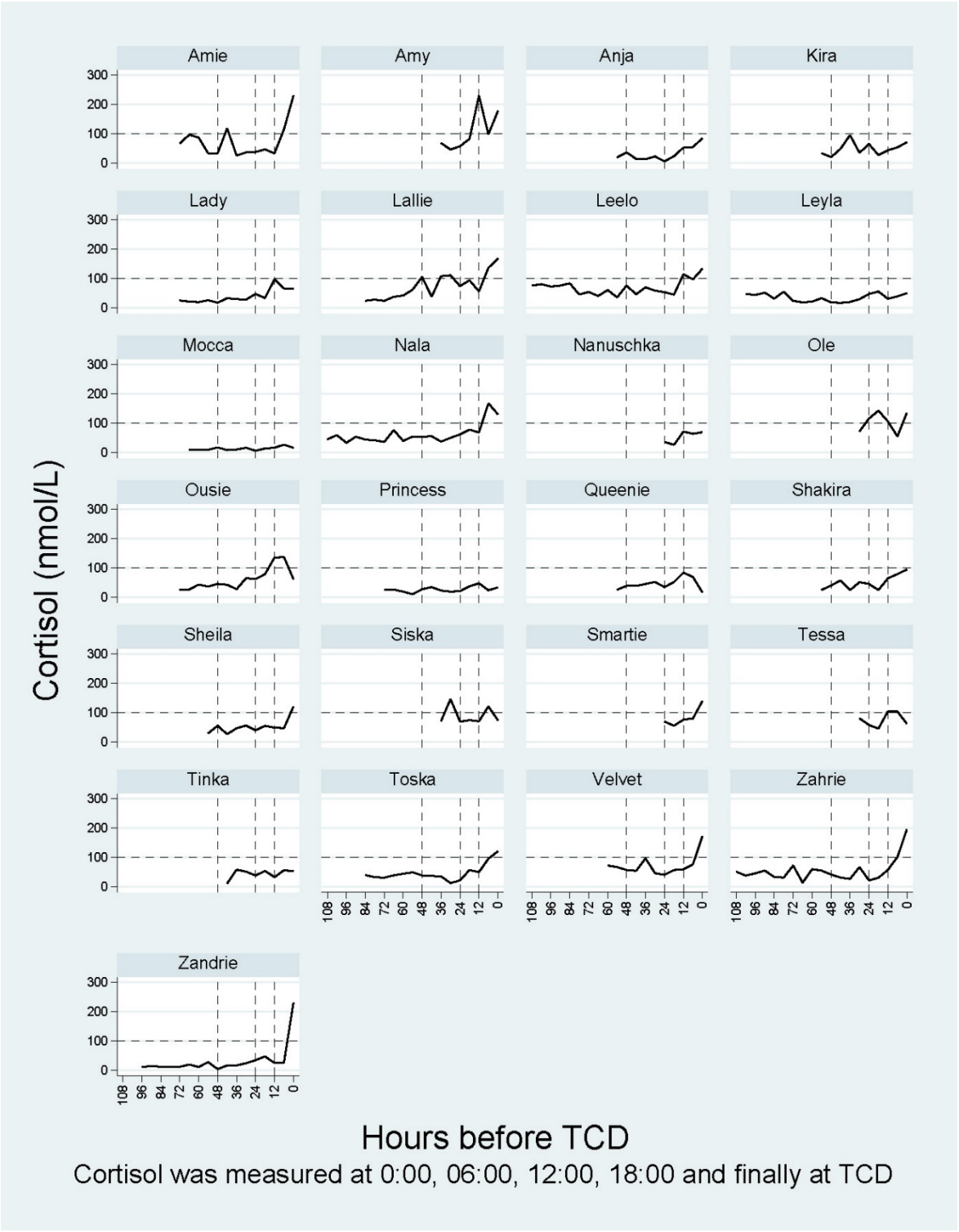

Figure 9.1

The concentration of cortisol in blood plasma of 25 bitches, measured six-hourly during the last few days before the time of cervical dilatation (TCD)

Notwithstanding the wide fluctuation in the concentration within bitches shown in Figure 9.1, Figure 9.2 shows an overall trend of increase in cortisol as TCD approaches.

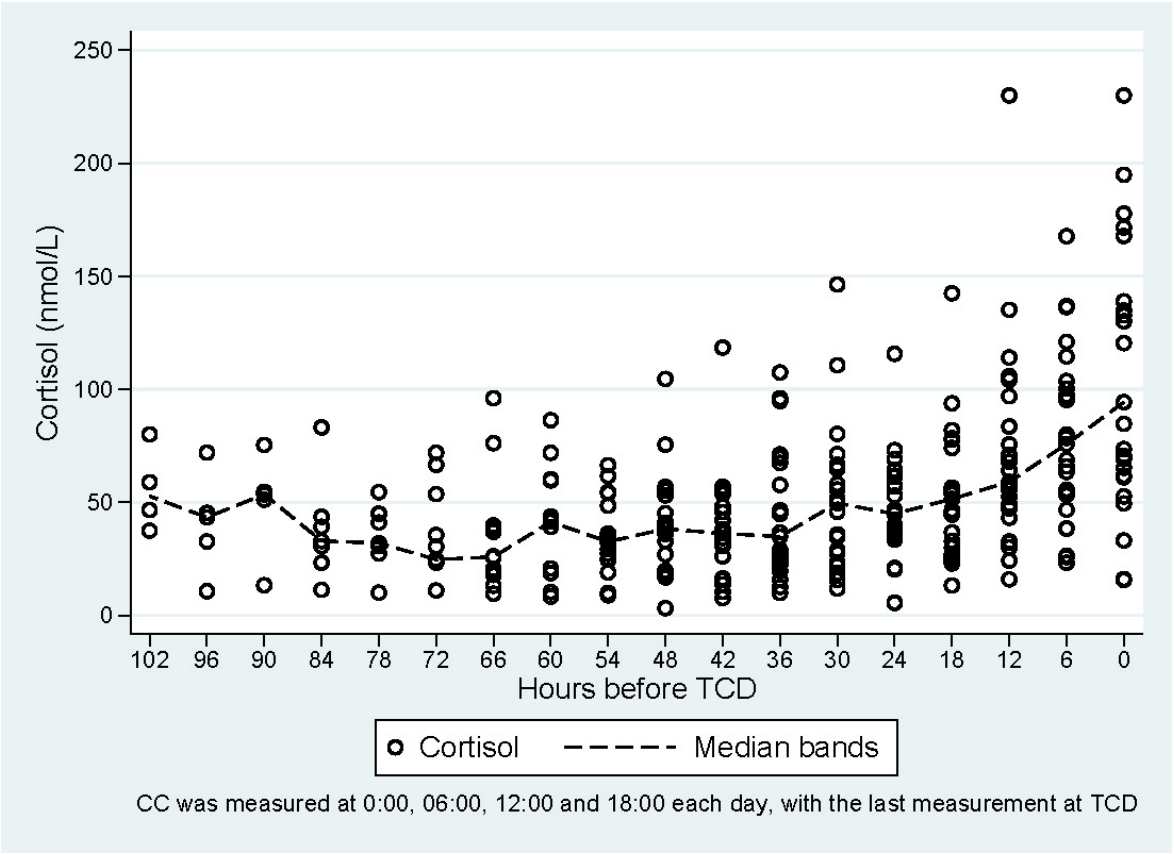

Figure 9.2

Concentrations of cortisol in the blood plasma of 25 bitches at restricted intervals before cervical dilatation (T0), with the solid line connecting the median concentrations

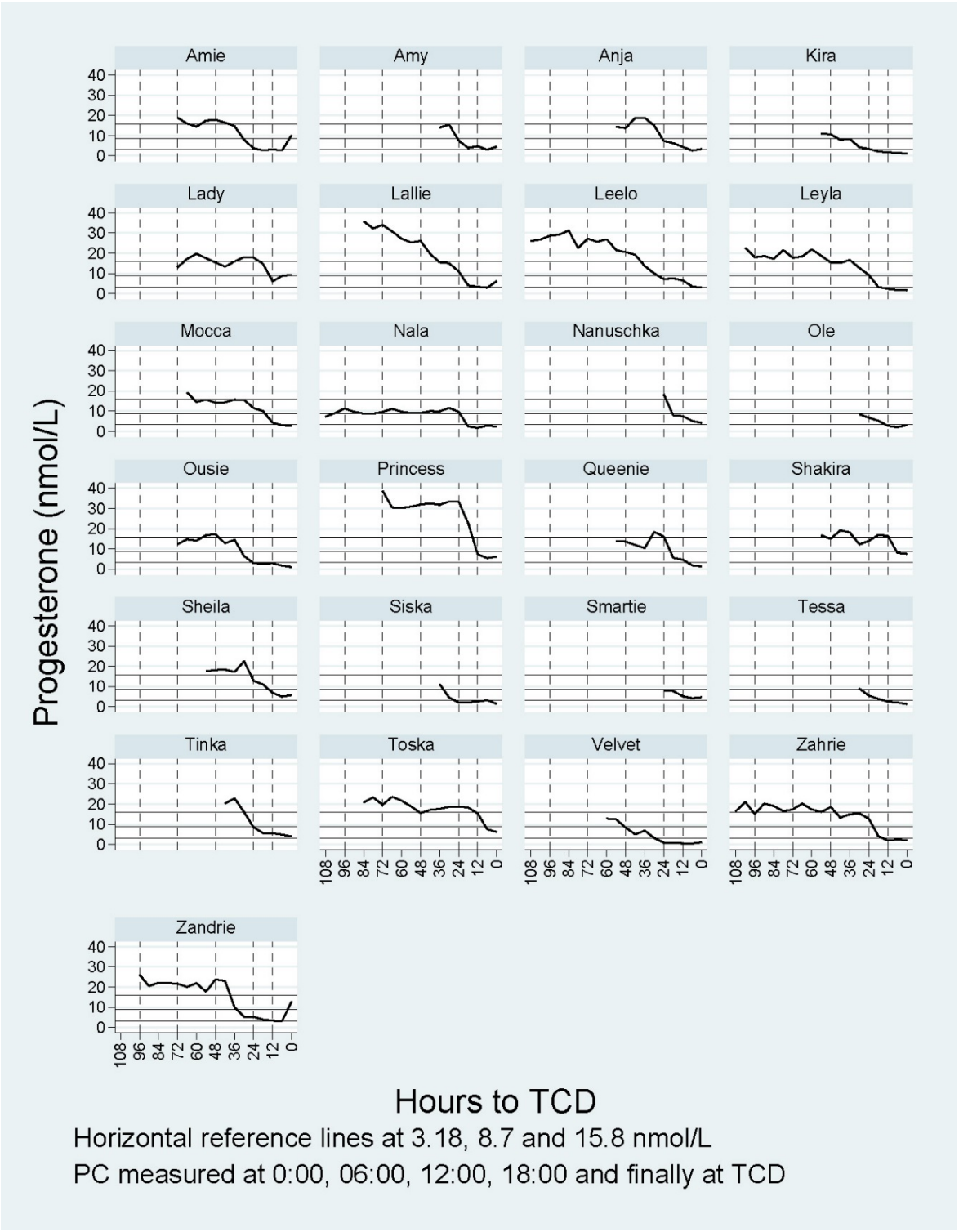

Figure 9.3

The concentration of progesterone (PC) in blood plasma of 25 bitches, measured six-hourly during the last few days before the time of cervical dilatation (TCD)

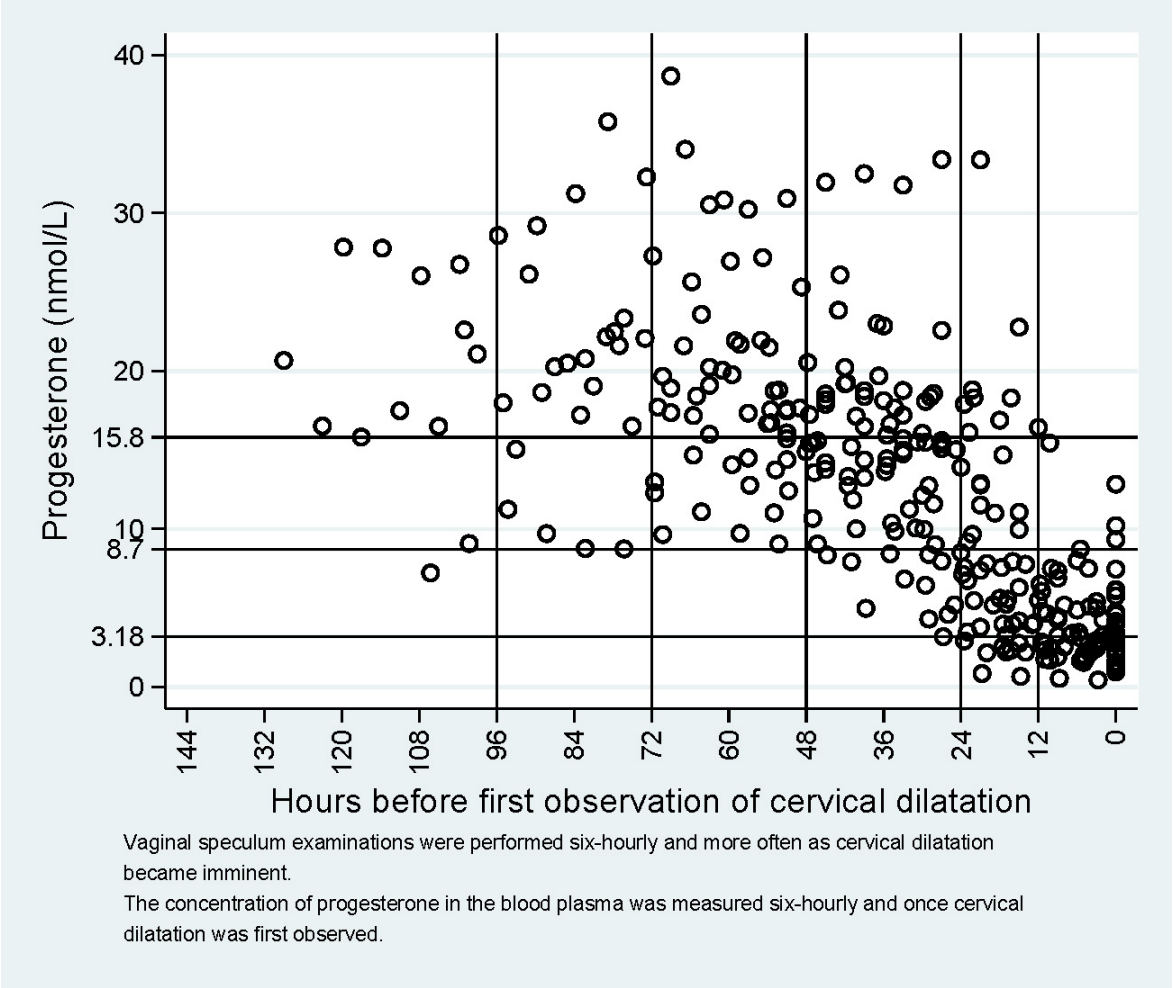

Figure 9.4

Scatterplot of all the progesterone concentrations of 25 bitches with reference lines used to identify possible crosspoints

c) Useful crosspoints

Applying the 12-, 24-, 48-, 72- and 96-hours timelines (Figure 9.4), revealed that PC lines of 3.18-, 8.7- and 15.8 nmol/L may identify useful crosspoints.

Table 9.1 shows the most useful crosspoints of which the top right quadrants were sparsely populated with PCs. Only 2% of bitches with PC above 15.8 nmol/L are expected to start undergoing cervical dilatation within 12 hours and 9% within 24 hours. Only 6% of bitches with PC above 8.7 nmol/L are expected to start undergoing cervical dilatation within 12 hours.

Table 9.1

Useful crosspoints by which the concentration of progesterone in blood plasma (PC) may aid in predicting the time of onset of cervical dilatation because few PCs occur in their top right quadrants

Crosspoint	FNF[a]	SE[a]	NPV[a]	Score[b]
12 h by 15.8 nmol/L	0.02 (0.00–0.10)	0.98 (0.90–1.00)	0.99 (0.95–1.00)	2.95
12 h by 8.7 nmol/L	0.06 (0.02–0.16)	0.94 (0.84–0.98)	0.98 (0.95–0.99)	2.86
24 h by 15.8 nmol/L	0.09 (0.05–0.16)	0.91 (0.84–0.95)	0.91 (0.85–0.96)	2.74

[a] 95% CI in parenthesis

[b] Score depicts weighting calculated as 1-FNF + SE + NPV

Table 9.2 shows the most useful crosspoints of which the bottom left quadrants were sparsely populated with PCs. All bitches with PC below 3.18 nmol/L are expected to start undergoing cervical dilatation within 24 h. Ninety nine percent of bitches with a PC below 8.7 nmol/L are expected to start undergoing cervical dilatation within 48 h. Ninety nine percent of bitches with a PC below 15.8 nmol/L are expected to start undergoing cervical dilatation within 96 h. Only 1% of bitches with PC below 8.7 nmol/L are expected to take longer than 48 h to start undergoing cervical dilatation and only 7.5% longer than 24 h.

Looking at Table 9.1 and Table 9.2 together, the PC line of 8.7 nmol/L joins crosspoints lying at clinically important timelines. If a bitch has a PC of 8.7 nmol/L or higher the probability is 98% that TCD will not occur within 12 hours (NPV, Table 9.1). If her PC is below 8.7 nmol/L, the probability is 86% that TCD will occur within 24 h and 99% that it will occur within 48 h (PPV, Table 9.2).

Table 9.2

The most useful crosspoints by which the concentration of progesterone in blood plasma may aid in predicting the time of onset of cervical dilatation because few PCs occur in their bottom left quadrants

Crosspoint	FPF[a]	SP[a]	PPV[a]	Score[b]
24 h by 3.18 nmol/L	0.00 (0.00–0.02)	1.00 (0.98–1.00)	1.00 (0.90–1.00)	3.00
48 h by 8.7 nmol/L	0.01 (0.00–0.06)	0.99 (0.94–1.00)	0.99 (0.94–1.00)	2.97
24 h by 8.7 nmol/L	0.07 (0.04–0.13)	0.92 (0.87–0.96)	0.86 (0.77–0.92)	2.71
96 h by 15.8 nmol/L	0.15 (0.04–0.42)	0.85 (0.58–0.96)	0.99 (0.96–1.00)	2.68

[a] 95% CI in parenthesis

[b] Score depicts weighting calculated as 1-FPF + SP + PPV

9.5. Discussion

The mean interassay coefficients of variation for both the progesterone and cortisol in the current study was sufficiently low not to have substantially influenced the results of this study. Our interassay coefficients were similar to those previously published for progesterone (Kutzler et al., 2003a).

