ULTRASONOGRAPHIC MAPPING OF CABERGOLINE-INDUCED OESTROUS CYCLE, GESTATION AND POSTPARTUM CHANGES; EFFECTS OF INSONATION ON NIGERIAN INDIGENOUS BITCHES AND PUPPIES

BY

MARUF LAWAL

DEPARTMENT OF VETERINARY SURGERY AND RADIOLOGY,
FACULTY OF VETERINARY MEDICINE
AHMADU BELLO UNIVERSITY,
ZARIA, NIGERIA

OCTOBER, 2018

ULTRASONOGRAPHIC MAPPING OF CABERGOLINE-INDUCED OESTROUS CYCLE, GESTATION AND POSTPARTUM CHANGES; EFFECTS OF INSONATION ON NIGERIAN INDIGENOUS BITCHES AND PUPPIES

BY

Maruf LAWAL (DVM 2004, MSc 2011 A.B.U, ZARIA)

(PhD/ VET-MED/2580/2011-12)

A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF DOCTOR OF PHILOSOPHY IN VETERINARY DIAGNOSTIC IMAGING

DEPARTMENT OF VETERINARY SURGERY AND RADIOLOGY,

FACULTY OF VETERINARY MEDICINE,

AHMADU BELLO UNIVERSITY,

ZARIA

OCTOBER, 2018

DECLARATION

I declare that the work in this thesis entitled "**ULTRASONOGRAPHIC MAPPING OF CABERGOLINE-INDUCED OESTRUS CYCLE, GESTATION AND POSTPARTUM CHANGES; EFFECTS OF INSONATION ON NIGERIAN INDIGENOUS BITCHES AND PUPPIES"** has been performed by me in the Department of Veterinary Surgery and Radiology Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria under the supervision of Professor A.Z. Hassan, Dr N. D. Chom and Professor I.U. Ate.

The information from the literature has been duly acknowledged and a list of reference provided. No part of this thesis has been submitted elsewhere for a degree or diploma at any Tertiary Institution.

…….....	……	……
Name of student	Signature	Date

CERTIFICATION

This thesis entitled "**ULTRASONOGRAPHIC MAPPING OF CABERGOLINE-INDUCED OESTROUS CYCLE, GESTATION AND POSTPARTUM CHANGES; EFFECTS OF INSONATION ON NIGERIAN INDIGENOUS BITCHES AND PUPPIES"** By Maruf LAWAL meets the regulations governing the award of the degree of Doctor of Philosophy of Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

……. ……...
Professor. A. Z.Hassan. DVM MSc, PhD, (FCVSN) Date
Chairman, Supervisory Committee.

…….. ……
Dr N.D. Chom. MBBS, FWACS Date
Member, Supervisory Committee.

…… ……
Professor I.U. Ate. DVM. MSc, PhD (FCVSN) Date
Member, Supervisory Committee.

……. ……..
Dr S.T. Fadason, DVM, MSc, PhD, (FCVSN) Date
Head, Department of Veterinary Surgery and Radiology

……. ……
Professor Sadiq. Z. Abubakar, PhD Date
Dean of Postgraduate School

DEDICATION

This thesis is dedicated to my family, Amie, Mujaheed, Rahamah-Adama, Sabeerah and in memory of Ahmatullah, HajiaKhadijahOgungboye and Prof. B.D. Remi-Adewunmi

ACKNOWLEDGEMENTS

I give thanks to Almighty Allah (SWT) for enabling me reach this phase of life, even against all odds.

I wish to express my profound gratitude and appreciation to my supervisors, Prof A. Z. Hassan, Dr N D Chom and Prof. I.U. Ate for their guidance and advice throughout the course of this study. To my "second set of supervisors" (second major Dr S N A Saidu, Profs O.O. Okubanjo, J.O. Ayo, and D.A.Y. Adawa) I say a huge thanks and May Allah (SWT) grant you the best of your wishes and needs.

To members of my group (Drs Abdurrahman Mohammed, E.O Abidoye, Audu Solomon, Rashida Mustapha, MunirSani Ari, M.N Bappah and A.A Bada), I say may Allah reward you abundantly for all your efforts. I also want to say thank you to the following people, Mals (YauHamza, Yusuf Danladi, Abdullahi, Mannir) Isa of Small Animal Clinic. To the Head Pharmacist Rukkayah Pharmacy, thanks a lot for your assistance.

To my Head of Department DR S.T. Fadason, mummy MrsAjibade may God reward you abundantly for all your encouragement. To members of my esteemed Department thank you all for everything.

To my esteemed parents The Lawal's and The Momodu's and my siblings on both sides may Allah (SWT) reward you abundantly. Only Allah (SWT) can reward you for all you have done, are doing and will do for me and my family.