In the preparturient period the best predictor of TCD would ideally give the veterinary obstetrician 12 h or more notice in advance of impending parturition and the result be available within hours. The current study shows that the concentration of cortisol in the blood plasma, as well as the pattern of change thereof over time during the few days preceding parturition, even when 6 hourly or 12 hourly sampling are considered, is too variable within and among bitches to be of use as a predictor of the time of onset of parturition. Observing a concentration of 100 nmol/L in a preparturient bitch may be of value in suggesting that parturient cervical dilatation is likely to start within 30 h. In the current study it cannot be determined whether increases in maternal cortisol resulted from imminent parturition only or perhaps complicated by manipulation. However, the bitches in the current study were a well habituated group of bitches used to travelling, showing and residing at the obstetric clinic for purposes of stud and management of parturition. The cortisol profile in the preparturient period may therefore be different in non-habituated and stressed bitches. This factor may limit the scope and restrict the value of any inference drawn from the current study with respect to the temporal relationship between cortisol and TCD.

It is interesting to note that of the two bitches that had a sharp rise in preparturient cortisol

(exceeding 240 nmol/L), also unexpectedly had a concomitant preparturient PC rise at TCD. This may be explained by progesterone being a precursor in the endocrine production pathway of cortisol. More research is required to establish the positive relationship between high cortisol and PC in the blood of preparturient bitches and the clinical significance of this finding on PC as a preparturient predictor of TCD. Assuming that a sudden unexpected preparturient PC rise does occur in some bitches when randomly sampled at TCD, the clinician would be able to identify such bitches during the vaginal speculum examination by observing a dilated cervix. This accentuates the value of clinical assessments during the management of parturition in the bitch.

In agreement with other studies (Concannon et al., 1975; England and Verstegen, 1996a; Onclin and Verstegen, 1997; Concannon et al., 1988), the current study demonstrated a consistent decrease in PC during the 36 h leading to spontaneous parturition. Although the means of PC reported at the onset of the second stage of parturition reported by Veronesi et al. (2002) was 3.2 nmol/L and that by Concannon et al. (1988) approximately 2.9 nmol/L, one may, from their standard deviation (Veronesi et al.) or standard error (Concannon et al.), conclude that concentrations as high as 5.5 nmol/L and 6.9 nmol/L at the birth of the first puppy, are feasible. Sixty eight percent (17 of 25) and 84% (21 of 25) of the PCs at the time when cervical dilatation was first noted in the current study, were below the likely high values of 5.5 nmol/L and 6.90 nmol/L that that Veronesi et al. found and Concannon et al. found, at the birth of the first puppy. Cervical dilatation precedes stage 2 of parturition by an unknown period, suggesting that the mean (or median) PC at first observation of cervical dilatation, may be higher than it would be at the onset of the second stage of parturition. The results of the current study supports this view.

It is suspected that incomplete luteolysis might have a role in the singleton enigma (Lopate, 2008; Johnson, 2008a). If so, preparturient PC may be of no use in alerting the veterinary obstetrician to imminent parturition in singleton pregnancies and singletons were therefore removed from the current study. Further research is required as to how best deal with the singleton pregnancy.

Figure 9.4 shows the PCs of 297 samples drawn from 25 bitches at various intervals, starting from 132 h before TCD until TCD. We interpreted those PCs as if they were random samples from 297 preparturient bitches, which they were not. They were repeated samples from each of 25 bitches. Therefore caution should be exercised in generalising the inference to all

bitches. Not all crosspoints need to be of similar value in any bitch. The wide spread in PCs at any given time before TCD shown in Figure 9.4, together with the expected pattern of decline towards TCD suggests that the 297 PCs used in this study may approximate 297 randomly taken PCs from a random sample of 297 parturient bitches that were at the time intervals before TCD as depicted in the figure, supporting our assumption.

The results of the current study show that no preparturient threshold in the concentration of progesterone exist below which absolute conclusions can be drawn about when parturient cervical dilatation would occur in all bitches (Figure 9.3). Our data did not identify a PC above which the probability would be high that a bitch would not enter spontaneous parturition within 48 h or not within 72 h or not within 96 h. This is because the PC may decline rapidly from above 15.8 nmol/L to below 3.18 nmol/L in 12–24 h. However, the selected crosspoints with their timelines and PC lines do provide the veterinary obstetrician with useful means of predicting TCD in large proportions of the obstetric population as indicated by the high NPV and PPV. Veterinary obstetricians need a crosspoint of which the timeline is such that a bitch is highly unlikely to enter spontaneous parturition sooner than its timeline suggests if her PC lies above the PC line of the crosspoint (crosspoint with a high NPV). Equally, veterinary obstetricians need a crosspoint such that a bitch is highly likely to enter spontaneous parturition within an interval shorter than that signified by the timeline, provided that her PC lies below the PC line of the crosspoint (crosspoint with high PPV).

The NPV for PC above 15.8 nmol/L at timeline 12 h is 0.99 (0.95–1.00) and indicates that if the bitch has a PC of above 15.8 nmol/L, there is a 99% chance that the bitch will not enter spontaneous parturition within the following 12 h. The NPV for PC above 8.7 nmol/L is 0.98, indicating that the probability of a bitch with a PC above 8.7 nmol/L not entering spontaneous parturition within the following 12 h is 98%. When below 15.8 nmol/L there is a 99% probability of parturition within 96 h. These predictions help the veterinary obstetrician in making the decision whether the bitch may be left unattended overnight or not. From a clinical standpoint, the crosspoint at 3.18 nmol/L and the 24 h timeline is valuable. Its PPV of 100% suggests that all bitches having a PC below 3.18 nmol/L are expected to enter spontaneous parturition within 24 h and are likely ready for immediate CS. The crosspoints on the 8.7 nmol/L PC line at the 12 h, 24 h and 48 h timelines are also of clinical importance. If a bitch has a PC above 8.7 nmol/L the probability is 98% that TCD

will not occur within 12 hours whereas, if her PC is below 8.7 nmol/L, the probability is 86% that TCD will occur within 24 h and 99% that it will occur within 48 h.

The clinical value of the crosspoints at timelines 12 h, 24 h, 48 h and 96 h, suggest that when using PC to predict the onset of spontaneous parturition in the bitch, blood should ideally be collected every 12 h. If the clinician would elect to collect only once a day, this single sample should then be collected just before closing time of the clinic. This is because during the day time-hours the bitch could be monitored and the in-house PC assay just prior to night time-hours would reveal the probability of the bitch entering spontaneous parturition or not in the following 12 h.

Being sure that the bitch is within the 48 h timeline of spontaneous parturition is particularly useful. This is because previous studies have shown that foetuses are viable when delivered within this critical period before the onset of spontaneous parturition (Baan et al., 2005; Levy et al., 2009; Vannucchi et al., 2012).

The attempt made in the current study to determine the precision with which PC in the preparturient bitch is able to predict TCD was crucial. This is because the risk associated with poor precision could lead to performing CSs too early or too late with catastrophic consequences for the foetuses and possible legal actions if retrospectively, intervention or lack thereof, is claimed to have reduced puppy survival. Despite the finding in the current study that the PPV and NPV approaches 100% for certain timelines below 48 h, further studies are required to establish whether it is safe to routinely perform CSs based on the PC having reached critical crosspoints. These results allow the veterinary obstetrician to make prompt decisions in the management of parturition.

9.6. Conclusions

In the preparturient period a random sample with a PC; of above 15.8 nmol/L indicates that there is a 99% probability that the bitch will not enter spontaneous parturition within the following 12 h, when above 8.7 nmol/L there is a 98% probability of not entering spontaneous parturition within the following 12 h. These predictions help the veterinary obstetrician in making the decision whether the bitch may be left unattended overnight or not. When the PC is below 15.8 nmol/L, there is a 99% probability of entering spontaneous parturition within 96 h, when below 8.7 nmol/L, 99% probability of entering spontaneous parturition within 48 h or less, and when below 3.18 nmol/l, 100% probability of entering

spontaneous parturition within 24 h or less. Preparturient PC in the blood of the bitch may allow the veterinary obstetrician to make prompt decisions in the management of parturition. The data did not allow for selecting a PC above which the bitch is highly likely to be further than 48 h or more from the onset of spontaneous parturition. The results of this study show that the concentration of cortisol in the blood plasma is too variable within and among bitches to be of use as a predictor of the time of onset of parturition. These results allow the veterinary obstetrician to make prompt decisions in the management of parturition.

Chapter 10. Performing preparturient caesarean section in bitches

K G M De Cramer*, J O Nöthling[2]

The content of this chapter will be submitted for publication in a different format as an article by J.O. Nöthling and K.G.M. De Cramer under the title "Preparturient caesarean section in bitches"

Abstract

Properly planned elective CS is considered a safe, effective and justified intervention for some breeds. At the time of onset of parturition there may already be foetal distress or demise in some bitches, inadvertently changing elective CSs to emergency CSs. Therefore planning a scheduled (fixed date and time) preparturient (before the onset of parturient cervical dilatation) CS in bitches where a CS is unavoidable, seems justified. The current study assumed that a foetus can be delivered and remain viable without assistance 48 h before the expected time of spontaneous parturition (Baan et al., 2005; Levy et al., 2009; Vannucchi et al., 2012). In a previous study by the authors the precision of the first day of cytological diestrus (D0) was found to be the most precise and practical predictor of the onset of cervical dilatation (Chapter 8). The aims of this study were to (1) compare the mortality of puppies delivered by preparturient CS to that of puppies delivered by parturient CS, (2) compare the probability of mortality of puppies delivered by preparturient CS to the mortality ratios of puppies delivered by CS at full term reported in the literature, (3) determine the extent to which a preparturient CS (done at 08:00 on D57, while the cervix is closed) would shorten gestation, (4) compare the concentration of progesterone in the serum of bitches at the time of preparturient CS to that at the time of parturient CS, (5) compare the change in haematocrit before and after CS for parturient- and preparturient CSs. Out of 99 gestations for which D0 was known and a preparturient CS planned for 08:00 on D57, the CS was performed at the scheduled time in 61%, before the scheduled time because cervical dilatation had started in 32% and before the scheduled time in 7% because the bitches had started showing signs of impending parturition, although their cervices were still closed. Therefore although the parturition observation was reduced for most of the bitches it was still necessary to observe bitches from the morning of D54 until the morning of D57. Finally, the study compared the results of 205 parturient CSs with 67 preparturient CSs. This study

showed high neonatal survival ratios after preparturient CS with 99% of puppies delivered by preparturient CS, born alive, 98.8% surviving to 2 hours and 88.6% to 7 d, suggesting that the protocol is safe with regards to puppy survival rates. The odds of stillbirth, dying before two hours and dying before 7 d were similar for parturient and preparturient CSs.The overall mortality of puppies delivered by preparturient CSs compared favourably to both those reported in the literature as well as to parturient CSs performed in the current study.

The extent whereby the gestations were shortened by performing preparturient CSs in this study ranged from 4–52 h and in 50% of the CSs, gestation would have been shortened by at least 10 hours. Progesterone concentration was significantly higher at the time of preparturient CSs than at the time of parturient CSs, with medians of 8.3 nmol/L (Q1 3.8, Q3 15.1) and 2.71 nmol/L (Q1 1.28, Q3 4.75), respectively. The current study concluded that hat performing a fixed date and fixed time (08:00 on D57) preparturient CS is safe for both the puppies and the dam and reduced the time spent performing parturition observation. The study provides the veterinary obstetrician with a protocol that can be used to safely perform elective preparturient CSs in a large proportion of the obstetric population but not all and that observation of parturition is still required for that proportion of the obstetric population that had not had a dilated cervix by the morning of D57. Further studies are required to timeously identify those bitches that enter spontaneous onset of parturition prior to our fixed date and time and to conclude whether it is routinely safe to perform preparturient CSs on D57.

10.1. Introduction

The veterinary community and dog breeding fraternities have thus far failed to reduce the high proportion of deliveries requiring CS in many breeds (Munnich and Kuchenmeister, 2009; Evans and Adams, 2010; Linde-Forsberg and Eneroth, 2000; Davidson, 2008; Trautmann and Nolte, 2003; Johnson, 2008a; Moon et al., 1998; Bergström et al., 2006b). In the Boston Terrier, English Bulldog and French Bulldog more than 80% of litters are delivered by CS (Evans and Adams, 2010; Munnich and Kuchenmeister, 2009; Wydooghe et al., 2013). In such breeds, properly planned elective CS is considered a safe, effective and justified intervention (Linde-Forsberg and Eneroth, 2000; Davidson, 2008). In addition, within the canine obstetric population, irrespective of breed, there exist subpopulations of pregnant bitches in which the prevalence of obstetric complications is likely to be higher than that of the general obstetric population. These may be defined as high-risk pregnancies

and include pregnancies with a history of dystocia (Stolla et al., 1999), history of prior CS and those with known large litters (Bennett, 1974). Considering that more than 60% of dystocia cases end up in CS anyway (Bergström et al., 2006b; Polster et al., 2005) and that delays due to failed medical management may lead to increases in foetal losses (Moon et al., 2000; Munnich and Kuchenmeister, 2009; Michel and Reichler, 2008a), a strong argument can be made for planned elective CS in this high-risk obstetric population.

Even when litters are delivered by elective CS, stillbirths may occur. Wydooghe (2013) reported that 13% of English Bulldog foetuses delivered by CS at full term were stillborn. Moon et al. (1988) reported puppy mortality data on a wide variety of breeds: 8% of foetuses delivered by CS at full term were stillborn, 13% died before they were 2 h old and 20% died before they were 7 d old.

Using the signs of parturition to time elective CSs in the bitch is problematic, as by then there may already be foetal distress or demise in some bitches. Therefore elective CSs may become emergency CSs associated with reduced survival of the offspring in the bitch (Moon et al., 2000). Preparturient CS may reduce the prevalence of parturient foetal demise and stillbirths in these high-risk pregnancies. The time of day during which CSs are performed influences the proportion of still born foetuses because a full staff complement during normal working hours help maximize puppy survival (Smith, 2007). These arguments form the basis for planning elective CSs as preparturient CSs, occurring at a fixed date and time before the onset of parturient cervical dilatation, in bitches where a CS is unavoidable. The management of parturition exposes clinicians to criticism if their intervention or lack thereof is thought to have reduced puppy survival. The concept of a reliable protocol allowing planned preparturient CS therefore resonates with clinicians' needs. Planned preparturient CS obviates the need for after-hour interventions and allows for timeous delivery of the puppies potentially reducing the prevalence of foetal compromise and still births.

In women, term is a well-defined safe period of four weeks (37 to 41 weeks of gestation), also known as the safe period of intervention, wherein a foetus may be delivered without having increased risk of complications compared to foetuses delivered at the time of spontaneous labour (Fleischman et al., 2010b). Planned term CSs are only possible in a species when the safe period of intervention as well as a method confirming that foetuses are within that safe period, are known. There is no literature on this period of intervention in the bitch and we do not know how long this period is. Performing a preparturient CS

requires some knowledge of the range of the safe period of intervention in the bitch. The current study assumed that a foetus can be delivered and remain viable without assistance 48 h before the expected time of spontaneous parturition. This assumption was based on current knowledge that foetuses born naturally or by CS as much as 48 h prior to the expected time of spontaneous parturition from bitches treated with aglepristone (Baan et al., 2005; Levy et al., 2009) or betamethasone (Vannucchi et al., 2012) survive without special assistance.

In a previous study by the authors the precision of the first day of cytological diestrus (D0) was found to be the most precise and practical predictor of the onset of cervical dilatation. The first day of cytological diestrus predicted the date of cervical dilatation with a precision of ± 1 d, ± 2 d and ± 3 d in 88%, 99% and 100% in 242 oestrous cycles (Chapter 8).

No study thus far, used D0 to time CS nor did any study perform preparturient CS without the antenatal maternal administration of either aglepristone or betamethasone.

The first aim of this study was to compare the mortality of puppies delivered by preparturient CS (CS performed on D57 or D56, while the cervix was still closed), to that of puppies delivered by parturient CS (CS performed once cervical dilatation has started). The second aim was to compare the probability of mortality of puppies delivered by preparturient CS to the mortality ratios of puppies delivered by CS at full term reported in the literature. The third aim was to determine the extent to which gestation of the bitches would be shortened by performing preparturient CSs at 08:00 on D57 if their cervices have not dilated by then or they have not yet shown signs of imminent parturition before that time. The fourth aim was to compare the concentration of progesterone in the serum at the time of CS for bitches undergoing preparturient CS to that of parturient CS. The fifth aim was to compare the change in haematocrit before and after CS of parturient CSs to preparturient CSs.

10.2. Materials and methods

The protocol was approved by the Animal Ethics Committee of the Faculty of Veterinary Science of the University of Pretoria (Project numbers v071-13, v010-14, v010-14 amend1, v048-14 and v079-15). The experimental animals were all housed and fed commercial dry pellets twice daily and had access to ad-lib water. They were privately owned English Bulldog and Boerboel bitches presented to a private veterinary clinic for routine oestrus monitoring, artificial insemination and elective CS. This choice of breed was in line with

the typical patient profile of the private clinic, which consisted mostly of English Bulldog and Boerboel bitches, although other breeds were also represented. The owners declined a trial of labour (attempt at spontaneous unassisted parturition) for all the bitches in this study.

During pro-oestrus and oestrus, vaginoscopy (Jeffcoate and Lindsay, 1989) was used to time AI and vaginal cytology was performed to establish D0 (Holst and Phemister, 1974). The vaginoscopy and vaginal cytology was performed at 08:00 a.m. This standardizing of time of collecting data for D0 was necessary to obtain accurate relevant time intervals between D0 and CS. In the pre-parturient period, all bitches were monitored for behavioural changes associated with impending parturition (sustained panting for > 2 h, restlessness, nesting behaviour, depression, vomition, anorexia, looking towards flanks). Based on the findings of Holst and Phemister (1974) and those reported in Chapter 8, the expected day of cervical dilatation was taken as D57 (D0 + 57 d). In all the bitches vaginal speculum examinations were performed every 6 h, starting at 08:00 on D54, to observe for the first sign of any degree of dilatation of the cervix.

10.2.1. Allocation of bitches and CSs to groups

First, 173 CSs were performed on 148 bitches (74 on 58 English Bulldog bitches and 99 on 90 Boerboel bitches) once cervical dilatation was first seen to have started. These CSs and bitches were the control group.

Following the CSs in the control group, the 99 experimental CSs were performed. They included 60 CSs performed on 45 bitches (17 on eight English Bulldog bitches and 43 on 37 Boerboel bitches) at 08:00 on D57, while their cervices were still closed. These 60 CSs were allocated to Group D57. The experimental CSs also included seven CSs on five bitches (two on one English Bulldog bitch and five on four Boerboel bitches) that were done on D56 while their cervices were still closed. The seven CSs in these five bitches were performed because the bitches showed what were considered signs of imminent parturition (restlessness, panting, displaying nesting behaviour, anorexia, abdominal discomfort, licking of the vulva, depression and looking at the flanks) whilst their cervices were still closed. These seven CSs were allocated to Group D56. Finally, the experimental CSs included 32 CSs on 26 bitches (four on one English Bulldog bitch and 28 CSs on 25 Boerboel bitches) done before D57, once the cervix of these bitches had started to dilate. These 32 CSs were assigned to the experimental parturient group.

The 173 CSs of the control group and the 32 CSs of the experimental parturient group were combined into the parturient group, consisting of 205 CSs in 174 bitches. The 60 CSs of Group D57 and the seven of Group D56 were combined to form the preparturient group, consisting of 67 CSs in 50 bitches.

Only healthy bitches destined for elective CS were included in the current study. No ovariohysterectomies were performed and the placentas were removed with each puppy. The bitches where weighed immediately prior to surgery and anesthetized using the standard anaesthetic protocol in the practice which included low dose alpha2-adrenergic agonist premedication (Medetomidine 7 µg/kg iv) (Zoetis Animal Health, Sandton, South Africa), propofol (1–2 mg/kg iv) (Fresenius Kabi, Midrand, South Africa) as induction agent and sevoflurane (1–2%) (Safeline Pharmaceuticals, Northcliff, South Africa) in oxygen for maintenance of anaesthesia (Chapter 4). The CS was performed in standard fashion as previously described (Gilson, 2003). The haematocrit was assessed before and after CS as previously described (De Cramer et al., 2016). At the time of CS, a serum sample was collected for some dogs of all groups, left at room temperature for 2 h and then centrifuged and transferred to a cryovial and frozen at -20°C for progesterone assay using chemiluminescent immunoassay (Immulite LKPW1 ®). Live puppies born with malformations were euthanized within 15 min of delivery.

Critical data collected in the periparturient period were progesterone concentration in the serum at the time of CS; haematocrit before and after CS; state of the cervix (dilating or closed); numbers of puppies that were normal, deformed, dead and euthanized; time that the CS was performed; maternal survival and puppy survival at 2 h and 7 d after CS.

10.3. Data analysis

The frequencies of puppies born alive, surviving to 2 h and surviving to 7 d were summarized and reported as percentages for English Bulldogs and Boerboels and both breeds combined. This was done for parturient CSs (those performed at the time of spontaneous cervical dilatation) and for preparturient CSs (those performed prior to the onset of cervical dilatation).

The percentages of litters with live and stillborn foetuses and the percentages of puppies that were born alive or were stillborn in such litters were summarised for both breeds.

A mixed-effects logistic regression was done to determine the effect of day of CS, breed and litter size on the log odds of foetuses being stillborn. Day of CS had three levels: Zero for CSs performed at the first observation of spontaneous cervical dilatation; one for CSs performed at 08:00 on D57, while the cervix was still closed and two for CSs performed on D56, while the cervix was still closed. Breed had two levels: Zero for English Bulldogs and one for Boerboels. Litter size stated the number of foetuses born, irrespective of whether they had died before or during CS. Data were clustered in litters.

The same mixed-effects logistic model was used to assess the effect on the log odds of puppies spontaneously dying before 2 h. Stillborn foetuses were included as losses prior to 2 h, but puppies that were born alive but euthanized due to serious congenital defects were excluded.

The same mixed-effects logistic model was also used to assess the effect on the log odds of puppies dying spontaneously before 7 d. Foetuses that were stillborn, as well as puppies that died spontaneously before 2 h were included as losses prior to 7 d.

For each mixed-effect logistic regression model the first-order interactions between day of CS and breed, between day of CS and litter size, and between breed and litter size were first included in the model, following which each model was finally run again without the interaction terms.

Following each final logistic regression model, the population-averaged predictive probability of stillbirth or death prior to 2 h or death prior to 7 d were, each with its 90% CI, respectively determined. In order to determine whether the mortality ratios found in the current study were lower than those reported in the literature for puppies delivered by full term CS (one-sided comparison, $P < 0.05$), the upper limit of the 90% CI of each mortality ratio found in the current study was compared to the probability of mortality of puppies delivered by CS at full term as reported in the literature.

A Kruskal-Wallis test was used to determine whether distributions of the concentrations of progesterone differed among the four subgroups. They were the parturient CS control subgroup, parturient CS experimental subgroup, preparturient D57 subgroup and the preparturient D56-D55 subgroup. Wilcoxon's rank sum test, with Bonferroni's correction was used for pairwise comparisons ($P < 0.05$).

Multiple regression was used to compare the declines in haematocrit during CSs performed before the onset of cervical dilatation (preparturient group) to those occurring during CSs performed once cervical dilatation had started (parturient group). Breed (Boerboel compared to English Bulldog) and litter size as well as the interaction between them were included as covariates. The interaction was first removed from the model, sequentially followed by the covariates that did not significantly affect the decline in haematocrit ($P > 0.05$). The five largest declines in haematocrit (one each of 13, 14 and 16 percentage points associated with CSs in parturient bitches and one each of 13 and 14 percentage points associated with CSs done on preparturient bitches) were removed from the model to avoid heteroskedasticity and residuals that were not normally distributed.

10.4. Results

Table 10.1 shows the numbers of puppies that were stillborn or died before two hours or before 7 d of life after having been delivered by parturient caesarean section or preparturient caesarean sections on D57 or D56.

Table 10.2 shows the numbers and percentages of puppies alive at birth, 2 h and 7 d following CS done upon first observation of a dilating cervix (parturient CS) or CS done before cervical dilatation had started (preparturient CS).

Table 10.1

Survival status of puppies delivered by parturient caesarean section (CS) or preparturient CS 57 days (D57) or 56 days (D56) after the onset of cytological dioestrus

	English Bulldog		Boerboel	
Day of CS	Survived	Died	Survived	Died
At birth				
Parturient	476	15	1189	26
Preparturient on D57	91	3	266	1
Preparturient on D56	14	0	44	0
Two hours after birth				
Parturient	431	28	1164	36
Preparturient on D57	80	4	263	1
Preparturient on D56	12	0	43	0
Seven days after birth				
Parturient	388	71	1090	110
Preparturient on D57	71	13	230	26
Preparturient on D56	10	2	39	4

Table 10.2

Frequency and percentage of puppies alive at birth, 2 h and 7 d following parturient and preparturient caesarean section in bitches

	Parturient CS group (dilating cervix)			Preparturient CS (closed cervix)		
	English Bulldog (78 litters from 59 bitches)	Boerboel (127 litters from 115 bitches)	Both breeds (205 litters from 174 bitches)	English Bulldog (19 litters from 9 bitches)	Boerboel (48 litters from 41 bitches)	Both breeds (67 litters from 50 bitches)
Born alive	476 of 491 (97.0)[a]	1189 of 1215 (97.9)	1665 of 1706 (97.6)	105 of 108 (97.2)	310 of 311 (99.7)	415 of 419 (99.0)
Alive at 2 hours[b]	431 of 459 (93.9)	1164 of 1200 (97.0)	1595 of 1659 (96.1)	92 of 96 (95.8)	306 of 307 (99.7)	398 of 403 (98.8)
Alive at 7 days[b]	388 of 459 (84.5)	1090 of 1200 (90.8)	1478 of 1659 (89.1)	81 of 96 (84.3)	269 of 299 (90.0)	350 of 395 (88.6)

[a] The first number in each cell shows the number of live puppies, the second the total number of puppies and percentages are between parentheses

[b] Numbers in these rows exclude puppies that were euthanized due to severe congenital defects

10.4.1. Effects of day of CS, breed and litter size on the odds of stillbirth

No interaction was significant ($P \geq 0.12$).

After controlling for breed and litter size, the log odds of still births for preparturient CSs performed on D57 was -1.18 (95% CI -2.46 to 0.09), which tended to be lower than the log odds of still births for parturient CSs ($P = 0.07$). No puppies were stillborn from preparturient CSs performed on D56 and the log odds were not determined for that group of CSs.

Controlling for day of CS and litter size, the log odds of stillbirths in Boerboels was -0.24 (95% CI -1.12 to 0.64), which was similar to that in English Bulldogs ($P = 0.60$).

After controlling for day of CS and breed, the log odds of stillbirth tended to decrease by 0.13 (95% CI 0.00 to 0.26) for each increase in one of litter size ($P = 0.06$). Transformed to odds, this means that, after controlling for day of CS and breed, the odds of stillbirth decreased by 12 percentage points (95% CI zero to 23 percentage points) for each increase in one of litter size ($P = 0.06$).

10.4.2. Effects of day of CS, breed and litter size on the log odds of neonatal death before 2 hours

No interaction was significant ($P \geq 0.17$).

After controlling for breed and litter size, the log odds of neonatal death before 2 h for preparturient CSs performed on D57 was -1.50 (95% CI -2.69 to -0.31), which was lower than the log odds of neonatal death before 2 h for parturient CSs ($P = 0.01$). No puppies delivered by preparturient CSs performed on D56 died before two hours and the log odds were not determined for that group of CSs.

Controlling for day of CS and litter size, the log odds of neonatal deaths before 2 h for Boerboels was -0.64 (95% CI -1.40 to 0.13), which was similar to that for English Bulldogs ($P = 0.10$).

After controlling for day of CS and breed, the log odds of neonatal death before 2 h tended to decrease by 0.11 (95% CI 0.00 to 0.22) for each increase in one of litter size, $P = 0.05$. Transformed to logs, this means that, after controlling for day of CS and breed, the odds of neonatal death before 2 h tended to decrease by 10.6 percentage points (95% CI zero to 20

percentage points) for each increase of one in litter size (P = 0.05).

10.4.3. Effects of day of CS, breed and litter size on the log odds of neonatal death before 7 d

No interaction was significant (P ≥ 0.38).

After controlling for breed and litter size, the log odds of neonatal death before 7 d for preparturient CSs performed on D57 was 0.08 (95% CI -0.39 to 0.55), which was similar to the log odds of neonatal death before 7 d for parturient CSs (P = 0.74). Similarly, the log odds of neonatal deaths before 7 d for preparturient CSs performed on D56 was 0.03 (95% CI -1.06 to 1.11), which was similar to that for parturient CSs (P = 0.96).

Controlling for day of CS and litter size, the log odds of neonatal deaths before 7 d was -0.54 (95% CI -0.95 to -0.12) in Boerboels, P = 0.01. Transformed to odds, this means that, controlling for day of CS and litter size, the odds of neonatal deaths before 7 d was 42 percentage points lower in Boerboels than in English Bulldogs (95% CI 12 to 61 percentage points lower), P = 0.01.

After controlling for day of CS and breed, the log odds of neonatal deaths before 7 d was independent of litter size (-0.008, 95% CI -0.066 to 0.050, P = 0.79).

Table 10.3

Predictive margins (population average probabilities) for stillbirths or neonatal deaths for parturient caesarean sections (performed once the cervix has started to dilate) or preparturient caesarean sections (performed while the cervix was still closed 57 or 56 d after the onset of cytological dioestrus)

	Stillborn	Died before 2 hours	Died before 7 days
English Bulldog			
Parturient	0.029 (0.015–0.044)[a]	0.057 (0.035–0.080)	0.148 (0.115–0.180)
Preparturient D57	0.010 (0.000–0.021)	0.015 (0.001–0.030)	0.157 (0.010–0.215)
Preparturient D56			0.151 (0.040–0.261)
Boerboel			
Parturient	0.024 (0.014–0.034)	0.033 (0.022–0.045)	0.095 (0.078–0.111)
Preparturient D57	0.008 (0.000–0.015)	0.008 (0.001–0.016)	0.101 (0.070–0.132)
Preparturient D56			0.097 (0.021–0.172)

[a] The first number shows the expected probability of death and the figures between parentheses its 90% (confidence interval)

10.4.4. Probability of puppy mortality with preparturient CSs compared to those reported in the literature

Table 10.3 shows the expected probabilities of stillbirth, death before 2 h and death before 7 d in English Bulldogs and Boerboels, with their 90% CIs.

The upper limits of the 90% CIs of stillborn foetuses of English Bulldogs delivered by parturient CS or preparturient CSs performed at 08:00 on D57 are below the 13% of stillborn English Bull Dog puppies delivered by CS at full term as reported by Wydooghe (2013). The upper limits of the 90% CIs of stillborn foetuses of English Bulldogs and Boerboels delivered by parturient CS or preparturient CSs performed at 08:00 on D57 are below the 8% of stillborn puppies delivered by CS at full term as reported by Moon et al. (1988).

The upper limits of the 90% CIs of English Bulldog- and Boerboel puppies delivered by parturient CS or preparturient CSs performed at 08:00 on D57 that died before they were 2 h old are below the 13% reported by Moon et al. (1988).

The upper limit of the 90% CI for the 7-day mortality ratio of English Bulldog puppies that were delivered by parturient CS was below the 20% for a variety of breeds reported by Moon et al. (1988), whereas the upper limits for English Bulldog puppies delivered by preparturient CSs were above 20%. The upper limit of the 90% CI for the 7-day mortality ratio of Boerboel puppies delivered by parturient CS or by preparturient CS at 08:00 on D57 or on D56 were below the 20% reported by Moon et al. (1988).

These results suggest that, apart from the 7-day mortality ratio of English Bulldog puppies delivered by preparturient CSs on D57 or on D56, the mortality ratios found in the current study are below those reported in the literature ($P < 0.05$).

10.4.5. The extent of shortening gestation by preparturient caesarean sections

Figure 10.1 shows the interval between D0 and caesarean section in the 173 parturient caesarean sections of the control group. The shaded bars represent the 88 caesarean sections that were performed later than they would have been if they were performed at 08:00 on D57. Half of these 88 CSs took place 4–10 h later than they would have if they were performed at 08:00 on D57 and the other half 10–52 h later. The central 50% of these 88 CSs (those lying between the 25th and the 75th percentiles), were performed between 4 and 24 h later than

would have if they were performed at 08:00 on D57.

Of the 99 experimental CSs, 69 (70%) took place at the scheduled date and time of 08:00 on D57, 92 (93%) took place within 24 h before that time and all occurred within48 h before that time.

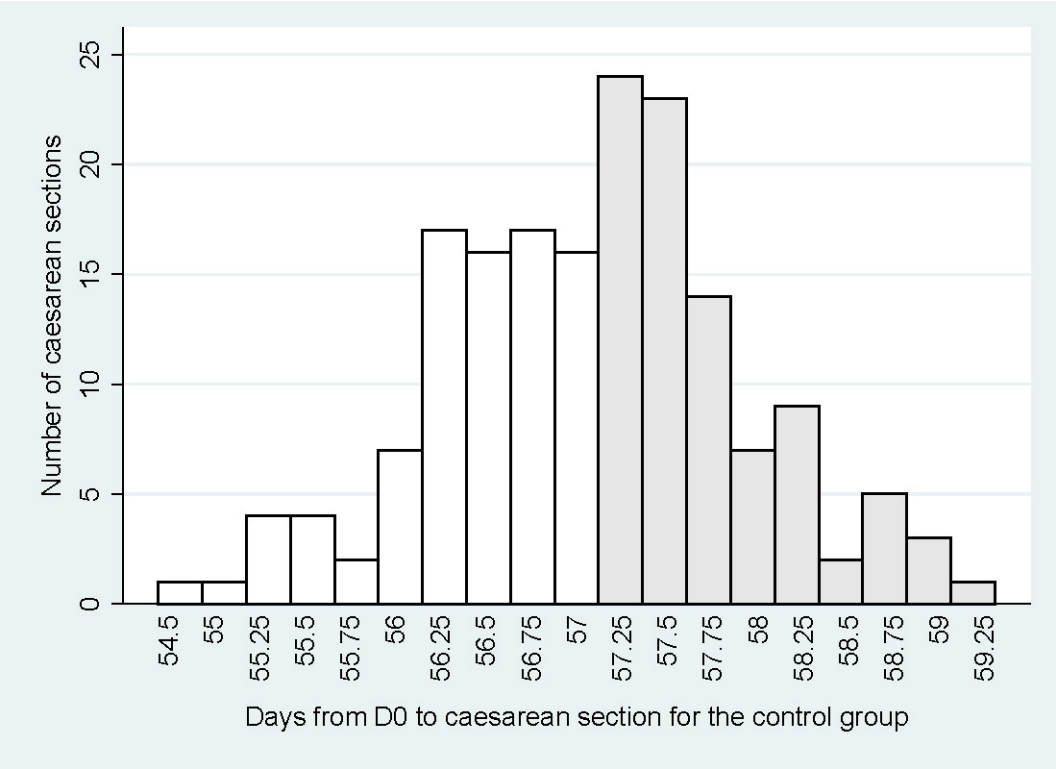

Figure 10.1
Interval between D0 and each of 173 caesarean sections from the control group, performed once cervical dilatation was first observed

10.4.6. Progesterone at the time of CS

Figure 10.2 shows the difference in PC at the time of CS in 50 parturient- and 52 preparturient CSs. Progesterone concentration was above 6.4 nmol/L in 31 (60% of) preparturient CS, compared to five (10% of) parturient CS ($P < 0.001$). Twenty three (44% of) preparturient CSs had PCs above 10 nmol/L compared to 4 (8% of) parturient CSs ($P < 0.001$).