ABSTRACT

Nine (9) apparently healthy adult Nigerian Indigenous Dogs (NIDs) were used to evaluate the ultrasonographic changes in the genitalia, foeti and neonates in cabergoline-induced oestrous. Sonostar C5® with a 6.5 MHz transcutaneous curve-linear probe was used to scan. All bitches were synchronised using cabergoline at 5µg/ kg body weight *per os* for twenty days and then mated naturally. At the start of gestation phase, bitches in group A were not insonated again until parturition. The other bitches were insonated once and twice a week for B and C respectively. Ultrasonography of the internal genitalia in bitches revealed that uterine structures were the ones easily visualised once the female was not in season. The baseline haemogram, sera parameters were all within normal limits. The mean time taken from initiation of treatment with cabergoline to onset of proestrus was found to be 15.75 ± 0 .68 days. The ovaries (right and left) started becoming visible. They appeared as roundish structures with isoechoic tissues. The Superoxide dismutase (SOD) ranged from 0.35 to 0.51 µgm /ml, Catalase between 5.71 to 7.12 µmol/min/ml, Glutathione peroxidase (GPX) between 9.47 -12.77 µmol/ml while Malondialdehyde (MDA) between 0.94 - 1.88 µmol/ml. Sonographic pregnancy diagnosis was between days 19-21 post last service and the maximum foetal poles recorded was 8.33 ± 0.67, 9.0 ± 0.58 and 9.0 ± 0.58 for groups A, B and C, respectively. The P4 decreased to 2.32 - 3.60 ng/ml. SOD increased to 1.05 to 1.47 µmol/ml, Catalase 10.11 to 19.12 µmol/ min/ ml, GPX 41.88 to 42.59 µmol/ml and MDA 2.31 to 3.43 µmol/ ml. The mean time for appearance of limb buds was 35.17 ± 0.48 days of gestation. The last structure to appear on ultrasound was the foetal intestines; on day 60.33 ± 0.61. Mean litter was 7. 33 ± 0.67, 6.67 ± 0.67 and 5.33 ± 0.33 puppies/ litter, while mean birth weight was 246.4 ± 8.42, 197.5 ± 11.55 and 189.7 ± 10.21

g. The percentage postpartum death was 4.55, 10 and 16.67 % for groups A, B and C respectively. The PCV was reduced to 42.92 ± 1.70, 40.92 ± 1.46 and 37.25 ± 1.57 % for bitches in groups A, B and C respectively, HGB concentration was 14.39 ± 0.55, 13.06 ± 0.80 and 13.68 ± 0.50 for groups A, B and C respectively, Leucocyte count to be 36.50 ± 3.17, 29.75 ± 2.00 and 32.75 ± 2.12 for groups A, B and C. WBC was 8.67 ± 0.87, 9.65 ± 1.11 and 9.13 ± 1.40 for groups A, B and C respectively. The TP was 6.40 ± 0.26, 6.10 ± 0.23 and 5.90 ± 0.16 A, B and C respectively. P4 increased to 6.54 ± 4.94, 45.19 ± 24.51 and 70.50 ± 31.50 ng /ml for groups A, B and C respectively. The concentration of SOD was 1.50 ± 0.124, 1.86 ± 0.21 and 1.90 ± 0.03, Catalase concentration was 26.86 ± 4.09, 27.95 ± 4.33 and 33.91 ± 5.83 µmol /min/ml, GPX was 60.87 ± 3.85, 60.91 ± 5.38 and 76.61 ± 8.98 µmol/ml MDA was 5.05 ± 0.79, 6.28 ± 2.09 and 7.41 ± 1.62 µmol/ml for groups A, B and C respectively. Triiodothyronine had means of 4.34 ± 0.48, 2.49 ± 0.20 and 1.16 ± 0.20 ng/ml and thyroxin with means of 22.84 ± 6.37, 16.16 ± 1.16 and 14.24 ± 2.19 µg/ml for puppies from bitches in groups A, B and C respectively. The concentration of SOD was 0.15 ± 0.05 µg/ ml, 0.25 ± 0.032 µg/ ml 0.63 ± 0.09 µg/ ml, Catalase was 27.03 ± 0.40, 25.11 ± 1.00 and 17.33 ± 5.62 µmol/min/ml, GPX was 74.68 ± 19.33, 71.12 ± 16.66 and 32.03 ± 2.51 µmol/ml while MDA was 10.33 ± 0.96, 7.04 ± 0.69 and 4.78 ± 0.85 µmol/ml for puppies from bitches in groups A, B and C respectively. During the first week postpartum the uterine diameter ranged from 3.510 - 4.51 with a mean of 3.94 ± 0.11 cm. uterine involoution led to a reduction of the diameter by 56.1 % by the third week postpartum. In conclusion, the numerical differences suggest the possibility that ultrasound could cause increased oxidative stress which resulted in intra-uterine growth retardation which lower birth weights especially with multiple insonation.