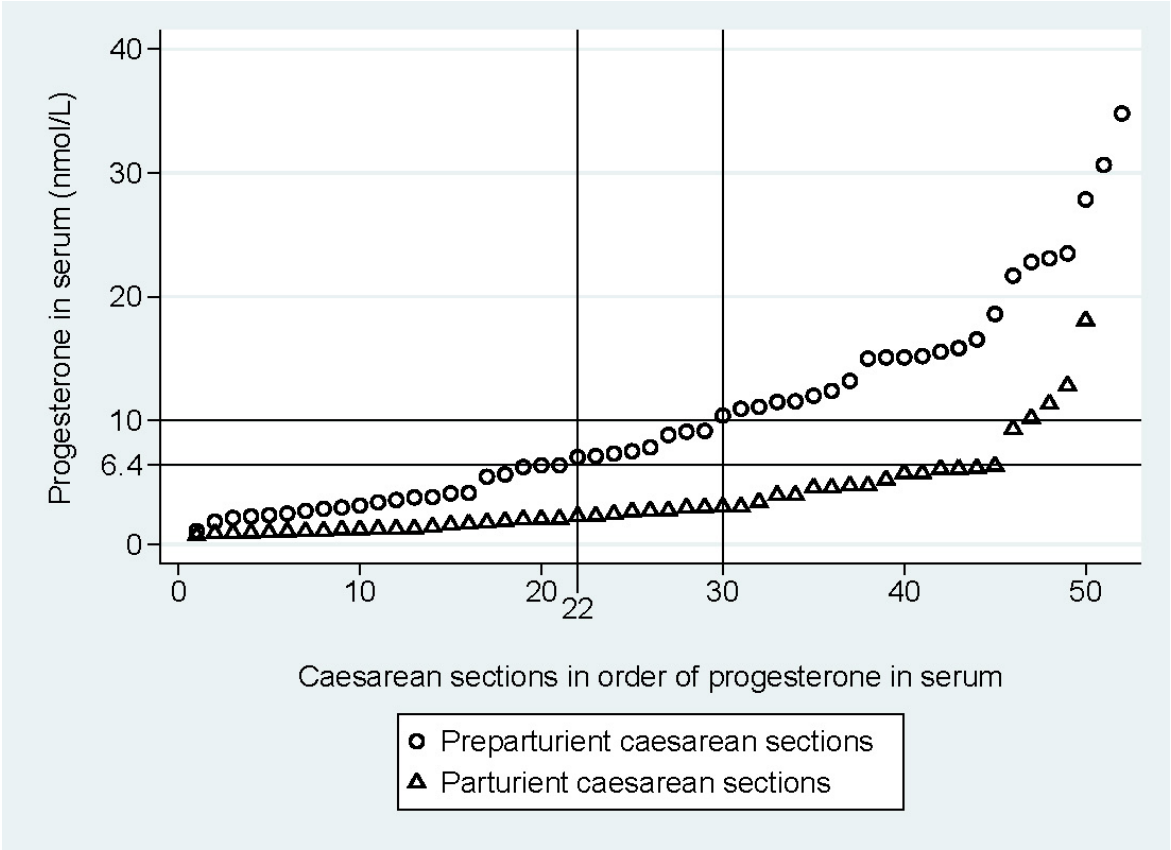

Figure 10.2

The progesterone concentration in the serum of bitches at the time of caesarean section, plotted in increasing order of concentration for 50 parturient- and 51 preparturient caesareans

Table 10.4 shows that the concentration of progesterone in the serum of bitches that underwent preparturient CS was higher than in bitches that underwent parturient CS. Table 10.4 also shows that all the data were positively skewed.

Considering preparturient CSs, the odds of stillbirth and of puppies dying at any time from birth to 7 d is independent of the concentration of progesterone in the serum at the time of CS ($P > 0.30$).

Table 10.4

Concentration of progesterone (nmol/L) in the serum at the time of CS

	Min	Q1	Med	Q3	Max	n	
Parturient CSs							
Control group	0.72	1.44	3.24	6.11	12.84	24	a
Experimental subgroup	0.90	1.27	2.19	3.98	18.10	26	a
Both subgroups combined	0.72	1.28	2.71	4.75	18.10	50	c
Preparturient CSs							
D57 (08:00) subgroup	1.08	3.58	7.52	15.10	34.80	47	b
D56 subgroup	3.79	11.50	13.20	15.55	27.85	5	b
Both subgroups combined	1.08	3.81	8.34	15.10	34.80	52	d

a, b Subgroups with different letters differ ($P < 0.05$)

c, d Groups with different letters differ ($P < 0.001$)

10.4.7. The effect of preparturient CS on haematocrit

After removal of the non-significant terms (interaction between breed and litter size ($P = 0.08$), breed ($P = 0.97$) and litter size ($P = 0.98$) from the model, CS on preparturient bitches resulted in a 1.29 (95% CI 0.55–2.02) percentage points smaller decline in haematocrit than CSs performed on parturient bitches ($P = 0.001$, $n = 259$). Haematocrit declined from 43.92 (SD 4.27) to 37.62 (SD 4.31) during the 193 CSs on parturient bitches and from 44.24 (SD 4.31) to 39.15 (SD 4.02) during the 66 CSs on preparturient bitches.

10.5. Discussion

The advantages of parturition induction protocols (Fontbonne et al., 2009; Fieni and Gogny, 2009) and preparturient CS (Levy et al., 2009) are similar. Both allow for a shorter period of supervision prior to the delivery of puppies and allows the clinician to expect a bitch to whelp or to perform a CS, on a predictable time. For high risk pregnancies, it is ideal to perform a preparturient CS to avoid foetal compromise. This notion concurs with the suggestion that a substantial proportion of pregnant brachycephalic bitches undergo elective CS before natural parturition begins (Evans and Adams, 2010). Once the decision has been made that an elective CS will be planned for a bitch, the problem of timing the elective CS manifests. Historically this decision was based on the display of imminent signs of parturition, having observed ruptured foetal membranes or having observed the presence of a dilated cervix (Smith, 2007).

The preparturient drop in PC to below 6.4 nmol/L in the last 24–36 h before parturition has been adequately demonstrated (Concannon et al., 1975; Concannon et al., 1978; England and Verstegen, 1996a). The only exception may be the singleton pregnancy (Johnson, 2008a). Although there is anecdotal evidence that preparturient PC can be used to confirm that the bitch is at term (Concannon, 2000), no controlled studies have been published showing that it is safe to perform a CS when the PC has decreased to below 6.4 nmol/L or that is unsafe to perform a CS when the PC is above 6.4 nmol/L.

Using D0 as predictor of the day of cervical dilatation (Chapter 8) or other methods of predicting the day of parturition (Concannon et al., 1977b; Kutzler et al., 2003a; Tsutsui et al., 2006), allows the clinician to predict the day of parturition to within 3 days on either side of the day on which parturition would actually occur. Because the current study performed preparturient CSs (n = 60) at a fixed time and date (08:00 on D57), parturition observation time was curtailed to a period of 72 h (from 08:00 on D54 until 08:00 on D57) or less. In addition, our protocol allowed for safe preparturient CS on D56 (n = 7) in bitches showing clinical signs of impending parturition. Out of 99 gestations for which D0 was known and a preparturient CS planned for 08:00 on D57, the CS was performed at the scheduled time in 60 (61%), before the scheduled time because cervical dilatation had started in 32 (32%) and before the scheduled time in 7 (7%) because the bitches had started showing signs of impending parturition, although their cervices were still closed. The time interval between first displays of behavioural signs of parturition to stage 2 of parturition may be very unreliable. Uterine contractions detected by electromyography associated with nesting behaviour was recorded 7 d before spontaneous parturition (van der Weyden et al., 1989) and therefore when no precise prediction date of parturition is available, behavioural signs cannot be used to time CSs. However, signs of imminent parturition at a time close to D57 (within 48 h of D57) may be more significant than when no prediction date is available.

Unless the interval between D0 and the day and time of cervical dilatation differ among breeds, this study provides a protocol that enables clinicians to perform CSs on a scheduled convenient time in approximately two thirds of bitches in which an elective CS has been planned. It also confirms the precision of the method of timing, using D0. However, the current study only examined two breeds and further research is required to establish whether the temporal relationship between D0 and the time of cervical dilatation differs among breeds.

Despite many decades of routine elective CSs in women, the timing of elective CSs in women is not perfect. The literature is nearly unanimous in recommending elective caesarean delivery for women at 39 weeks of gestation because of lower rates of neonatal respiratory complications compared to 38 weeks (Salim and Shalev, 2010). However, when the elective CS is scheduled at exactly 39 weeks, approximately 10% to 14% of women go into spontaneous labour (Smith, 2001b); meaning that a considerable number of women scheduled for an elective caesarean delivery at 39 weeks will deliver earlier in an unscheduled, frequently emergency, caesarean delivery (Salim and Shalev, 2010). Similarly, in the current study, our timing protocol for scheduled fixed time CS is not perfect. Although it was possible to perform scheduled preparturient CSs in 61% of the gestations with good outcome, it was still necessary to perform speculum examinations on all bitches every six hours. Thirty two percent of the CSs in the current study, also with good outcome, were performed because they were observed to have a dilated cervix before 08:00 on D57. It may be speculated that if the interval between vaginal speculum examinations be increased from every 6 h to every 12 h, there may be foetal compromise, poorer puppy survival and the need for some emergency CSs. The use of preparturient PC having reached certain crosspoints and timelines as discussed in Chapter 9, alludes to possible answers to mitigate this problem and help identify pregnancies which are likely to enter spontaneous parturition in the following 12 h, thereby negating the necessity to observe those bitches overnight.

This study showed high neonatal survival ratios after preparturient CS with 99% of puppies delivered by preparturient CS, born alive, 98.8% surviving to 2 hours and 88.6% to 7 d, suggesting that the protocol is safe with regards to puppy survival rates. The overall probability of puppy mortality with preparturient CSs compared favourably to those reported in the literature (Moon et al., 2000; Wydooghe et al., 2013) The mortality rate for the English Bulldog puppies delivered by both preparturient and parturient CSs was lower than those for Boerboel puppies which is in concurrence with the literature (Moon et al., 2000; Wydooghe et al., 2013).The odds of stillbirth, dying before two hours and dying before 7 d were similar for parturient and preparturient CSs.

The high neonatal survival rates obtained in this study confirms that our timing of the preparturient CSs using our protocol was good and resulted in delivery of the foetuses before foetal demise and compromise could occur during onset of parturition. More data on preparturient CSs performed on D57 in bitches carrying litters larger than one and on D56 in

bitches showing impending signs of parturition are required to conclude whether this is routinely safe for the English Bulldog and Boerboel breeds and whether this preparturient protocol is safe for other breeds.

This study shows that larger litters have lower stillbirth ratios and ratios of puppies dying before they are two hours old than smaller litters, but that there is no such an effect of litter size on ratio of puppies dying before 7 d. This suggests that once puppies have survived beyond two hours, factors other than those relating to CS, are the main determinants of their survival at 7 d and that stillbirth ratios and 2 h survival ratios are better suited to determine CS outcome than is the 7-day survival.

The current study showed that following CS, the English Bulldog puppies have higher odds of dying before 7 d than Boerboel puppies, which is in agreement with the English Bulldog having lower survival rates than other breeds after CS (Wydooghe et al., 2013). The number of puppies dying before 7 d per puppy surviving until 7 d is expected to be 42% lower for Boerboels than for English Bulldogs.

The timing of the preparturient CS is important. Performing preparturient CSs too early may yield premature, non-viable puppies, cause failure of placental release and increase the risk of serious uterine haemorrhage (Smith, 2007) and death of the dam (Pretzer, 2008). In the current study both, preparturient CSs and parturient CSs yielded high survival ratios with similar odds of puppy survival, suggesting that the preparturient CSs in the current study were performed in the so-called safe period of intervention (what is referred to as “term” in women). Also, our results suggest that significant lung maturation, allowing normal extra-uterine survival, occurs in the dog foetuses some time before spontaneous parturition and that our fixed-time preparturient intervention occurred after foetal lung maturation.

The current study shows that a scheduled preparturient CS, performed at 08:00 on the morning of D57, while the cervix is still closed, shortens gestation by 4–52 h relative to what would have been the case if it was performed upon first noticing cervical dilatation. In 50% of preparturient CSs, gestation would have been shortened by at least 10 hours. However, the current study does not allow one to determine by how long gestation was curtailed in each preparturient CS. More research is required to better define the safe period of intervention “term” in the bitch.

In the current study, PC was significantly higher at the time of preparturient CSs than at the time of parturient CSs, with medians of 8.34 nmol/L and 2.71 nmol/L, respectively. The proportions of preparturient CSs performed when the concentration of progesterone in the serum was higher than 6.4 nmol/L and 10 nmol/L, respectively, were substantially and highly significantly larger than those for parturient CSs. These differences confirm that, as a group, gestation of the preparturient group has not progressed as far by the time of CS as it did in the parturient group. The concentration of progesterone at the time of preparturient CS had no effect on the odds of stillbirth or of puppies dying before 7 d. The current study shows that it is not a prerequisite for the concentration of progesterone in the serum to decrease below 6.4 nmol/L in order to safely deliver foetuses by preparturient CS. This study also shows that five of the 50 parturient cases still had PC above 6.4 nmol/L (as high as 18 nmol/L) at the time of cervical dilatation.

The studies that did use predicted parturition dates to time CS in the bitch or induce parturition, all used the concentration of progesterone in blood plasma or serum either on its own or with that of LH during the peri-ovulatory period as the means of timing (Baan et al., 2005; Levy et al., 2009; Vannucchi et al., 2012; Fontbonne et al., 2009). Unfortunately, none of these studies are large or convincing enough to conclude that performing CS based on their timing method is safe for routine use in clinical practice. Also, these studies do not show whether it is safe not to use priming. Priming is the preparturient treatment of the dam in an attempt to stimulate foetal maturation. Aglepristone (Baan et al., 2005; Fontbonne et al., 2009; Levy et al., 2009) and betamethasone (Vannucchi et al., 2012) have been administered to preparturient bitches as priming agents but it is not known whether they are beneficial and, if they are, at what time before the onset spontaneous parturition. In contrast, the scientific basis for using prenatal corticosteroid therapy in preparing the human foetus for premature delivery (before 37^{+0} weeks) is extensive and convincing (Ballard and Ballard, 1995; Brownfoot et al., 2007). The current study achieved good results without priming. It is not known whether priming may influence the outcome of preparturient CSs performed earlier than was the case in the current study.

Fixed date CSs have advantages over current parturition induction protocols as it requires no time to monitor the delivery of puppies and no ecbolic support to complete parturition (Baan et al., 2005; Fieni and Gogny, 2009; Fontbonne et al., 2009).

Preparturient CS did not lead to complications in the dam and the maternal survival rate was 100%. The decline in haematocrit during CS for preparturient was slightly smaller (1.29 percentage points) than for parturient CSs.

Performing a CS while the cervix is closed poses a potential risk of retained placentas and metritis if the cervix does not dilate adequately to allow for placentas and lochia to escape. Retained placenta was avoided in the current study as each placenta was removed together with its foetus and no post-operative uterine infections were noted during the 7 d following CS. The removal of placentas was not associated with complications and renders it more likely to identify placental abnormalities and foetuses sharing placental sites (Joonè et al., 2015; Joonè et al., 2016). This does however not mean that routine removal of placentas can be advocated in all circumstances. This is because it cannot be excluded that the use of the alpha 2-agonist, medetomidine, may have influenced uterine tone and placental site haemorrhage.

10.6. Conclusions

For bitches destined for CS, the results of this study showed that performing a fixed date and fixed time preparturient CS based on the predicted day of cervical dilatation using the first day of cytological dioestrus, is safe for both the puppies and the dam and reduced the time spent performing parturition observation. The study provides the veterinary obstetrician with a protocol that can be used to safely perform elective preparturient CSs in a large proportion of the obstetric population. Further studies are required to timeously identify those bitches that enter spontaneous onset of parturition prior to our fixed date and time and to conclude whether preparturient CSs on D57 are routinely safe and safe in in all breeds

Chapter 11. Dizygotic monochorionic canine foetuses with blood chimaerism and suspected freemartinism

The content of this chapter has been published in a slightly different format as a case report. "Joonè*, C.J., De Cramer*, K.G.M., Nöthling, J.O., 2015. Dizygotic monochorionic canine fetuses with blood chimaerism and suspected freemartinism. Reproduction, Fertility and Development 29**,** 368-373."

*These authors contributed equally to this work.

Abstract.

Two full-term canine foetuses were found to share a placenta during CS. The foetuses were of discordant gender, with apparently normal male and female external genitalia. Genetic analysis of whole-blood samples obtained from each foetus revealed identical DNA profiles, with more than two alleles detected at six loci. Subsequent genetic analysis of myocardial tissue samples revealed dissimilar DNA profiles, with at most two alleles detected per locus. Superimposition of the tissue-derived profiles matched that derived from the blood samples exactly, except for two loci failing to amplify, and hence demonstrated blood chimaerism. Dissection of the abdomen of the male foetus revealed delayed descent of the testes towards the inguinal canals. Macroscopically, the gonads, uterus and vagina were not identifiable on dissection of the female foetus, although vestigial ovarian tissue and a vagina were detected microscopically. The hypoplastic internal reproductive tract of the female foetus was suggestive of freemartinism and is believed to be the first report of this condition in the canine.

keywords: disorder of sexual development, dog, DNA, shared placental site, twin, vascular anastomoses.

11.1. Introduction

The detection of monozygotic twinning in the canine remains elusive. Similarly, the syndrome of freemartinism, defined in cattle as a sterile female born as a co-twin to a male, has not previously been documented in the canine. A report published in 1946 described the discovery of two canine embryos within one placental site (Duke, 1946). These embryos shared both a chorion and yolk sac, but had separate amniotic and allantoic sacs. The author proposed them to be monozygotic twins due to both a shared chorion and, in particular, a shared yolk sac. More recently, Urhausen et al. (2013) similarly reported two canine foetuses within separate amnions but with a shared chorion. Genetic testing of the two foetuses ruled out monozygotic twinning by revealing dissimilar genetic profiles and genders. This report describes a case of dizygotic, monochorionic canine foetuses with blood chimaerism and suspected freemartinism.

11.2. Case report

A 2.5-year-old, primiparous Boerboel bitch artificially inseminated using fresh semen from a Boerboel male underwent an elective CS at a veterinary facility. The surgery was performed following vaginoscopic visualisation of a dilated cervix. Fifteen puppies were delivered, of which 11 were alive and vigorous. One puppy was subsequently euthanized due to *atresia ani* and the absence of a tail. The stillborn 12th and 13th foetuses were small and autolysed. Upon removal of the 14th foetus and its foetal membranes (already detached from the endometrium), a second umbilicus, originating from the 15th foetus, was seen extending from the same placenta (Figure 11.1). Both foetuses, with their common placenta, were removed *in toto* from the uterus. The amnions had ruptured during delivery, precluding their identification. Both foetuses were classified as full term, based on the vigour of their surviving litter mates, the presence of a fine hair coat extending to the tips of the ears and the paws (Mosier, 1978) and absence of autolysis (Figure 11.1).