TABLE OF CONTENTS

Title Page……i

Declaration v

Certification..... vi

Dedication vii

Acknowledgements..... viii

Abstract ix

Contents..... xi

List Of Tables xvi

List Of Figures..... xvii

List Of Plates xvii

List Of Appendices xx

Chapter One……1

1.0 Introduction 1

1.1 Preamble ……...1

1.1.1 Definition and Application Of Ultrasound 1

1.1.2. Oestrus Cycle And Oestrus Induction In Bitches 2

1.1.3. Ultrasound Safety.....3

1.2 Statement of Research Problems 4

1.3 Justifiaction.....4

1.4. Aim of The Study.....5

1.5.Objectives of The Study 5

1.6.Research Questions 6

Chapter Two 7

2.0 Literature Review.....7

2.1 Anatomy and Physiology 7

2.1.1 Sexual Maturity In Bitches 7

2.1.2 The Female Reproductive Organ 7

2.1.3 The Oestrous Cycle. 8

2.1.4 Oestrus Regulation And Oestrus Synchronisation....10
2.1.5 Ovulation....11
2.1.6 Fertilisation....13
2.1.7 Canine Stages Of Pregnancy....14
2.1.8 Reproductive Hormone....15
2.1.9 Impact Of Multiple Conceptuses Per Follicle On Fecundity In The Bitch....17
2.1.10 Gestation Period And Parturition In The Bitch....19
2.2 Ultrasound And Ultrasonography....21
2.2.1 Historical Overview Of Ultrasonography....23
2.2.2 General Principles Of Ultrasonography....24
2.2.3 Transducers....27
2.2.4 Types Of Transducers....27
2.2.5 Transducer Frequencies....28
2.2.6 Ultrasound Image Formation....28
2.2.7 Modes Of Sonography....29
2.2.8 Ultrasound Artefacts....30
2.3 Doppler Ultrasonography....35
2.3.1 Coloured Ultrasonic Imaging....36
2.3.2 Evaluation Of The Reproductive Tract Of Bitches By Doppler Ultrasonography....42
2.4 Contrast-Enhanced Ultrasonography....43
2.4.1 The Use Of Bubbles In Ultrasound Imaging And Therapy....44
2.4.2 The Basic Principles Of Micro Bubbles As Echo Contrast Agents....45
2.4.3 Types Of Micro Bubble Agent....46
2.4.4 Use Of Micro Bubbles As Quantitative Imaging....48
2.4.5 Using Micro Bubbles To Target Tissues And Molecular Imaging....48
2.4.6 Therapeutic Applications Of Contrast Agents....49
2.5 Contrast Harmonic Imaging....52
2.5.1 Principle Of Contrast Harmonic Imaging....53
2.5.2 Second Harmonic Imaging....54
2.5.3 Ultrasound Tissue Harmonic Imaging (Thi)....54
2.5.4 Future Perspective Of Contrast-Enhanced Harmonic Eus....55

2.6 Other Imaging Modalities....56
2.6.1 Advanced Diagnostic Imaging (Computed Tomography, Magnetic Resonance Imaging)....57
2.7 Application Of Ultrasound In Pregnant Bitches....58
2.7.1 Doppler USS Evaluation Of Uterine Blood In Bitches....58
2.7.2 Indication For Ultrasonography During Paturition....60
2.7.3 Appearance Of Embryonic/Foetal Structures Using Ultrasonographic Or Radiographic Examination....61
2.7.4 Ultrasonographic Measurement Of Extra-Foetal Or Foetal Structures....61
2.7.5 Ultrasonographic Evaluation Of Heart Rate Or Foetal Flux....62
2.7.6 Use Of B-Mode Ultrasonography For Foetal Sex Determination In Dogs....63
2.7.7 Bidimensional And Doppler Ultrasonographic Evaluation Of Postpartum Uterine Involution....64
2.8 Oestrus Induction In Canine Patients....65
Chapter Three....68
3.0 Materials And Methods....68
3.1. Study Area....68
3.2. Research Considerations....68
3.3. Materials....68
3.3.1 Experimental Animals....68
3.3.2 Consumables....69
3.3.3 Equipment....69
3.4 Methodology....69
3..4.1 Experimental Design....69
3.4.2. Phase I (Acclimatization)....71
3.4.3 Phase Ii (Induction And Breeding)....71
2.4.4 Phase III (Gestation)....73
3.4.5 Phase Iv (Postpartum)....74
3.4.6 Determination Of Haematological Parameters....75
3.4.7. Puppies Postpartum....75
3.4.8. Hormonal Assay....76
3.4.9 Data Analysis....76

Chapter Four…….77

4.0 RESULTS……...77

4.1 Sonographic features of the reproductive tract of bitches……..77

4.1.1. Sonographic features of the reproductive tract of bitches during phase I…….77

4.1.2. Sonographic features of the reproductive tract of bitches during phase II…….80

4.1.3. Sonographic features of the reproductive tract of bitches during phase III……..84

4.1.4. Sonographic features of the reproductive tract of bitches during phase IV……..86

4.2. Haematological parameters of bitches during the reproductive phases……..104

4.2.1. Haematological parameters of bitches during phase I…….104

4.2.2. Haematological parameters of bitches during phase II……...104

4.2.3. Haematological parameters of bitches during phase III……..105

4.2.4. Haematological parameters of bitches during phase IV……..105

4.3. Serum parameters of the of bitches during the reproductive phases……..109

4.3.1. Serum parameters of bitches during phase I……109

4.3.2. Serum parameters of bitches during phase II……...109

4.3.3. Serum parameters of bitches during phase III……..109

4.3.4. Serum parameters of bitches during phase IV…….110

4.4. Puppies physical and Serum parameters……116

4.4.1. Physical parameters postpartum……..116

4.4.2. Serum parameters of puppies…….116

Chapter five……..124

5.0 Discussion……..124

Chapter six……137

6.0 Conclusions and recommendation……137

6.1 Conclusions…….137

6.2 Recommendations……137

6.3 Limitations……..137

REFERENCES……138

Appendices 17271

LIST OF TABLES

Table 2.1: Depth and axial resolutions obtained by different transducer frequencies…….26

Table 4.1 Showing the duration of the anoestrus with reproductive structures visualized on ultrasound examination……..…….78

Table 4.2. Results of the response of the bitches to Cabergoline treatment……81

Table 4.3.The summary of the first appearance of various ultrasonographic foetal and extrafoetal features in the bitches post mating…….87

Table 4.4. Showing the various means and SEM for the other haematologicals parameters bitches at different phases of the study……..108

LIST OF FIGURES

Fig.3.1 shows the overview of the experimental design……..……70

Fig.4.1**.** Means of maximum foetal poles visualized at second trimester of gestation….96

Fig.4.2. Mean litter size at whelping by the bitches in various groups……...97

Fig.4.3. Comparism of the Maximum Fetal poles counted and the mean number of puppies whelped……..……..……..98

Fig.4.4. Percentage of post-parturient death recorded two week post whelping……..….99

Fig 4.5 The trend in mean diameter of the uterus during part of postpartum (involution) phase of the study plotted against time……..103

Fig.4.6. Showing the PCV of the bitches at the various phases of the study…….107

Fig.4.7. Concentration of progesterone of the bitches at the various phases of the study.112

Fig.4.8. Concentration of superoxide dismutase in bitches at various phases of the study……...113

Fig.4.9. Concentration of glutathione peroxidase in the bitches at various phases of the study……...114

Fig.4.10 Column graph showing concentration of catalase of the bitches at various phases of the study...……115

Fig.4.11.Column graph showing concentration of malondialdehyde of the bitches at various phases of the study……..116

Fig 4.12. Mean birth weights of puppies from the various groups of bitches……118

Fig 4.13 Concentration of triiodothyronine of puppies of various bitches……119

Fig 4.14 Concentration of thyroxin of puppies of various bitches……120

Fig 4.15 Concentration of superoxide dismutase in the puppies from various bitches…..121

Fig 4.16 Concentration of catalase in the puppies of various bitches……122

Fig 4.17 Concentration of glutathione peroxidase of puppies of various bitches……..123

Fig 4.18 Concentration of glutathione peroxidase of puppies of various bitches……..124

LIST OF PLATES

Plate 2.1 Ultrasonic images collection of female reproductive tract in various USS modes……38

Plate 2.2 Spectral Doppler evaluation of mesometrial attachment artery…….40

Plate 4.1. Sonographic image of the uterine body…….……79

Plate4.2.Ultrasound appearance of the ovary at late proestrus…….….82

Plate 4.3:Sonograph of ovary post ovulation…….…...83

Plate 4.4: Sonograph showing early pregnancy between 19-21 days of gestation……88

Plate 4.5:Sonograph of foetal poles between days 27-29……89

Plate 4.6Sonogram of the foetal structures in pregnant bitches Days 28-32 of gestation.90

Plate 4.7 Sonograms of the foetal structures in pregnant bitches between days 34-37 of gestation……...……91

Plate 4.8.Sonogram of the foetal structures in pregnant bitches Days 35-39 of gestation.92

Plate 4.9: Sonograms of the foetal structures in pregnant bitches Days 39-42 of gestation…….93

Plate 4.10: Sonograms of the foetal structures in pregnant bitches Days 39-42 of gestation…….……..94

Plate 4.11 Sonogram showing some foetal structures which was taken between days 41-45post mating…….95

Plate 4.12 Sonographic image of postpartum uterus 3 days after parturition……...100

Plate4.13 Sonographic image of postpartum uterus following one month of involution……...101

Plate 4.14 Sonograph the urinary bladder (UB) and a mixed echogenic uterus (UT) five weeks postpartum……102

LIST OF APPENDICES

Appendix 1. Concentration of Superoxide dismutase at various phases for the bitches 171

Appendix 2. Concentration of Malondialdehyde at various phases for the bitches..... 173

Appendix 3. Concentration of Catalase at various phases for the bitches 175

Appendix 4. Concentration of Glutathione peroxidase at various phases for the bitches 177

Appendix.5 Showing the uterine diameters of the bitches from week 1 to week 8 postpartum 179

CHAPTER ONE

1.0 INTRODUCTION

1.1 Preamble

Ultrasound has a frequency greater than 20 kHz, which is above the range of human hearing. It allows communication, food acquisition or orientation in bats and other animals, but can also be used as a technique of diagnostic medical two- and three-dimensional or flow imaging (Meinecke-Tillmann, 2016). Meanwhile, ultrasonography (from Latin words 'ultra': more and 'sono': sound and Greek word 'graphein' ['˛ϕε´ ']: to write) or ultrasound imaging is one of the most widely used powerful tools in animal diagnostics and research, which allows the real-time visualisation of internal organs, tissues, structures or fluid accumulations and the investigation of blood flow without any radiation risk. It depends on the computerised analysis of reflected ultrasound waves. Due to its non-invasive character and apparent high safety, it has become one of the most rapidly advancing technologies in the clinical fields (Meinecke-Tillmann, 2016).