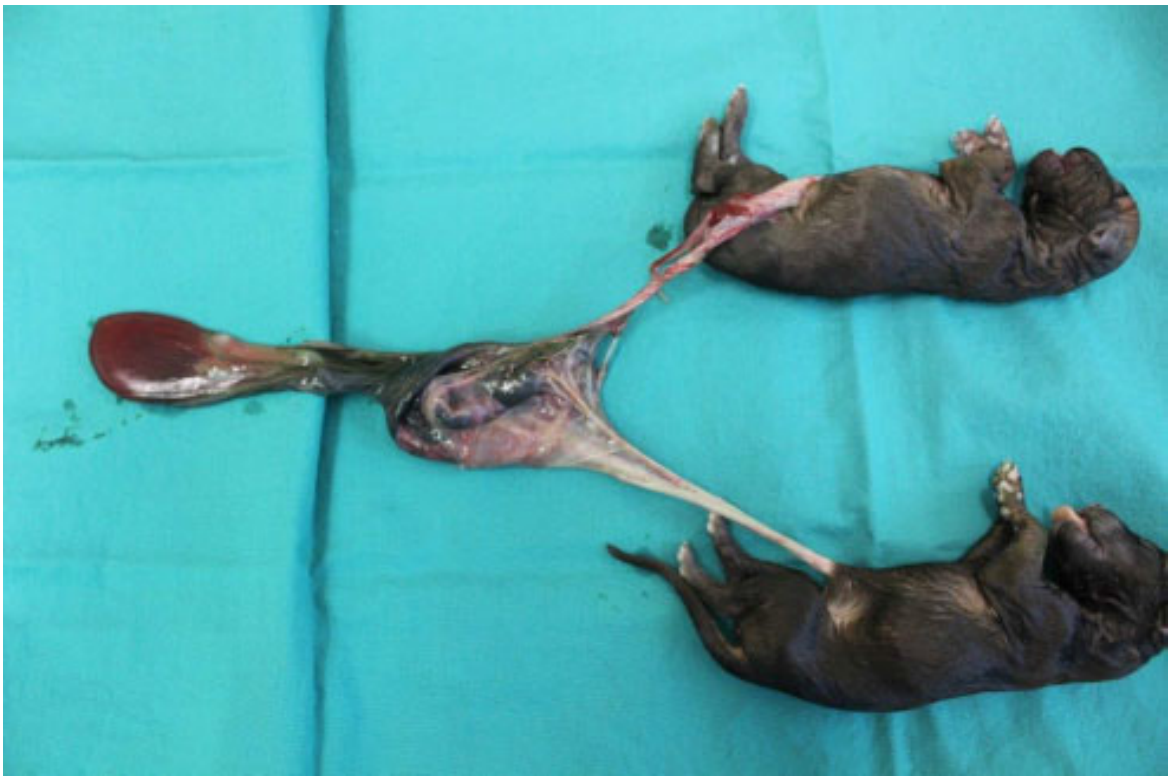

Figure 11.1
Photograph of male (MF) and female (FF) foetuses showing sharing of the placenta

The 14th and 15th foetuses, hereafter referred to as MF (male foetus) and FF (female foetus), were similar in size and of discordant gender. The external male and female genitalia appeared normal. Blood samples were taken from the heart of each foetus, via direct cardiac puncture, using aseptic technique and taking care to prevent cross-contamination between the foetuses. The samples were stored in ethylenediamine tetraacetic acid blood collection tubes before their transfer to FTA paper (Whatman, Maidstone, UK). Both foetuses were immediately preserved by immersion, as a whole, in 10% buffered formalin.

Genetic analyses were performed by the Veterinary Genetics Laboratory (University of Pretoria, Pretoria, South Africa). Following initial DNA profiling of the blood samples, additional samples of myocardium, obtained by dissection of each foetus, were submitted for genetic analysis.

Samples were profiled using a panel of 23 microsatellite markers recommended by the International Society for Animal Genetics (ISAG) and used routinely for canine DNA profiling and parentage verification, as well as the amelogenin marker for the determination of gender (Table 11.1).

Table 11.1

Results of genetic analyses of blood and myocardial tissue samples obtained from male (MF) and female (FF) foetuses with shared placenta

Data shows DNA fragment lengths, in base pairs, produced for microsatellite markers following polymerase chain reaction and capillary electrophoresis. –, marker that failed to amplify

Locus	Whole blood				Myocardium			
	Male and female foetus				Male foetus		Female foetus	
	Allele 1	Allele 2	Allele 3	Allele 4	Allele 1	Allele 2	Allele 1	Allele 2
AHT121	96	100			96	100	96	
AHT137	131	141	147		131	147	131	141
AHTh130	119	125	129		125	129	119	125
AHTh260	244	246			244	246	–	–
AHTk211	89	91			89	91	89	91
AHTk253	290				290		290	
CXX279	118	120			118	120	118	120
FH2001	132				132		132	
FH2054	148	160	176	180	148	176	160	180
FH2328	192	200	204		192	204	192	200
FH2848	236				236		236	
INRA21	95	101			95	101	101	
INU005	124	126			124		124	126
INU030	144	146			146		144	146
INU055	210	216	218		210	216	210	218
LEI004	–	–	–	–	95	107	95	105
REN105LO3	235	239			235	239	235	239
REN162C04	202	206			202	206	202	206
REN169D01	212	216	220		216	220	212	216
REN169O18	168	170			168	170	168	170
REN247M23	270	272			270	272	270	
REN54P11	222	236			222	236	222	236
REN64E19	–	–	–	–	145	147	147	153
AMEL	Y	X			Y	X	X	

Extraction of DNA from whole blood stored on FTA paper was performed as described

previously (Smith and Burgoyne, 2004). Tissue samples were processed using a phenol–chloroform DNA extraction protocol. Polymerase chain reaction by a 9800 Fast Thermal Cycler (Life Technologies, Johannesburg, South Africa) was followed by capillary electrophoresis on an ABI 3130 XL Genetic Analyser (Life Technologies). Fragment sizes for each marker were evaluated using the software program STR and Version 2.4.49 (University of California, Davis, CA, USA;(Toonen and Hughes, 2001).

Following temporary removal from formalin storage, the abdomen of each foetus was opened and the reproductive organs examined *in situ* before their extraction *in toto*. Transverse sections of the reproductive tract from each foetus were embedded in paraffin, sectioned at 6 mm and stained with haematoxylin and eosin (H&E) for histopathological examination. Images of sections were acquired using a Zeiss Axiostar Plus microscope, AxioCam ICc1 camera and AxioVision software (Carl Zeiss, Jena, Germany).

For comparison purposes, two unrelated, full-term, stillborn foetuses – one male and the other female, hereafter referred to as MC (male control) and FC (female control) – were similarly preserved in formalin soon after removal from their dams' uteri. These foetuses, which were of breeds similar in size to the Boerboel, were also dissected and their reproductive organs submitted for histopathological examination.

11.3. Results

Genetic analysis of the whole blood samples from MF and FF revealed identical DNA profiles, in terms of the peaks present as well as their relative fluorescence intensities. A notable finding was that of three alleles at five loci (AHT137, AHTh130, FH2328, INU055 and REN169D01) and four alleles at one locus (FH2054; Table 11.1). The amelogenin marker, located on the sex chromosomes, showed a heterozygous genotype consistent with a genetic male for both foetuses.

Profiling of the myocardial tissue samples from MF and FF generated two dissimilar genotypes, with no more than two alleles identified at each locus. Foetus MF showed an XY genotype at the amelogenin marker while FF was homozygous for the X allele. Superimposition of the tissue-derived DNA profiles of MF and FF provided an exact match to the DNA profile generated from the foetal blood samples, with the exception of two loci from the foetal blood samples that failed to amplify (Table 11.1).

The bladder and urachus extending to the umbilicus were clearly identified upon abdominal dissection of MF and FF.

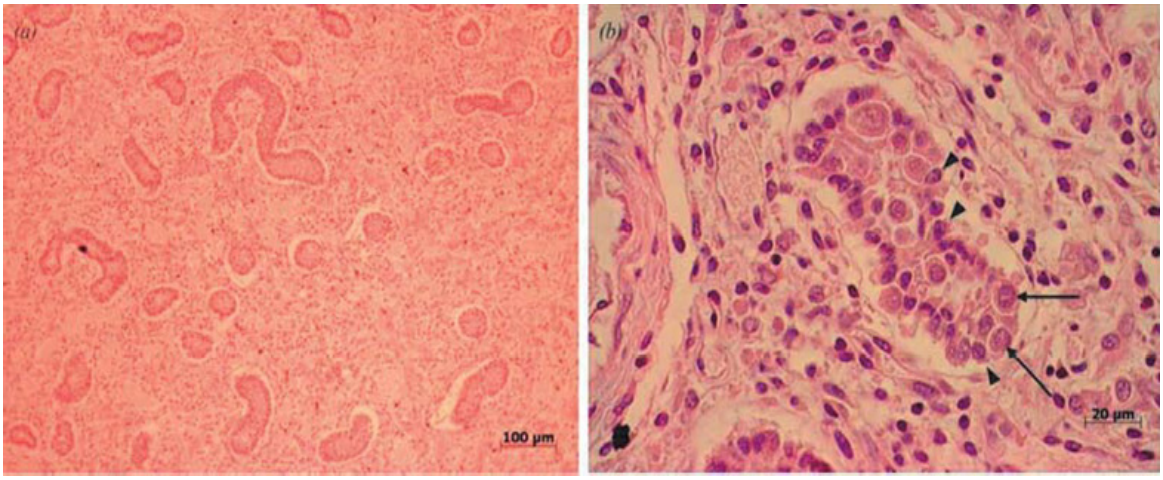

Figure 11.2
Photomicrographs of a gonad from MF, showing (a) seminiferous tubules, containing (b) spermatogonia (arrows) and Sertoli cells (arrowheads). H&E staining

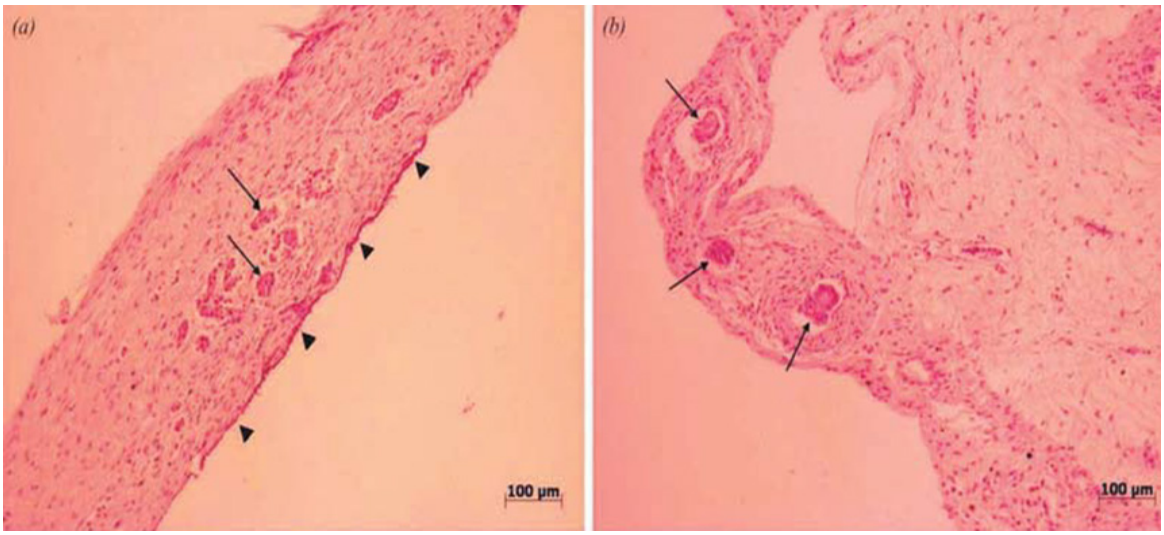

Figure 11.3
Photomicrographs of sections of tissue obtained from the caudal pole of FF's left kidney, showing (a) ovarian tissue with germ-cell nests (arrows) and germinal epithelium (arrowheads) and (b) structures resembling uterine tubes or hypoplastic uterus (arrows). H&E staining

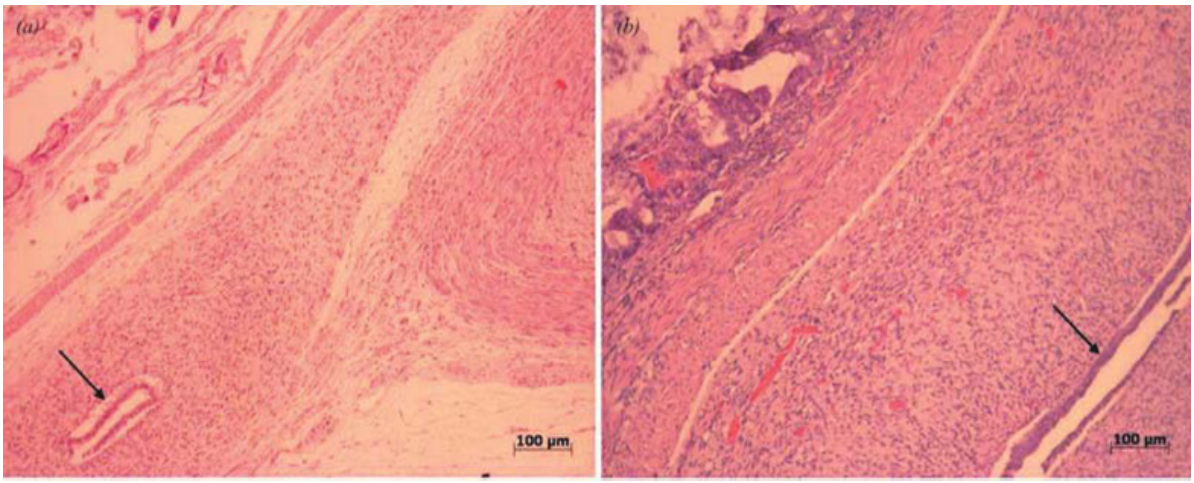

Figure 11.4
Photomicrographs of a transverse section through rectum and vagina from (a) FF and (b) FC. Note the size disparity between the vaginas of FF and FC, both identified by arrows. H&E staining

Dissection contrasted with dissection of MC showing gonads situated immediately within the internal openings of the left and right inguinal canals. On histopathology, MF's gonads were confirmed to be testes, identified as seminiferous tubules (Figure 11.2a) containing spermatogonia and Sertoli cells (Figure 11.2b). The prostate surrounding the urethra was identified microscopically. Testicular, epididymal and prostatic tissues were similar to the histological structure of these tissues derived from MC.

Gonads were not identified macroscopically within the abdominal cavity of FF. A band of tissue without a discernible uterus or vagina extended from the caudal pole of each kidney to the bladder. The round ligament was identified extending into the inguinal canals. In contrast, dissection of FC revealed a readily identifiable vagina and uterus with gonads clearly identified caudal to each kidney.

Histopathology of tissues associated with the caudal pole of FF's left kidney demonstrated a small section of ovarian tissue consisting of germ cells within germ-cell nests, bordered by typical ovarian germinal epithelium (Figure 11.3a). Structures resembling uterine tubes or a hypoplastic uterus were also identified within this region (Figure 11.3b). The vagina of FF was seen adjacent to the rectum but this was significantly smaller than that of FC (Figure 11.4a, b). In FC, gonads, uterine tubes, uterus and vagina were microscopically identifiable.

11.4. Discussion

Reports of the localisation of two foetuses within one placental site in the canine are rare. Duke (1946) described two canine embryos located within one embryonic site; each embryo had its own amnion and allantois and they shared a single chorion without a line of fusion between placentae. The embryos were 9–10 mm in crown–rump length, with visible limb buds and not yet sexually differentiated, correlating to a gestational age of 31 d from the pre-ovulatory luteinising hormone (LH) surge (Lopate, 2008; Yeager et al., 1992). More recently, Urhausen et al. (2013) reported observing two foetuses within one placental site on Day 40 post-LH surge during transabdominal ultrasonographic examination of a German Shepherd bitch. Following CS at Day 54, these foetuses were found to be of discordant genders; DNA profiles confirmed their origin from individual zygotes. Aberrant spacing of embryos before fixation, such that two embryos are closely juxtaposed within the uterine lumen, could be an explanation for the sharing of placental sites. Although the well- organised spacing of embryos in the uterus has been noted for over a century, the cellular and molecular mechanisms involved remain undefined (Chen et al., 2013). Current understanding, derived largely from studies in mice and rabbits, includes the mixing action of peristaltic uterine contractions and the timely resorption of intrauterine fluid to 'lock' embryos into position, both of which are under the control of a complex balance between progesterone and oestradiol. The existence of a signal gradient, due either directly or indirectly to the embryo, has also been proposed as a mechanism of uterine spacing. In addition, prostaglandins and noradrenalin may play essential roles (Chen et al., 2013).

Few studies have investigated the mechanisms of intrauterine spacing in the canine. A study by Tsutsui et al. (2002) demonstrated the trans-cornual migration of canine embryos. However, mixing of embryos (the bypassing of embryos along the length of the uterus), a feature of porcine embryonic migration (Dziuk et al., 1964), was not found, suggesting differences in the mechanisms of embryo migration between the two species.

Assuming that failure of uterine spacing mechanisms resulted in the described placental anomaly, it would seem logical that a large litter size might predispose to the condition. In the case study here described, the litter size was 15. In the report of Duke (1946), the litter size was 10 and in the report by Urhausen et al. (2013), only two, with evidence of three resorbing placental sites. No link to large litter size is evident at this stage. It is possible that these bitches

had more embryos originally but experienced a degree of early embryonic loss before implantation. None of the reports provide information on the number of corpora lutea present. This information would suggest embryo numbers before implantation. An added complication in the bitch is the occurrence of multi-ovular follicles; however, it has been suggested that, in general, a cohort of oocytes from a multi-ovular follicle includes only one oocyte of good quality (Chastant-Maillard et al., 2011). Monochorionic dizygotic twinning was first confirmed in the human in 2003 (Souter et al., 2003). Reports of this rare phenomenon indicate a link to the use of assisted reproductive techniques, particularly *in vitro* fertilisation (Chen et al., 2013). Souter et al. (2003) considered the possibility of the chorions having fused during early pregnancy, with subsequent degeneration of the area of fused membranes. Histological examination of the placental membranes in this case study revealed no evidence of remnant chorionic tissues between the closely apposed amnions. The authors therefore hypothesise that fusion occurred at the blastocyst stage of development, before implantation. Redline (2003) speculates that the fusion of blastocysts, surrounded by the developing trophoblast – a tight epithelial layer – is unlikely, unless fusion occurs during a brief period immediately before the embryo's transition to the blastocyst stage. However, the zona pellucida would be expected to prevent this occurrence, unless this structure has been removed during the *in vitro* fertilisation process (assisted hatching). Interestingly, fusion of mouse blastocysts leading to the development of two inner cell masses within one blastocoele was reported to be induced in the presence of inactivated Sendai virus, although no progress on this method seems to have followed the preliminary report (Tarkowski and Wojewodzka, 1982). Other mechanisms suggested include the fertilisation of two oocytes within one zona pellucida (binovular zona pellucidae) and the fertilisation of the mitotic products of a single oocyte by two spermatozoa (Chen et al., 2013). The latter was ruled out in at least one case of monochorionic dizygotic twins in humans through analysis of allele frequencies (Souter et al., 2003).

Similarly, one can speculate as to when fusion of the extraembryonic structures, or foetal membranes, occurred in the present case study. The canine embryo remains mobile within the uterus until 16–20 d after the LH surge (Days 16 to 20). Hatching from the zona pellucida occurs on Days 19 or 20, with attachment occurring as late as Days 21 to 22 (Concannon et al., 2001). The abnormal sexual differentiation of the female foetus implies the formation of vascular anastomoses before the completion of sexual differentiation. Testicular tissue is first

recognised histologically at Day 34 post-LH surge, while regression of the Müllerian ducts begins at Day 36 with completion by Day 46 (Meyers-Wallen et al., 1991; Meyers-Wallen, 2003).