1.1.1 Definition and application of Ultrasound

Ultrasound is an oscillating sound pressure wave with a frequency greater than the upper limit of the human hearing range. It is thus not separated from 'normal' (audible) sound by differences in physical properties, but rather only by the fact that humans cannot hear it. Although this limit varies from person to person, it is approximately 20 kilohertz (20,000 hertz) in healthy, young adults. Ultrasound devices operate with frequencies from 20 kHz up to several gigahertz (Novelline and Robert, 1997). Ultrasound is used in many different fields for example; ultrasonic devices are used to detect objects and measure distances. Furthermore ultrasonic imaging (sonography) is used in both veterinary and human

medicine. In the non-destructive testing of products and structures, ultrasound is used to detect invisible flaws (Novelline and Robert, 1997). Industrially, it is used for cleaning and for mixing, as well as to accelerate chemical processes. Animals such as bats and porpoises use ultrasound for locating prey and obstacles (Novelline and Robert, 1997).

1.1.2. Oestrus cycle and oestrus induction in bitches

Bitches have been classified as non-seasonal monoestrus mammals which have a long period of anoestrus (Verstegen *et al*., 1999). Cyclicity is exhibited only once or twice in a year in normal reproductive physiology of domestic bitches (Verstegen *et al*., 1999). Even with the aforementioned uniqueness, the oestrous cycle of the bitch is a unique physiological period that contains four stages. These stages are proestrus, oestrus, dioestrus, and anoestrus (Sudson, 2011). Proestrus typically lasts for about 9-11 days and then directly leads to oestrus. At this stage, ovarian follicles mature and oestradiol concentration in the blood increases resulting in the swelling of vagina and uterus. The changes in the vaginal epithelium, vulvar oedema and diapedesis of erythrocytes from capillaries of the reproductive tract also occur from the hormonal influence (Sudson, 2011). At this stage the bitch still refuses to stand for mating. Peak concentrations of oestradiol in late proestrus prepare the pituitary and hypothalamus for an ensuing surge-release of luteinizing hormone (LH). Progesterone level begins to increase in late proestrus at the onset of the LH surge. Then, mating is accepted at oestrus that lasts for about 6-11 days. During this period the progesterone gradually rises. This causes the softening of the vulva resulting in mating behaviour. Ovulation takes place spontaneously at the beginning of oestrus. Subsequently, dioestrus starts and the bitch rejects mating. This stage is

predominated by progesterone release thereby inhibiting uterine contraction throughout the period of pregnancy. Dioestrus varies among bitches and can be approximately 9-11 weeks. Anoestrus is a quiescent period for reproductive organs and sexual behaviour. It is a prolonged stage, lasting for 3-5 months (Noakes *et al*., 2001).

Induction of fertile oestrus in the bitch has been achieved using dopamine agonist such as bromocriptine which is traded as a human drug and is not recommended for animals, while Cabergoline is a veterinary drug for treatment of pseudo pregnancy in dogs. Bromocriptine administered orally once or twice per day (50 μg/kg) induces proestrus in 17-28 days (Concannon, 1993) while using cabergoline (5 μg/kg) daily induces fertile proestrus in 15-25 days, 10-20 days, or 4-10 days when administered to bitches in early, middle or late anooestrus, respectively (Verstegen *et al*., 1994). Dopamine agonist may cause unpleasant side effects such as anorexia and vomiting (Concannon, 1993), and may not successfully induce proestrus in cat (Kutzler, 2007).

1.1.3. Ultrasound safety

Present day diagnostic ultrasound machines use such low levels of energy that they appear to be safe, but the safety threshold levels for different tissues, may vary and with the development of more powerful and sophisticated apparatus, the levels may be exceeded for some tissues (Donald, 1979). Despite decades of ultrasonic investigation, the adverse effects of in-utero insonation have not been pinned to a particular point of higher incidence during pregnancy (Newnham *et al*., 1991). The cumulative effect of ultrasound exposure, it's relation to the output of a particular machine, the length of the examination, high frequency of exposures and the mechanisms of its effects on intrauterine growth are yet to

be elucidated through as large-scale study (Newnham *et al*., 1991). As even the relative lower birth weights showed after intra uterine insonation of foetuses, a later study suggested the offspring's soon made up the deficit. Numerous studies on rats, mice and monkeys showed reduced foetal weight in litters that were exposed to in-utero insonation as compared to the controls (Tarantal *et al*., 1993).

1.2 Statement of Research Problems

There are breeding outfits for dogs springing up all over the country, however, there is a shortage of concrete ways of promptly determining pregnancy and possible reproductive problems in the bitches. To the best of our knowledge, no method of accurately determining litter size in-utero has also been established sonographically in our indigenous breed. No records of the effect of insonation on oxidative stress and reproductive parameters in Nigerian Indigenous Dogs (NIDs) have also been established. Likewise thyroid hormone levels in neonates and progesterone profile in the dam,as well as the safety levels following multiple in-utero exposure is yet to be established in NIDs.

1.3 Justification

There is a surge in the use of ultrasonography in clinical veterinary practice due to its affordability and reliability which eases the clinicians' duties. There is therefore an urgent need to establish information on conception, pregnancy and foetal status for clinician on the dominant dog breed in our environment; sonographic features of the non-gravid uterus; data on the sonographic features of the gravid uterus at various stages of foetal development. There is also the need to establish a procedure to determine number of foetuses in-utero so as to help clinicians advise the client who may make such request. Effect of multiple exposures of the foetuses to ultrasound in-utero needs to also be

elucidated. There is also a dearth of information on the association between multiple insonation and clinically observed differences in neonatal puppies.

1.4. Aim of the Study

This study aims to determine the sonographic changes that occur in the reproductive tracts during oestrus cycle, gestation and postpartum and foetuses (during development) in Nigerian Indigenous dogs (NIDs), and the effects of multiple insonation on the bitches in-puppy and the puppies postpartum.

1.5.Objectives of the Study

1. To identify the various sonographic changes that occurs during different phases of the induced oestrus cycle.
2. To sonographically determine the developmental changes that occurs during embryonic and foetal stages in-utero.
3. To provide an accurate method of predicting the number of puppies in-utero sonographically and gestational aging in NIDs
4. To assess the measureable effects of multiple insonation on the biomarkers of oxidative stress in the bitches and puppies.
5. To assess the effects of multiple insonation on birth weight and thyroid hormones profile of the puppies.
6. To assess the quantifiable effects of multiple insonation on the bitches and puppies.
7. To map out various sonographic changes that occurs postpartum in the reproductive tract of NIDs

1.6 Research questions

1. Are there any sonographic changes that occur in the genitalia in induced oestrus?
2. Can insonation map changes during foetal development?
3. Can insonation help in predicting possible litter size?
4. What are the measurable effects of insonation on puppies?
5. What are the measurable effects of insonation on biomarkers of oxidative stress in bitches and puppies?
6. What are the effects of multiple insonation on birth weight and thyroid hormones?
7. Are there any changes in the reproductive tract postpartum?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Anatomy and Physiology

2.1.1 Sexual maturity in bitches

The age of sexual maturity in the bitch may vary with breed.This could however range from approximately 6 months to 2 years, with most reaching maturity at 8–12 months (Kumi- Diaka and Adeyanju,1986; Peter, 2001; Feldman and Nelson, 2004).

2.1.2 The female reproductive organ

The female reproductive organ in bitch comprises of two ovaries, which contain oocytes and the tubular genitalia duct including the oviduct, bicornuate uterus with a short body, the cervix, vagina and vestibule (Linde-Forsberg, 2007). The latter is quite large in this species to be able to accommodate the *bulbusglandis* of the male during the copulatory tie (Linde-Forsberg, 2007).

Unlike in the human and mouse, canine oocytes are dark due to high lipid content making it difficult to identify intracellular structures by light microscopy and to cryopreserve functional eggs and embryos (Chastant-Maillard *et al*., 2010). Canid oocytes are also ovulated at an immature stage compared with other mammalian species (pre-germinal vesicle breakdown), and require 48–72 hrs in the oviduct postovulation to complete nuclear maturation (Reynaud *et al*., 2005).

2.1.3 The oestrous cycle.

The reproductive behaviour of the domestic dog is commonly described as monoestrous with a uniquely prolonged interoestrous interval of approximately seven months and a range of 3.5–13 months (Rowlands, 1950; Olson and Nett, 1986; Feldman and Nelson, 2004). The oestrus cycle of the bitch is classically divided into four stages: the proestrus, oestrus, metoestrus and anoestrus (Heape 1900). It is known that the development of the tertiary follicles, *corpora hemorrhagica* and *corpora lutea* appeared to be related to minimal hormonal variations even within the same stage of the reproductive cycle (Groppetti *et al*., 2015a). The tertiary follicles distribution (small, medium and large) is different among all the stages of the reproductive cycle, except for small follicles (<2 mm), which is always observed from proestrus to anoestrus (Reynaud *et al*. 2009; Groppetti *et al*., 2015a). The number and distribution of large follicles significantly differed between early oestrus (E1), periovulatory oestrus (E2) and oestrus after ovulation (E3) (Groppetti *et al*., 2015a). Moreover, large follicles with a diameter greater than 4 mm characterize the early oestrus up to ovulation (Groppetti *et al*., 2015a). Dioestrus is sometimes used to refer to the metoestrus for the luteal period (Linde-Forsberg, 2007).

2.1.3.1 Proestrus: This is considered to begin on the day when the vaginal haemorrhage appears from the turgid vulva (Linde-Forsberg, 2007). It can last for average of 9 days with a range of 3-27 days (Linde-Forsberg, 2007). The beginning of proestrus is gradual. Firstly the bitch is inviting the male but is not ready to mate. The vulva turgidity and haemorrhage subside towards the end of the proestrus (Linde-Forsberg, 2007).

2.1.3.2 Oestrus: It is usually lasts for an average of 9 days with a range of 2-21 days. The bitch allows mating during this period (Linde-Forsberg, 2007). The end of the fertile period, which coincides with the beginning of dioestrus (D1) is characterized by a mean value of blood progesterone concentration greater than 36.9 ng/mL (Groppetti *et al*., 2015a). The precise identification of the range of the fertile period is essential to plan programs of assisted reproduction technology (ART) in the bitch (Groppetti *et al*., 2015a).