The canine placenta is zonary in the form of a girdle between avillous cranial and caudal polar extensions (Evans and De Lahunta, 2013). This arrangement has led to the suggestion that fusion of the allantochorion is unlikely in this species (Kuiper et al., 2010). However, at Day 28 post-LH surge, the embryo occupies only a small section of the cranio–caudal extent of the placental girdle, with the as-yet underdeveloped avillous poles not extending beyond the margins of the placental girdle (Kuiper et al., 2010). At this stage of development, it is conceivable that two foetuses in close apposition could undergo placental fusion.

While it is unknown whether the embryos in the report of Duke (1946) were viable before hysterectomy of the dam, it is interesting to note that neither the foetuses in the present case report, nor those in the report by Urhausen et al. (2013), survived to term. An unpublished finding of two embryos or foetuses within one placental site, detected during abdominal ultrasound of a bitch, was similarly associated with intrauterine death (C. Lopate, pers. comm.). In the dog foetus, the last third of gestation is associated with significant development in cellular complexity of the foetal membranes, as well as increased density of capillaries, related to the rising nutritional requirements of the foetus during a period of rapid growth (Miglino et al., 2006; Aralla et al., 2013). A possible explanation for the failure of these cases to reach full term may be a failure of the placenta to cope with the increased nutritional requirements of two foetuses.

Of particular interest in the present case report is the finding of a hypoplastic reproductive tract in the female foetus, suggestive of freemartinism. This phenomenon has not previously been reported in the canine, although it has been described in cattle, sheep, goats, red deer and camels (Padula, 2005). Freemartins arise following the formation of vascular anastomoses between the placentae of twins of discordant gender before or during the period of sexual differentiation, leading to the female showing varying levels of masculinisation of the reproductive tract (Padula, 2005). As a result of mixing of the foetal blood supplies, haemopoietic progenitor cells are also able to migrate from one foetus to another, resulting in persistent blood cell chimaerism (Seguin et al., 2000).

Of interest, too, in the present case report is preliminary evidence of bilateral cryptorchidism

in the male foetus. Whether this is an incidental finding or linked to the foetus's shared blood supply with a female foetus, is unknown. Bulls born co-twin to a freemartin are generally phenotypically normal, with descended testes, although such bulls may be more likely to exhibit poor fertility than singletons (Dunn et al., 1979; Long, 1979).

In conclusion, this report describes the finding of blood chimaerism in monochorionic canine foetuses of discordant gender, with evidence of freemartinism. To the best of our knowledge, this is the first report of suspected freemartinism in the canine. This case provides an interesting comparison to human monochorionic twins of discordant gender, in which aberrant sexual differentiation as a result of blood chimaerism has not been described (Chen et al., 2013). Further research on monochorionic twinning in the canine, if it does indeed exist, as well as freemartinism in this species, is warranted.

Chapter 12. The first case of genetically confirmed monozygotic twinning in the dog

The content of this chapter has been published in a slightly different format as a short communication "Joonè*, C.J., De Cramer*, K.G.M., Nöthling, J.O., 2016. The first case of genetically confirmed monozygotic twinning in the dog. Reproduction in Domestic Animals 51**,** 835-839."

*These authors contributed equally to this work.

Abstract

Monozygotic twinning has not previously been genetically confirmed in the dog. This case report describes the finding of two viable male monozygotic foetuses within one placental site during CS. Their umbilical cords attached to a single placenta. Genetic profiling using a total of 38 microsatellite markers, as well as amelogenin and SRY for sex determination, revealed identical DNA profiles, whether derived from blood or tissue (buccal swabs) samples. To the best of our knowledge, this is the first report of monozygotic twinning in the dog confirmed using DNA profiling.

Keywords: canine, genetic, monozygous, monochorionic

12.1. Introduction

Monozygotic twinning has been reported in the horse (Govaere et al., 2009), cow (Del Rio et al., 2006) and pig (Bjerre et al., 2009), and is presumed to be extremely rare in the mouse (McLaren et al., 1995) and rabbit (Bomsel-Helmreich and Papiernik-Berkhauer, 1976). In contrast, the nine-banded armadillo (*Dasypus novemcinctus*), and possibly other species of the genus *Dasypus* (Loughry et al., 2015), consistently produces genetically identical quadruplets through binary fission events, lending itself to the study of the mechanism behind monozygotic twinning which is currently poorly understood (Blickstein and Keith, 2007). In humans, spontaneous monozygotic twinning occurs at the rate of approximately one in 330 livebirths (Hall, 2003).

Monozygotic twinning has not previously been genetically confirmed in the dog. Duke (1946)

described two dog embryos within one placental site. A presumptive diagnosis of monozygotic twinning was based on the finding of a single chorion and yolk sac; each embryo having possessed its own amnion. The embryos had not yet undergone sexual differentiation.

Conjoined twinning has been reported rarely in the dog (Mainland, 1929; Mazzullo et al., 2007; Nottidge et al., 2007; Paquet et al., 2011; House et al., 2012). Furthermore, the sharing of a single placental site by dizygous dog foetuses has been described rarely (Urhausen et al., 2013; Joonè et al., 2015).

12.2. Case report

A four-year-old, multiparous Irish wolfhound bitch was presented to a veterinary facility during second-stage labour. The bitch had had one previous litter of 10 puppies, the last five of which were delivered by emergency CS. At presentation, the owner reported that the bitch had been showing tenesmus for two hours without the expulsion of a foetus. No vulvar discharge was present. Due to the extended period of unproductive tenesmus, a CS was performed.

Upon exposure of the uterus, the surgeon noticed a bulge near the base of one of the uterine horns, approximately the length of a single foetus. Via a longitudinal incision into the body of the uterus, one foetus (twin A) was delivered from this section of uterus. A second foetus (twin B) was immediately noticed within the same chorionic bag. Without rupturing either puppy's umbilical cord, the second puppy and the placenta were delivered from the uterus. Both pups' umbilical cords, which were similar in length to the rest of the litter's, attached to the same placenta (Figure 12.1). Five more live, normal puppies were delivered with different placentae.

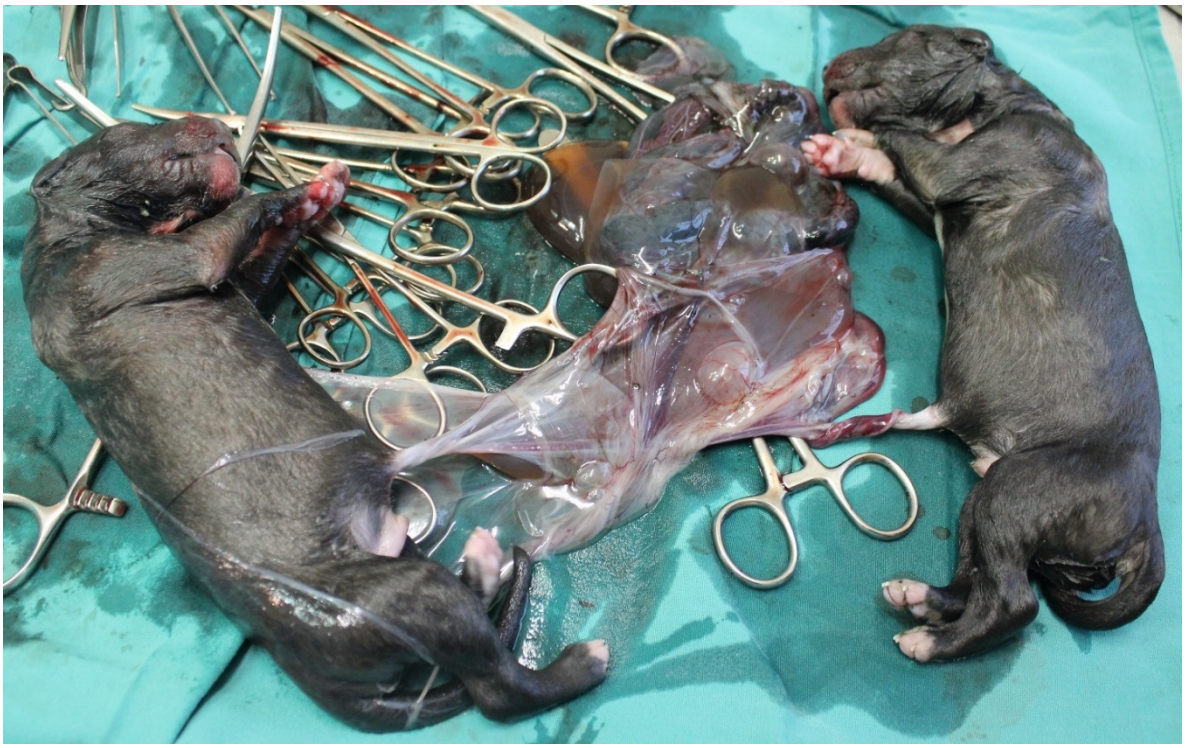

Figure 12.1
Monozygotic twins A and B photographed after delivery while still connected to the single placenta via their umbilical cords

At two weeks of age, blood samples from twins A and B were collected via jugular venipuncture into ethylenediamine tetraacetic acid vacutainer tubes for genetic analysis. At six weeks of age, blood was similarly collected from the five non-twin members of the litter. In addition, buccal swabs were collected from twins A and B by twirling a dry swab against the inside of the cheeks for at least 15 s.

Genetic analyses were performed by the Veterinary Genetics Laboratory (VGL; University of Pretoria, South Africa). Extraction of DNA from whole blood and buccal swabs was performed using the Prepfiler™ Forensic DNA Extraction Kit (Applied Biosystems, Foster City, USA) and the Gentra Puregene Tissue Kit (Qiagen, Valencia, USA), respectively, according to the manufacturers' instructions. Genetic profiles were generated using a panel of 24 short tandem repeat (STR) microsatellite markers and the amelogenin marker for sex determination. Twenty-one of these markers and the amelogenin marker are recommended by the International Society of Animal Genetics (ISAG; http://www.isag.us/Docs/consignment forms/2005ISAGPanelDOG.pdf, accessed 3 June 2016) for dog parentage verification. A further three markers augmented the panel. Primer design, chromosome

position, number of alleles and fragment size ranges have been described previously (Pedersen et al., 2012). Polymerase chain reaction (PCR) for this panel consisted of an initial activation step of 10 min at 95°C, followed by 30 cycles of 95°C for 60 s, 56°C for 30 s and 72°C for 60 s. A further panel consisting of 14 tetranucleotide STR microsatellite markers and a marker for the SRY gene was also utilised. Primer design and PCR conditions were as previously described (Wictum et al., 2013). Polymerase chain reaction was performed using a 9800 Fast Thermal Cycler (Life Technologies, Johannesburg, South Africa), followed by capillary electrophoresis by an ABI 3500 XL Genetic Analyser (Life Technologies). Fragment sizes for each marker were evaluated using the software program STRand Version 2.4.49 (University of California, Davis, USA; (Toonen and Hughes, 2001))

12.3. Results

Twins A and B were phenotypically normal males. At birth, twins A and B weighed significantly less (*t* test; $P < 0.001$) than their five littermates, however this difference had lost statistical significance by the age of six weeks ($P = 0.32$; Table 12.1). Although remarkably similar in physical appearance, they showed slight differences in terms of the size and shape of white markings on the chest, lower legs and the tip of the tail (Figure 12.2).

Table 12.1
Weights of twins A and B and their littermates, at birth and at the age of six weeks.

Puppy	Weight (g) at birth	Weight (kg) at six weeks of age
Brindle male	755	6.0
Brindle female	743	5.9
Light female	723	5.5
Dark brindle male	790	6.9
Dark brindle female	777	6.1
Twin A	450	5.5
Twin B	530	5.8
Mean (Twins A and B)	490[a]	5.7[a]
Mean (Non twins)	758[b]	6.1[a]

Means bearing different superscripts within a column differ significantly ($P < 0.05$)

Figure 12.2
Monozygotic twins A and B photographed with their dam at six weeks of age. Note the differences in the white markings on the chest and paws

The DNA profile derived from whole blood matched that derived from tissue (buccal swabs) for each twin, A and B. Further, the DNA profiles of twins A and B were identical at all 40 genetic markers. The DNA profiles of all seven littermates are shown in Table 12.2. Excluding the comparison between twins A and B, at which no loci were different, the genetic profiles of the littermates differed at a median of 14 loci (range 8–20), excluding amelogenin and SRY.

Table 12.2
Genetic profiles derived from seven littermates including monozygotic twins A and B

Locus	Light female	Brindle male	Brindle female	Dark brindle male	Dark brindle female	Twin A*	Twin B*
AHT121	104	96,104	96,104	96,104	96,104	96,104	96,104
AHT137	131	131	131	–	131	131	131
AHTh130	129	129	129	129	129	129	129
AHTh171	219	219	219	219	219	219	219
AHTh260	244	244	244	–	244	244	244
AHTk211	91	91	91	91	91	91	91
AHTk253	288,292	288,292	288,292	288,292	288,292	288	288
CXX279	118,122	122,124	122	122	122,124	122	122
FH2001	136,148	148	136,148	136,148	136,148	148	148
FH2054	156,172	156,172	156,172	156,172	172	172	172
FH2328	200	200,204	200	200,204	200	200	200
FH2848	–	–	–	–	–	238,242	238,242
INRA21	99,101	99,101	99,101	99,101	99,101	99,101	99,101
INU005	124,132	124,132	124,132	132	124,132	132	132
INU030	144,152	144,152	144	–	144,152	144,152	144,152
INU055	214,218	214,220	214,220	–	214,220	218,220	218,220
LEI004	95	95	95	–	95	95	95
REN105LO	231,241	231	231,241	–	231,241	231	231
REN162C04	202	202	202	202	202	202	202
REN169D01	216	216	216	–	216	216	216
REN169O18	164,168	162,164	164,168	164,168	162,164	164,168	164,168
REN247M2	268,278	268,278	278	–	268,278	278	278
REN54P11	228,236	228,240	228,236	228,236	228,240	228,240	228,240
REN64E19	147,153	145,149	145,149	145,149	149,153	145,147	145,147
VGL0760	21.1	21.1	21.1	21.1	21.1	21.1	21.1
VGL0910	17.1	17.1	17.1	17.1	17.1	17.1	17.1
VGL1063	17.3,18.3	13,18.3	13,18.3	13,18.3	13,18.3	13,17.3	13,17.3
VGL1165	29,30	16,30	29,30	29,30	29,30	16,30	16,30
VGL1541	18	17,18	17	17,18	18	17	17
VGL1828	20	20,21	20	20	20,21	20,21	20,21
VGL2009	9	9,15	9,15	9	9	15	15
VGL2136	15	15,16	15,16	15	15	15,16	15,16
VGL2409	19	18,19	19	18,19	19	18,19	18,19
VGL2918	21,22	22,24	21,23	23,24	21,22	21,23	21,23
VGL3008	12	12	12	12	12	12	12
VGL3112	14	13	13	13	13	14	14
VGL3235	13,16	13,16	12,13	12,13	13,16	12,13	12,13
VGL3438	14	14,17	14,17	14	14	14,17	14,17
AMEL	XX	XY	XX	–	XX	XY	XY
SRY	–	Y	–	Y	–	Y	Y

Data shows DNA fragment lengths, in base pairs, produced for 40 genetic markers including

amelogenin and SRY for sex determination. *The profiles generated from blood and tissue samples for twins A and B were identical, therefore no distinction is made between blood or tissue samples for these individuals. The hyphen character "–", indicates a marker that failed to amplify.

12.4. Discussion

The current study describes the finding of viable, monochorionic, monozygotic littermates in the dog. In polytocous species such as the dog, all littermates are essentially twins, triplets, quadruplets and so on, depending on the size of the litter. Thus, the term "twin", herein used to refer to the monozygotic "twins" only, should be used with care in these species.

This study made use of 38 STR microsatellite markers as well as markers for amelogenin and SRY, exceeding the eight and twelve microsatellite markers previously used to determine monozygosity in bovine and equine twins, respectively (Del Rio et al., 2006; Govaere et al., 2009). All 40 loci showed absolute identity between twins A and B. This, together with the finding of both foetuses within one placental site during CS, provides strong evidence for monozygosity.

The profiling of DNA derived from buccal swabs, essentially tissue samples, ruled out the possibility of blood chimaerism as an explanation for identical genetic profiles derived from two blood samples. In a previous report of blood chimaerism in two dog foetuses, the finding of more than two alleles at multiple loci on DNA profiles derived from blood samples alerted workers to the possibility of cross-foetus mixing of the blood supplies *in utero*. Subsequent profiling of tissue samples provided dissimilar genetic profiles, with no more than two alleles present per marker (Joonè et al., 2015). In the current study, the blood- and tissue-derived profiles for each individual were identical. In addition, no loci in either the blood- or tissue-derived profiles showed more than two alleles.

In human monozygotic twins, examination of the foetal membranes has been suggested to indicate the timing of the twinning event (Hall, 2003). Due to time constraints involved in the delivery of living puppies, the surgeon was unable to assess whether twins A and B were within a single amnion at delivery—precluding any useful estimation of the timing of embryonic fission in the current study.

Conjoined monozygotic twins are believed to arise from the incomplete splitting of an embryo

after formation of the primitive streak has begun. In humans, one in 400 monozygotic twins are reportedly conjoined (Hall, 2003). According to (Gupta et al., 2001), one to two percent of human conjoined twins are asymmetric (referred to as heteropagus). Logrono et al. (1997), found that, in a case of human heteropagus conjoined twinning, the parasite and autosite were dizygous; presumably resulting from the fusion of two conceptuses. Thus, conjoined twins may be monozygotic due to fission, but need not be. Conjoined twinning has been reported rarely in the dog (Mainland, 1929; Mazzullo et al., 2007; Nottidge et al., 2007; Paquet et al., 2011; House et al., 2012) and no DNA analyses were performed in the described cases. Nevertheless, the small number of cases of conjoined twins in dogs reported in the literature, most of which describe symmetrical conjoined twinning involving a degree of posterior duplication, suggest that monozygotic twinning in the dog is rare or that splitting events giving rise to conjoined monozygotic twins are rare in this species.

The monozygotic puppies described in the current study were viable and vigorous at birth, despite having shared a placental site. This finding contrasts to previous reports of two dog foetuses within one placental site, where death of the foetuses was detected 52 d after ovulation (Urhausen et al., 2013) and at term (Joonè et al., 2015). Therefore, the sharing of a placental site may not be incompatible with survival to term and beyond, as suggested previously (Joonè et al., 2015).

Of interest in this case report is the slight differences observed between the monozygotic twins in the white markings on the paws, the tip of the tail and the chest. Similar findings have been described in monozygotic twin horses and cattle (Ozil, 1983; Allen and Pashen, 1984), as well as in cloned dogs (Hossein et al., 2009). Woolf. (1995) concluded that stochastic events during development resulted in different white colour markings among the legs of horses despite the legs having had the same genotype and having developed in the same environment (Woolf, 1995). We do not know whether such stochastic events caused the phenotypic differences between the twins of the current case. Wong et al. (2005) concluded that variation in phenotype due to epigenetic differences is smaller in monozygotic twins than in isogenic dizygotic twins because monozygotic twins share an oocyte and, thereby, have a larger shared epigenomic background than isogenic dizygotic twins (Wong et al., 2005). Wong et al. nevertheless, concluded that epigenetic differences between monozygotic twins do occur. It is not known whether epigenetic differences would explain the colour differences between the monozygotic twins in the current case. Given that dog littermates often look strikingly

similar, slight phenotypic differences between monozygotic dogs would effectively mask their monozygosity, and may have played a role in this phenomenon having gone undetected until now.

For genetic identification and parentage analysis purposes, this study shows that dogs with identical genetic profiles, although likely rare, do exist. Bitches may have more conceptuses in the litter than they have corpora lutea (Andersen A.M. and Simpson M.E., 1973; Bysted et al., 2001). One cause for this may be multiovular follicles (Telfer E. and Gosden R.G., 1987; Reynaud et al., 2009) from which more than one oocyte may be fertilised. The current case confirms that monozygotic twins is another possible reason for finding more conceptuses than corpora lutea in bitches

12.5. Conclusion

This report describes the finding of monozygotic twinning in the dog, confirmed by DNA profiling. To the best of our knowledge, this is the first report of confirmed monozygotic twinning in the dog.

12.6. Acknowledgements

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12.7. Author contributions

C J. Joonè wrote the manuscript. K G M De Cramer and J O Nöthling assisted in drafting manuscript up to the final drafts. K G M De Cramer performed data collection.

12.8. Conflicts of interest

Conflicts of interest: none

Chapter 13. Discussion and conclusions of the thesis

13.1. Overarching discussion of the thesis

13.1.1. Factors increasing the odds of stillbirth and caesarean section

Performing elective CSs routinely for only the convenience of the owners and veterinary obstetricians is not ethically just. Routine elective CSs should be reserved for only that subpopulation of bitches whose pregnancies are considered high-risk. There is consensus that for breeds in which the prevalence of dystocia is unacceptably high, routine elective CSs is considered the only safe way of delivery (Linde-Forsberg and Eneroth, 2000; Davidson, 2008). In addition, this study identified bitches having undergone a previous CS in the immediate pregnancy before the current pregnancy, small litters (<8) and large litters (>11) in the Boerboel breed as obstetric risk factors (increasing the odds of emergency CSs and stillbirths). The current study showed that there was a curvilinear relationship between the odds of stillbirth and litter size. This confirms previous findings by others that both large litters (Bennett, 1974), and small litters (Darvelid and Linde-Forsberg, 1994; Lopate, 2008; Johnson, 2008b) are associated with reduced puppy survival at birth. To date no study has reported on the outcome of trial of labour after CS in the bitch or investigated the need for repeat CSs. The current study showed that a trial of labour after CS (TOLAC) was associated with considerable obstetric risks which exemplifies the need to avoid unnecessary CSs in bitches that have not yet undergone a CS before. The probability of a bitch requiring a repeat CS in her next pregnancy was 0.62 (CI 0.571–0.68). The odds for German Shepherd Dog bitches requiring a repeat CS was 11.8 (95% CI 4.9–28.7) times higher than for German Shepherd Dog bitches of which the first litter was born naturally ($P < 0.001$). Furthermore, this study showed that TOLAC was associated with a high proportion of stillbirths (40% more than with non-TOLAC litters ($P = 0.029$)). However, it may be speculated that the number of reported failed attempts at spontaneous whelp after a prior CS in the current study may have been over-reported. This is because fear, instilled by prior experience of stillbirths, may have prompted both the owner of the bitch and the veterinary obstetrician to opt too quickly for CS in some cases. This study confirmed that cephalic index is a risk factor for CS. Even when the English Bulldog was excluded from the data, brachycephalic bitches had 2.25 (95% CI 1.19–4.27) times more bitches ever having had a CS per bitch that did not, than was the case for non-brachycephalic bitches ($P = 0.013$). Survey 2 in section 3.2.3, shows that the

probability of CS in the German Shepherd Dog breed tends to increase with parity. This study confirmed that parity (increasing number of pregnancies) is a risk factors for CS ($P \leq 0.05$).

This study also showed that the method of delivery may impact on the probability of stillbirths. This study showed that the probability of stillbirths in the Boerboel bitches allowed to whelp normally was 24% compared to the 3% probability of stillbirths in Boerboel bitches (CI 0.02–0.05, n = 1379) that underwent elective CS.

The local relevance of this study was confirmed. The two main breeds represented in this thesis are the Boerboel and the English Bulldog breeds which combined, represented 25% of all puppies registered in South Africa for 2016. These two breeds ranked number one and number three respectively with 4222/22173 (19%) and 1315/22173 (6%) puppies registered out of a total of 22173 puppies registered with the three dog breeding registries in South Africa (GSD Federation of SA, 2016; Kennel Union Of South Africa, 2016; South African Boerboel Breeder Society, 2016). Both these breeds are considered high obstetric risk. The English Bulldog based on breed and the Boerboel based on the high stillbirth rate associated with natural whelp.

Whether the CS was performed as elective surgery or as an emergency impacted on the proportion of stillbirths delivered. The odds of stillbirths when the foetuses were delivered by emergency CS was significantly ($P < 0.001$), higher than was the case for elective CSs (OR, 7.3, 95% CI 5.16–10.36). The probability of stillbirths, for 458 foetuses, belonging to a variety of breeds and delivered by emergency CS was 23% (95% CI 0.18–0.27) compared to the 4% (95% CI 0.02–0.05) probability of stillbirths for 2233 foetuses delivered by elective CS. This finding confirms the findings that the likelihood of all the puppies being alive if the CS was performed on an emergency basis was decreased (Moon et al., 2000) and concurs with findings in human obstetrics (Ben-Meir et al., 2005). Avoiding an emergency CS should therefore be a priority with the veterinary obstetrician.

More research is required to identify the anatomical or other features that correlate to dystocia and to explore means of effectively selecting against them. Based on current knowledge, brachycephaly is such a feature (Trautmann and Nolte, 2003; Johnson, 2008b), as is the relative size of the vertical diameter of the maternal pelvic canal to the shoulder width of the offspring, in some breeds (Eneroth et al., 1999). Changing these features in dogs may threaten the existence of the breed, perhaps explaining the reluctance to change the breed standards.

The veterinary fraternity should therefore do more work in combatting the high prevalence of CSs in dogs. It is of interest to see if punitive measures by kennel clubs (The Kennel Club UK, 2012), will achieve its goal by discriminating against repeat CSs. This is because selection against the bitch that has had a CS may not resolve the underlying problem in the breed as a whole. It appears logic that changes should be made to the breed standards.

13.1.2. Parturient caesarean sections in bitches

The ultimate aim of this study was to perform preparturient CSs in bitches carrying high-risk pregnancies in which an elective CS was unavoidable. Preparturient intervention was planned to avoid foetal demise and stillbirth associated with parturition in high-risk breeds. Furthermore, a high priority was to perform these CSs at a convenient time of day with a full staff compliment to perform a professional service.

In order to have a base for future comparison (between parturient and preparturient CSs), we first had to evaluate the outcome (survival ratios in dams and puppies at 2 h and 7 d) of routine parturient elective CSs in significant numbers of bitches for our model. We also had to establish that the outcome of our obstetric interventions compared favourably to the outcome published in the literature. The outcome of CS depends greatly on a safe anaesthetic protocol as well as timeous intervention. In this study, the individual contribution of these two variables could not be determined. This study confirmed the safety of a protocol consisting of medetomidine hydrochloride (7 µg/kg iv) as premedicant, propofol, (1–2 mg/kg iv) as induction agent and sevoflurane, at 2% in oxygen for maintenance of aesthesia. The considerable reduction in the induction dose of propofol is of particular interest to the clinician. The proportion of puppies that survived for Boerboel, English Bulldog and other purebred bitches was respectively 97.4%, 96.7% and 91.7% at delivery, 95.4%, 88.4% and 89.8% by 2 h and 89.2%, 79.1% and 84.0% by 7 d. After correction for foetuses found dead on ultrasound examination and malformed euthanized puppies, 98.2%, 95.6% and 94.3% of Boerboel-, English Bulldog- and other purebred puppies survived until 2 h and 91.8%, 87.2% and 88.36% until 7 d. These results compare favourably to data on survival of puppies following the use of other anaesthetic protocols for CS in the bitch (Metcalfe et al., 2014; Doebeli et al., 2013). By eliminating puppy mortality unrelated to anaesthesia from the data, a true reflection of puppy survival rates was obtained in the current study and seems a more accurate way to compare anaesthetic protocols with each other. This correction is particularly

useful when comparing puppy survival rates of the English Bulldog breed to those of others. English Bulldog puppies not only have poorer survival rates at 2 h and at weaning age but also an increased risk of being stillborn or being born with defects requiring euthanasia (Wydooghe et al., 2013; Batista et al., 2014).

The good outcome associated with our elective CSs also proved that the method we used to time the CSs was satisfactory. Our study showed that 6 hourly visual inspection of the vagina by speculum examination starting 72 h prior to the predicted parturition date based on first day of cytological diestrus, was associated with good outcome for neonates and dams when cervical dilatation was used to indicate readiness for CS. Therefore, this study confirms that the first observation of the cervix having dilated (with not more than 6 hourly examinations), is a good indicator of readiness for CS in the bitch.

Overall, the Apgar scores achieved in the current study using the medetomidine protocol for parturient CSs were good and compared favourably to those reported in other studies (Doebeli et al., 2013; Veronesi et al., 2009; Groppetti et al., 2010; Metcalfe et al., 2014). The time at which Apgar scores are measured after birth is important. If there is too short a time lapse (5 min) from delivery to taking the Apgar score, then the evaluation is less predictive of survival than those done at 15 minutes and 60 minutes after delivery (Veronesi et al., 2009; Groppetti et al., 2010; Doebeli et al., 2013). This may be because more time could elapse for removal of depressive effects of the anaesthetic agents before an Apgar score was evaluated. In the current study the delay from delivery until Apgar score evaluation may have allowed for complete reversal of medetomidine in the puppy and for the concentration of propofol in the central nervous system to diminish by redistribution which is reported to take 15–20 min (Funkquist et al., 1997; Short and Bufalari, 1999). Another factor that may have contributed to the good Apgar scores achieved in the current study may be the reversal of the medetomidine with atipamezole as well as the low induction dose of propofol used (1 – 2 mg/kg).

Because at the inception of this study there was a concern that a preparturient CS might haemorrhage more than a parturient CS, we planned a study that compared the blood loss between the two. The fluid therapy had to be standardised between the two groups as fluid therapy affects haemoconcentration. The mean haematocrit was 44.2% (95% CI 43.8–44.6%) before CS and 37.8% (95% CI 37.3–38.2%) following CS and fluid therapy, with a mean

decrease of 6.4 percentage points (95% CI 6.1–6.7%) over all 406 CSs. This haematocrit is higher than those reported by Hayashi (1974), Concannon et al. (1977), Kaneko et al. (1993) and Dimço et al. (2013) but the time of collection during gestation was not standardised in these studies as blood was collected at different times in the last third of gestation. It may well be that there is an increase in haematocrit towards onset of spontaneous parturition. In our study, all the haematocrits were collected at the time of CS. Our study provided us with a percentage change in haematocrit to use for comparison in the study that would later follow (preparturient CS). It also provides the clinician with an estimate of normal range in haematocrit change before and after CSs.

13.1.3. Predicting the date and time of dilatation of the cervix (onset of spontaneous parturition) in the bitch

During the planning of our study, Batista et al. (2014) published a report suggesting that readiness for CS could be established by the biparietal diameter measured by ultrasound, having reached a minimum value. This appeared a promising method to determine readiness for CS but validation of the method has not been done. Many bitches present for the management of parturition in late gestation with mating dates only, making the accurate prediction of the parturition impossible. This prompted us to collect data on the variation in the biparietal diameters of the English Bulldog and the Boerboel breeds. In order to eliminate the errors that can be made using ultrasound, we decided to measure the BPD and birth weight of the puppies with a digital caliper and electronic scale within 15 min after delivery by CS. This study concluded that even with the most reliable measurement of BPD and birthweight, they are too variable within and among litters to be useful as a means of determining readiness for CS. Our study therefore suggests that foetal biometric measurements should be critically evaluated for usefulness in predicting readiness for CS and should then be put to test in a sufficient number of CSs, to be convincing.

Although prior to the current study, the Immulite® 1000 LKPG1 chemiluminescence immunoassay had been validated against the then “golden standard” Coat-A-Count® radioimmunoassay, we had sufficient reasons to repeat this study. This is because the Coat-A-Count® radioimmunoassay became unavailable during our study and the Immulite® 1000 LKPG1 changed to the Immulite® 1000 LKPW1 assay. Most important of all we had to ensure that the Immulite® 1000 LKPW1 was accurate at concentrations below 25 nmol/L as

this was our range of interest. This study concluded that on average, the value of Immulite® was 85% of that of Coat-A-Count® radioimmunoassay (95% CI 58% to 112%, n = 110). Immulite® exceeded 5.1 nmol/L or 13.6 nmol/L on the same day that Coat-A-Count® radioimmunoassay first exceeded 6 nmol/L or 16 nmol/L or the day before in 90% (n = 31) and 97% (n = 35) of oestrous periods. Immulite® first exceeded 5.4 nmol/L on LH1 or the day before in 95% (n = 25) of oestrous periods. The mean of Coat-A-Count® radioimmunoassay was 1.5 and 1.2 nmol/L higher than the 10.1 and 16.7 nmol/L of Immulite® 2 and 3 d after LH1 ($P \leq 0.001$). In conclusion, Immulite® underestimated Coat-A-Count® radioimmunoassay, and the days on which Immulite® first exceeded 5.1 nmol/L, 13.6 nmol/L and 5.4 nmol/L were effective in estimating the days on which Coat-A-Count® radioimmunoassay reached 6 nmol/L or 16 nmol/L, or first or only day of the LH surge measured using Witness® LH test (Synbiotics Europe, Lyon, France).

As one of the prime objectives was to avoid CS at inconvenient times, it was crucial that we could precisely predict the date of onset of parturition in the bitch. This study compared the precision with which four peri-oestrous predictors predict the date of onset of parturition (taken as the date of onset of cervical dilatation, measured to the nearest 6 h). The predictors evaluated in 24 bitches were; the date of the first or only day of the LH surge, the date on which the concentration of progesterone in the blood plasma, as measured with the Coat-A-Count radioimmunoassay, first exceeded 6 nmol/L, the date on which the concentration of progesterone in the blood plasma first exceeded 16 nmol/L and the date of onset of cytological dioestrus. Among the 24 bitches, the date of onset of cytological dioestrus predicted the date of onset of parturition with greater precision than the other three predictors. Following the evaluation of another 218 intervals between the onset of cytological dioestrus and the date of onset of parturition, it showed that the onset of cytological dioestrus predicted the date of onset parturition with a precision of ± 1 d, ± 2 d and ± 3 d in 88%, 99% and 100% of the 242 pregnancies. This study concluded that the first day of cytological dioestrus is a useful and precise predictor of the date of onset of parturition.

As most parturition management cases in practice are presented in the last third of gestation and are ones for which only mating dates are available, it was of clinical importance to have available a precise method to predict the time of onset of parturition in the bitch based on observations in the preparturient period. The literature alluded to both preparturient PC and cortisol concentrations being worthy candidates for investigation in this regard. Subsequently,

this study compared the precision of the ability of PC and cortisol concentration to predict the time of onset of parturition. The temporal relationship between the respective decrease in progesterone and increase in cortisol concentrations and the time of onset of parturition were evaluated in 25 bitches in the preparturient period.

In the preparturient period a random sample with a PC of above 15.8 nmol/L indicates that there is a 99% probability that the bitch will not enter spontaneous parturition within the following 12 h, when above 8.7 nmol/L there is a 98% probability of not entering spontaneous parturition within the following 12 h. These predictions help the veterinary obstetrician in making the decision whether the bitch may be left unattended overnight or not. When the PC is below 15.8 nmol/L, there is a 99% probability of entering spontaneous parturition within 96 h, when below 8.7 nmol/L there is a 99% probability of entering spontaneous parturition within 48 h, and when below 3.18 nmol/l there is a 100% probability of entering spontaneous parturition within 24 h. The data did not allow for selecting a PC below which the bitch is further than 48 h or more from the onset of spontaneous parturition. The results of this study show that the concentration of cortisol in the blood plasma is too variable within and among bitches to be of use as a predictor of the time of onset of parturition. These results allow the veterinary obstetrician to make prompt decisions in the management of parturition.

There was an overall trend of increase in the concentration of cortisol in the blood plasma as the time of cervical dilatation approached. In spite of this trend, cortisol concentrations were too erratic were too erratic to be of value in predicting the onset of parturition and require further studies.

For bitches in which an elective CS is unavoidable, the results of this study showed that using D0 as predictor of the day of cervical dilatation (Chapter 8) or other methods of predicting the day of parturition (Concannon et al., 1977b; Kutzler et al., 2003a; Tsutsui et al., 2006), allows the clinician to predict the day of parturition to within 3 days on either side of the day on which parturition would actually occur. Because the current study performed preparturient CSs at a fixed time and date (08:00 on D57), parturition observation time was curtailed to a period of 72 h (from 08:00 on D54 until 08:00 on D57) or less. In addition, our protocol allowed for safe preparturient CS on D56 (n = 7) in bitches showing clinical signs of impending parturition. Out of 99 gestations for which D0 was known and a preparturient CS planned for 08:00 on D57, the CS was performed at the scheduled time in 60 (61%), before

the scheduled time because cervical dilatation had started in 32 (32%) and before the scheduled time in 7 (7%) because the bitches had started showing signs of impending parturition, although their cervices were still closed. The time interval between first displays of behavioural signs of parturition to stage 2 of parturition may be very unreliable. Uterine contractions detected by electromyography associated with nesting behaviour was recorded 7 d before spontaneous parturition (van der Weyden et al., 1989) and therefore when no precise prediction date of parturition is available, behavioural signs cannot be used to time CSs. However, signs of imminent parturition (restlessness, panting, displaying nesting behaviour, anorexia, abdominal discomfort, licking of the vulva, depression and looking at the flanks) at a time close to D57 (within 48 h of D57) may be more significant than when no prediction date is available.

Unless the interval between D0 and the day and time of cervical dilatation differ among breeds, this study provides a protocol that enables clinicians to perform CSs on a scheduled convenient time in approximately two thirds of bitches in which an elective CS has been planned. It also confirms the precision of the method of timing, using D0. However, the current study only examined two breeds in sufficient numbers and further research is required to establish whether the temporal relationship between D0 and the time of cervical dilatation differs among breeds.