*2.1.3.3 Metoestrus or dioestrus:*The bitch rejects the male during this period (Linde-Forsberg, 2007). The progesterone stimulated uterine epithelium desquamates as the progesterone concentration subsides over 2-3 months (Linde-Forsberg, 2007). The endometrial repair processes is completed after 4.5-5 month (Linde-Forsberg, 2007). Dioestrus is characterized by the development of corpus luteum (CL) the size of which has been reported to be 3–12 mm in diameter (Rehm *et al*., 2007) and 5–6 mm (Groppetti *et al*., 2015a). Small and large luteal cells have been described in the pig (Richards *et al*., 1994), ovine (Fitz *et al*., 1982) and cattle (Cools *et al*., 2013) to originate from theca and granulosa cells, respectively (Baithalu *et al*., 2013). The sizes of small luteal cells (12–23 μm) and large luteal cells (25–55 μm) in cattle increases as the stage of oestrus cycle progressed and during pregnancy (Baithalu *et al*., 2013). This was similarly described in the dog with small luteal cell between 10 and 20 μm of diameter and large luteal cell between 20 and 40 μm of diameter while the size was unchanged between early and late dioestrus (Groppetti *et al*., 2015a).

*2.1.3.4 Anoestrus:*Dogs experience obligatory prolonged ovarian inactivity or anoestrus (Concannon, 2011), and as a result, ovulation only occurs once or twice annually

(Nagashima *et al*., 2015). This lasts for 1-9 months depending on whether the bitch has 1, 2 or 3 cycles per year (Linde-Forsberg, 2007). The interval between two oestrus periods is usually around 2 months longer after a pregnant cycle (Linde-Forsberg and Wallen, 1992). During anoestrus some studies record only follicles smaller than 1 mm in diameter (Reynaud *et al*., 2012), 1.3 ± 0.3 mm of diameter (Groppetti *et al*., 2015a) or ranging from 4 to 6 mm (Reynaud *et al*., 2009).

2.1.4 Oestrus regulation and oestrus synchronisation

It is generally accepted that day length is the main factor regulating breeding cycles in many species including the Basenji breed of dog (Fuller, 1956; Tedor and Reif, 1978; Gunn and Doney, 1979; Rhind and McNeilly, 1986; Forcada *et al*., 1992). For instance, in seasonal breeders such as sheep, the length of anoestrus is synchronised generating seasonal cycles of oestrus in the population. Anoestrus is regulated through exposure of the pineal gland to light, mediating the release of melatonin and, in turn, gonadotrophin-releasing hormone (Wigham *et al*., 2017). This same mechanism is known to drive annual oestrous cycles in the Basenji breed of dogs (Fuller, 1956; Tedor and Reif, 1978; Gunn and Doney, 1979; Rhind and McNeilly, 1986). Controlled studies have demonstrated that manipulating day length using artificial light can modulate the interoestrous period and, therefore, breeding cycles in ewes (Ducker *et al*., 1970; Vesely, 1978; Legan and Karsch, 1980). Although, similar controlled studies have not been carried out in domesticated dogs it may be reasonable to assume that exposure to artificial light, especially during the winter months, may also interfere with the regulation of reproduction in dogs, dampening any normal seasonal variations in oestrus (Wigham *et al*., 2017).

Although it is widely known that photoperiod is the main factor that synchronises oestrus in many species, seasonality in oestrus has also been attributed to other regulatory factors, such as ambient temperature (Chatdarong *et al*., 2007; Ortega-Pacheco *et al*., 2007) and breed (Sokolowski *et al*., 1977). Pregnancy and parity may also influence the length of the interoestrous interval and, thus, subsequent oestrous start dates (Sokolowski *et al*., 1977; Linde-Forsberg and Wallén, 1992; Mutembei *et al*., 2000).

2.1.5 Ovulation

The broad classification of species as either spontaneous or induced ovulators is based on the type of stimulus responsible for eliciting GnRH release from the hypothalamus (Adams and Ratto, 2013). Most canids including domestic dog (Concannon, 2011), blue fox (Moller, 1973) and red fox (Mondain-Monval *et al*., 1977), have been defined as spontaneous ovulators. The known induced ovulating canid is the maned wolf (Johnson *et al*., 2014). In addition, ovulation can be physically induced in most felids (Brown, 2011; Stewart *et al*., 2012) or chemically induced in maned wolf (Johnson *et al*., 2014). There is possibility of male effect such as scent signaling from urine or feces, playing a role in ovulation when female and male were housed together (Xu and Feng, 2016). All ova are not released simultaneously as ovulation may take 24 to 96 hours (Linde-Forsberg, 2007). Boyd *et al*. (1993) using ultrasound notice that the ova were released from one ovary at a time with the whole process taking about 36 hours. Unlike other mammals, dog ovulates primary ooctyes that are at the beginning of the meiotic division at metaphase I (MI) and the germinal vesicle break down shortly thereafter (Linde-Forsberg, 2007). Canine oocytes mature *in vivo* in the oviduct and there is a multilayered and tight cumulus mass around the

oocyte which expands as the oocyte matures (Holst and Phemister, 1971; Mahi and Yanagimachi, 1976; Tsutsui, 1989). The transportation of oocyte in the oviduct takes 5-10 days (Andersen and Simpson 1973; Tsuitsui, 1989). The circulating and local progesterone (P4) levels begin to rise prior to the luteinizing hormone (LH) surge and subsequently leading to ovulation (Olson *et al*., 1982). Canine oocytes are ovulated at an immature stage compared with other mammalian species, and require 48–72 and 96–120 h post ovulation in the oviduct to complete unclear maturation and cytoplasm maturation, respectively (Reynaud *et al*., 2005; Nagashima *et al*., 2015). Previous studies on canine ovarian activity are based on the oocyte evaluation and ultrasonographic measurements of the follicular populations (Payan-Carreira and Pires, 2008; England *et al*., 2009; Reynaud *et al*., 2009; Bergeron *et al*., 2013). However, luteinisation of follicles in the bitch occurs before ovulation and follicles do not collapse immediately after ovulation (Bouchard *et al*., 1991; Concannon, 2009; England *et al*., 2009), making it difficult to distinguish between preovulatory follicles and/or corpora lutea by ultrasound (Bergeron *et al*., 2013). Studying the canine ovary *in situ* is also complicated by some anatomical challenges, especially an encapsulating ovarian bursa that interferes with the ultrasonographic view of the gonads (Wildt *et al*., 1978; England *et al*., 2009; Songsasen *et al*., 2011).