Despite many decades of routine elective CSs in women, the timing of elective CSs in women is not perfect. The literature is nearly unanimous in recommending elective caesarean delivery for women at 39 weeks of gestation because of lower rates of neonatal respiratory complications compared to 38 weeks (Salim and Shalev, 2010). However when the elective CS is scheduled at exactly 39 weeks, approximately 10% to 14% of women go into spontaneous labour (Smith, 2001b); meaning that a considerable number of women scheduled for an elective caesarean delivery at 39 weeks will deliver earlier in an unscheduled, frequently emergency, caesarean delivery (Salim and Shalev, 2010). Similarly, in the current study, our timing protocol for scheduled fixed time CS is not perfect. Although it was possible to perform scheduled preparturient CSs in 61% of the gestations with good outcome, it was still necessary to perform speculum examinations on all bitches every six hours. Thirty two percent of the CSs in the current study, also with good outcome, were performed because they were observed to have a dilated cervix before 08:00 on D57. It is not known what would happen if the interval between the vaginal speculum examinations is increased from every 6 h

to every 12 h. It is likely that extending the interval between speculum examinations may prevent the clinician from identifying foetal compromise in time, which would lead to poorer puppy survival and the need for some emergency CSs. The use of preparturient PC having reached certain crosspoints and timelines as discussed in Chapter 9, alludes to possible answers to mitigate this problem and help identify pregnancies which are likely to enter spontaneous parturition in the following 12 h, thereby negating the necessity to observe those bitches overnight.

13.1.4. Preparturient caesarean sections in bitches

This study showed high neonatal survival ratios after preparturient CS with 99% of puppies delivered by preparturient CS, born alive, 98.8% surviving to 2 hours and 88.6% to 7 d, suggesting that the protocol is safe with regards to puppy survival rates. The overall probability of puppy mortality with preparturient CSs compared favourably to those reported in the literature (Moon et al., 2000; Wydooghe et al., 2013). The mortality rate for the English Bulldog puppies delivered by both preparturient and parturient CSs was lower than those for Boerboel puppies which is in concurrence with the literature (Moon et al., 2000; Wydooghe et al., 2013). The odds of stillbirth, dying before two hours and dying before 7 d were similar for parturient and preparturient CSs.

The high neonatal survival rates obtained in this study confirms that our timing of the preparturient CSs using our protocol was good and resulted in delivery of the foetuses before foetal demise and compromise could occur during onset of parturition.

This study shows that larger litters have lower stillbirth ratios and ratios of puppies dying before they are 2 hours old than smaller litters, but that there is no such an effect of litter size on ratio of puppies dying before 7 d. This suggests that once puppies have survived beyond two hours, factors other than those relating to CS, are the main determinants of their survival at 7 d and that stillbirth ratios and 2 h survival ratios are better suited to determine CS outcome than is the 7-day survival.

The current study showed that following CS, the English Bulldog puppies have higher odds of dying before 7 d than Boerboel puppies, which is in agreement with the English Bulldog having lower survival rates than other breeds after CS (Wydooghe et al., 2013). The number of puppies dying before 7 d per puppy surviving until 7 d is expected to be 41% lower for

Boerboels than for English Bulldogs.

The timing of the preparturient CS is important. Performing preparturient CSs too early may yield premature, non-viable puppies, cause failure of placental release and increase the risk of serious uterine haemorrhage (Smith, 2007) and death of the dam (Pretzer, 2008). In the current study both, preparturient CSs and parturient CSs yielded high survival ratios with similar odds of puppy survival, suggesting that the preparturient CSs in the current study were performed during the so-called safe period of intervention (what is referred to as "term" in women). Also, our results suggest that significant lung maturation, allowing normal extra-uterine survival, occurs in the dog foetuses some time before spontaneous parturition and that our fixed-time preparturient intervention occurred after foetal lung maturation.

The current study shows that a scheduled preparturient CS, performed at 08:00 on the morning of D57, while the cervix is still closed, shortens gestation by 4–52 h relative to what would have been the case if it was performed upon first noticing cervical dilatation. In 50% of preparturient CSs, gestation would have been shortened by at least 10 hours. However, the current study does not allow one to determine by how long gestation was curtailed in each preparturient CS. More research is required to better define the safe period of intervention "term" in the bitch.

In the current study, PC was significantly higher at the time of preparturient CSs than at the time of parturient CSs, with medians of 8.3 nmol/L (Q1 3.8, Q3 15.1) and 2.71 nmol/L (Q1 1.28, Q3 4.75), respectively. The proportions of preparturient CSs performed when the concentration of progesterone in the serum was higher than 6.4 nmol/L and 10 nmol/L, respectively, were substantially and highly significantly larger than those for parturient CSs. These differences confirm that, as a group, gestation of the preparturient group has not progressed as far by the time of CS as it did in the parturient group. The concentration of progesterone at the time of preparturient CS had no effect on the odds of stillbirth or of puppies dying before 7 d. The current study shows that it is not a prerequisite for the concentration of progesterone in the serum to decrease below 6.4 nmol/L in order to safely deliver foetuses by preparturient CS. This study also showed that five of the 50 parturient cases still had PC above 6.4 nmol/L (as high as 18 nmol/L) at the time of cervical dilatation.

The studies that did use predicted parturition dates to time CS in the bitch or induce parturition, all used the concentration of progesterone in blood plasma or serum either on its

own or with that of LH during the peri-ovulatory period as the means of timing (Baan et al., 2005; Levy et al., 2009; Vannucchi et al., 2012; Fontbonne et al., 2009). Unfortunately, none of these studies are large or convincing enough to conclude that performing CS based on their timing method is safe for routine use in clinical practice. Also, these studies do not show whether it is safe not to use priming. Priming is the preparturient treatment of the dam in an attempt to stimulate foetal maturation. Aglepristone (Baan et al., 2005; Fontbonne et al., 2009; Levy et al., 2009) and betamethasone (Vannucchi et al., 2012) have been administered to preparturient bitches as priming agents but it is not known whether they are beneficial and, if they are, at what time before the onset spontaneous parturition. In contrast, the scientific basis for using prenatal corticosteroid therapy in preparing the human foetus for premature delivery (before 37 completed weeks of gestation) is extensive and convincing (Ballard and Ballard, 1995; Brownfoot et al., 2007). The current study achieved good results without priming. It is not known whether priming may influence the outcome of preparturient CSs performed earlier than was the case in the current study.

Fixed date CSs have advantages over current parturition induction protocols as it requires no time to monitor the delivery of puppies and no ecbolic support to complete parturition (Baan et al., 2005; Fieni and Gogny, 2009; Fontbonne et al., 2009).

Preparturient CS did not lead to complications in the dam and the maternal survival rate was 100%. The change in haematocrit before and after CS for preparturient was slightly smaller (1.29 percentage points) than for parturient CSs.

Performing a CS while the cervix is closed poses a potential risk of retained placentas and metritis if the cervix does not dilate adequately to allow for placentas and lochia to escape. Retained placenta was avoided in the current study as each placenta was removed together with its foetus and no post-operative uterine infections were noted during the 7 d following CS. The removal of placentas was not associated with complications and renders it more likely to identify placental abnormalities and foetuses sharing placental sites (Joonè et al., 2015; Joonè et al., 2016). This does however not mean that routine removal of placentas can be advocated in all circumstances. This is because it cannot be excluded that the use of the alpha 2-agonist, medetomidine, may have influenced uterine tone and placental site haemorrhage.

The road towards elegant management of parturition in an obstetric clinic has been paved.

Bitches for which we have a known D0, can be admitted on the morning of D54. During day-time they can be observed for signs of impending parturition and vaginal speculum examinations can be performed once every 6 h to observe for the first signs of any extent of dilatation of their cervixes. If there is any bitch that starts displaying signs of impending parturition other than dilatation of the cervix, a CS can be safely performed on her. One hour before the day-time staff leaves, the PC in the blood of the bitch can be determined and if the PC is below 8.7 nmol/L, a CS could potentially be safely performed on her but further research is required to confirm this. Also if the PC was above 15.8 or 8.7 nmol/L respectively it would be 99 and 98% sure that the bitches would not enter spontaneous parturition within the next 12 h which is very helpful in making the decision whether the bitch may be left unattended or not overnight. If the PC is above the 8.7 nmol/L crosspoint, it means that the bitch still needs to be observed but is unlikely to enter spontaneous parturition in the following 12 h (before 8: a.m. the next morning). Thereafter the monitoring process continues until the morning of D57 when a CS is performed if the bitch has not yet by then been observed to have a dilated cervix.

For bitches with an unknown D0, day-time vaginal speculum examinations can be performed and one hour before closing the clinic, the PC in the blood of the pregnant bitch can be measured and the protocol followed as above.

During the execution of large numbers of CSs, we were fortunate to make two interesting novel incidental findings. The author of this thesis in this study removes each placenta with the puppy at CS delivery with no ill effect on the dam. This practice allowed us to identify two puppies sharing the same placenta on two separate occasions. In the first incidence of sharing one placenta, the puppies were of discordant gender. Further analysis revealed that they were dizygotic, that blood chimaerism had occurred and the female puppy was suspected to be a freemartin. The second set of twins were of the same gender and were confirmed the first ever case of monozygotic twinning in the dog. It is not known how common it is for dog foetuses to share a placenta and it is also not known how common monozygotic twinning in the dog is or how frequently freemartinism occurs in the dog. The implication is that there may be more puppies than placental sites in the bitch.

The demand by owners for a favourable outcome following parturition in the bitch is driven by both financial and emotional considerations (Smith, 2007). The concept of a reliable

protocol allowing planned preparturient CS resonates with the veterinary obstetrician for numerous reasons. Planned and timed CS obviates the need for after-hour interventions when staff shortages are a serious impediment to the execution of a professional service. Some high-risk pregnancies do not show signs of impending parturition or the signs are subtle and difficult to recognise, leading to foetal compromise. Parturition management is very time consuming and expensive. Parturition management exposes veterinary obstetricians to criticism if, retrospectively, intervention or lack thereof is suspected to have reduced puppy survival. As is the case with medical obstetricians (Ben-Meir et al., 2005; Bergholt et al., 2004; Husslein and Wertaschnigg, 2002), preparturient CS allows veterinary obstetricians to perform CSs at times convenient to them and advantageous to the puppies and bitch owner. Owners with experience of bitches with dystocia and related puppy mortality demand timeous intervention. Some owners are a long distance away from veterinary care and fear the extended transit time may compromise the litter in case of dystocia. Travelling at night to veterinary facilities in some parts of the world is a serious security risk resulting in both clients and veterinary staff to avoid after-hour service at all cost. In addition, after-hours veterinary facilities available to clients may not be adequately equipped to appropriately deal with dystocia cases or to perform a CS. Some bitches and their litters are considered particularly valuable (Smith, 2007). The life style factors such as working-hours, night-hours commitments, planned holidays or business trips, may make it impossible for the owner to supervise the parturient bitch. Finally, some owners insist on a CS for their bitch and it has been proposed that as veterinary obstetricians we should be prepared and allowed to counsel, treat and respect a client's right to elect a CS for their bitch (Smith, 2007) as is the case with women (Ben-Meir et al., 2005; Bergholt et al., 2004; Husslein and Wertaschnigg, 2002).

Despite all these arguments in favour of CS for the bitch, the veterinary obstetrician should only concede to CSs where there is a medical emergency or plan elective CSs in only those bitches belonging to an obstetric population with a known increased obstetric risk relative to bitches in general. This study provides the veterinary fraternity with evidence of such risks as well as a protocol to perform elective preparturient CSs in a large proportion of this population and elective parturient CSs in the other. More research is required to ensure fixed time and date preparturient CS on the entire obstetric subpopulation in which preparturient CS is justified.

More work needs to be done to combat the high prevalence of dystocia thereby negating the

need for major invasive surgery to deliver puppies from an unacceptable high proportion of the domestic dog population.

13.2. Final conclusion

This thesis found that it is justified to perform preparturient CSs in that subpopulation of the general obstetric population in bitches that have high-risk pregnancies. This thesis provides the veterinary obstetrician with a protocol that can be used to safely perform elective preparturient CSs in a large proportion of the obstetric population at a convenient time of the day. Further studies are required to conclude whether preparturient CSs on D57 are routinely safe and safe in in all breeds.

Chapter 14. Future research needs

14.1. Time lag between onset of dilatation of the cervix and stage 2 (delivery of the first puppy) in the bitch and progesterone and cortisol levels at that time

It is of interest to know what the time interval is between the first signs that the cervix has dilated and the delivery of the first puppy. It is also of importance to know what the concentrations in the blood of both progesterone and cortisol are in bitches at that point.

14.2. Singleton phenomena

We do not know why some singleton pregnancies fail to progress normally and others not. It might be that the sidedness of the foetus and sidedness of corpora lutea and or number of corpora lutea may have an influence on whether preparturient progesterone decline fails to occur. Then there is the relative size of litter and size of bitch or indeed combined luteal mass that may influence singleton phenomena.

14.3. A need to further investigate the safe period of intervention in the bitch

Although this study showed that preparturient CS performed on D57 is safe and that term is at least 48 h, we do not know whether it is routinely safe to perform CSs on D57 and what the range and extent of the safe period of intervention is in the bitch. We also do not know whether pharmacologic intervention in late gestation may increase the period (window) of safety for intervention as cortisone does in man. Also, it is unknown whether priming in the bitch may extend the safe period of intervention.

14.4. Effect of anaesthetic protocol on placental detachments and extent of haemorrhage following caesarean section in the bitch

All the placentas were removed simultaneously with the foetus at delivery per CS in the current study. Anecdotal evidence suggests that removal of placentas may result in excessive uterine haemorrhage necessitating blood transfusion. No excessive haemorrhage was ever encountered the current study and it is speculated that the use of medetomidine as premedicant in this study may have contributed to this finding. It may therefore be that anaesthetic protocol may influence uterine blood loss via a variety of mechanisms.

14.5. Objective assessment of maturity, prematurity and dysmaturity

This study showed that at least in the English Bulldog and Boerboel breeds, the extent of hair cover over face and distal part of the limbs may indicate that the foetus is term. This study collected many images from parturient and preparturient foetuses of two breeds. However, further research is required to accurately quantify hair coverage and its correlation to maturity or investigate other possible indicators of maturity in the foetus. Clinical experience by the prime investigator in this study suggested that breed and breed size appears to affect hair density and may have an influence on assessing maturity. We also have no way of knowing whether a foetus with apparent sparsity of hair is premature or dysmature. To date this distinction is made by presence of fully mature puppy or puppies in the presence of an immature puppy or puppies in the same litter.

14.6. Growth curve in last third of gestation of gestation

The growth curve in the last few days of pregnancy is unknown and knowledge of the growth curve may help interpret gestational age curves of foetal biometric measurements.

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Addendum (research approval certificates)

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Validation of a chemiluminescent immunoassay (Immulite 1000 Progesterone) as a reliable progesterone concentration assay at low values (<25 nmol/L) by comparison to a RIA assay (Coat-A-Count ® Progesterone assay)**
PROJECT NUMBER	**V071-13**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. KGM de Cramer**

STUDENT NUMBER (where applicable)	**812 670 46**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SPECIES	**Domestic dogs**	
NUMBER OF ANIMALS	**40**	
Approval period to use animals for research/testing purposes		**October 2013 –January 2014**
SUPERVISOR	**Prof. JO Nothling**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date	**28 October 2013**
CHAIRMAN: UP Animal Ethics Committee	Signature	

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UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Predicting parturition dates base on observations made in peri-ovulatory, mid-gestational and pre-parturient periods**
PROJECT NUMBER	**V010-14**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. KBM de Cramer**

STUDENT NUMBER (where applicable)	**8126 7046**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SPECIES	**Dogs**	
NUMBER OF ANIMALS	**56**	
Approval period to use animals for research/testing purposes		**April 2014 - July 2015**
SUPERVISOR	**Prof. JO Nothling**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date	24 February 2014
CHAIRMAN: UP Animal Ethics Committee	Signature	

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UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Predicting parturition dates base on observations made in peri-ovulatory, mid-gestational and pre-parturient periods**
PROJECT NUMBER	**V010-14 (Amendment 1)**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. KBM de Cramer**

STUDENT NUMBER (where applicable)	**8126 7046**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SPECIES	**Dogs**	
NUMBER OF ANIMALS	**56**	
Approval period to use animals for research/testing purposes		**April 2014 - July 2015**
SUPERVISOR	**Prof. JO Nothling**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date	26 May 2014
CHAIRMAN: UP Animal Ethics Committee	Signature	

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Anaesthesia experiment: Puppy survival associated with use of low dose mededomitine premedication and significance of preoxygenation for caesarean section in the bitch**
PROJECT NUMBER	**V048-14**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. KGM de Cramer**

STUDENT NUMBER (where applicable)	**8126 7046**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SPECIES	**Dogs**	
NUMBER OF ANIMALS	**250**	
Approval period to use animals for research/testing purposes		**August 2014 – August 2015**
SUPERVISOR	**Prof. JO Nothling**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date	30 June 2014
CHAIRMAN: UP Animal Ethics Committee	Signature	

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Anaesthesia experiment: Puppy survival associated with use of low dose mededomitine premedication and significance of preoxygenation for caesarean section in the bitch**
PROJECT NUMBER	**V048-14 (Amendment 1)**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. KGM de Cramer**

STUDENT NUMBER (where applicable)	**8126 7046**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SPECIES	**Dogs**	
NUMBER OF ANIMALS	**250**	
Approval period to use animals for research/testing purposes		**January 2015-January 2016**
SUPERVISOR	**Prof. JO Nothling**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date	4 February 2015
CHAIRMAN: UP Animal Ethics Committee	Signature	

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Haematocrit experiment: Haematocrit changes in peripartuirient bitches undergoing caesarean section**
PROJECT NUMBER	**V021-15**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. KGM de Cramer**

STUDENT NUMBER (where applicable)	**81267048**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SPECIES	**Canine (Female)**	
NUMBER OF ANIMALS	**To be reported**	
Approval period to use animals for research/testing purposes		**March 2015 –March 2016**
SUPERVISOR	**Prof. JO Nothing**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date	30 March 2015
CHAIRMAN: UP Animal Ethics Committee	Signature	

S4285-15

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**PREPARTURIENT CAESAREAN SECTION IN BITCHES**
PROJECT NUMBER	**V079-15**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr KGM de Cramer**

STUDENT NUMBER (where applicable)	**UP_81267046**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SPECIES	**Canine**	
NUMBER OF ANIMALS	**67**	
Approval period to use animals for research/testing purposes		**October 2015 – October 2016**
SUPERVISOR	**Prof. J Nothling**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date	9 September 2015
CHAIRMAN: UP Animal Ethics Committee	Signature	