The LH surge commences the first day of the oestrus (E1) (Wright, 1990; Johnston *et al*., 2001a; Kutzler *et al*., 2003a; Michel *et al*., 2011). In the canine species the LH surge is associated with a preluteinisation of the granulosa cells that begin to secrete progesterone (de Gier *et al*., 2006; Concannon, 2009). Early signs of luteinisation became more marked in E1 together with folded and vascularized granulosa layers. This time is characterized by the mucification of the cumulus in the granulosa cells, the intensity of which depends on

the follicular maturity (Reynaud *et al*., 2006). Preliminary histological studies suggested early canine luteal cells may originate primarily from theca in growth, with dispersed mid-cycle luteal cells of a single type as in some rodents (Concannon, 2012).

Ovulation is thought to occur when blood progesterone concentration is between 4 and 10 ng/mL (Wright, 1990; Johnston *et al*., 2001a; Michel *et al*., 2011; Groppetti *et al*., 2015a). In polytocous species not all follicles ripen at the same time and in the bitch ovulations of the various follicles are spread over a 24–36 h period (Boyd *et al*., 1993; Groppetti *et al*., 2015a).

Vaginal cytology alone cannot be used to detect the precise periovulatory events, such as the day of LH surge or ovulation, but together with blood progesterone evaluation, the combination of which allows accurate definition of the stage of the reproductive cycle (Wright, 1990; Johnston *et al*., 2001a; Kutzler *et al*., 2003a; Fontbonne, 2010; Groppetti *et al*., 2010; Michel *et al*., 2011).

2.1.6 Fertilisation

This occurs during the passage of the ovum in the distal part of the oviduct (Linde-Forsberg, 2007). Mature canine ova may remain alive and fertilisable for 2-4.5 days (Concannon *et al*., 1989; Tsutsui, 1989). Canine spermatozoa can fuse immature oocyte (Mahi and Yanagiachi, 1976) whereas, embryonic cleavage between 2 and 16 cells occur more rapidly following fertilization of more mature oocyte (Linde-Forsberg, 2007).

Optimal fertility in the bitch occurs between 2 and 5 days after ovulation when mature oocytes are present (Wildt *et al*., 1978; Concannon, 1993). The ovulation is assumed to

occur approximately 2–3 days after the LH surge (Wildt *et al*., 1978; de Gier *et al*., 2006). Unfortunately, the time of LH surge is highly variable in dogs and takes a relatively long time, ranging from approximately 24 to 60 h (Wildt *et al*., 1978; Concannon, 1993, 2009; Onclin *et al*., 2002). The rapid rise in blood progesterone concentration at the LH surge and ovulation makes its measurement, together with vaginal cytology, the most reliable marker to detect periovulatory events in clinical practice (Okkens *et al*., 2001; Fontbonne, 2010; Moxon *et al*., 2012).

2.1.7 Diagnosis and staging of pregnancy in Canine Spp

The three well-defined stages of pregnancy defined by Wiebe and Howard, (2009) are as discussed below:

2.1.7.1 Stage 1: Canine fertilization to implantation: Positive pregnancy is difficult to confirm at this stage (Wiebe and Howard, 2009). The implantation takes place at approximately 18 days from the LH surge (Wiebe and Howard, 2009). Progesterone is secreted for initiation and maintenance of pregnancy (Wiebe and Howard, 2009). This maintains endometrial integrity and placental attachment, and inhibits myometrial contractility (Wiebe and Howard, 2009).

2.1.7.2 Stage 2: Implantation to Foetal Ossification: Pregnancies are more easily confirmed at this stage (approximately 25-30 days with ultrasound). Embryogenesis and foetal growth and development occur rapidly (Wiebe and Howard, 2009). The foetal ossification similarly occurs at 40 to 42 days.

*2.1.7.3 Stage 3: Foetal Ossification to Parturition:*This is the stage in which the fetus is well developed (Wiebe and Howard, 2009).Prostaglandins are the natural inhibiting factors causing luteal functional arrest before parturition (Wiebe and Howard, 2009).

2.1.8 Reproductive hormone

Luteinizing hormone (LH) plays a major role in reproductive processes (De los Reyes *et al*., 2017). This pituitary gonadotropin regulates follicle/oocyte maturation, ovulation, as well as optimal fertilization and corpus luteum (CL) development (Assidi *et al*., 2010). In the ovary, granulosa, theca, and luteal cells are capable of secreting steroid hormones, such as estrogens, testosterone and progesterone in response to LH (Fernandes *et al*., 1987; De Gier *et al*., 2006). The type and amount of hormone released vary according to the reproductive physiological status of the follicle and CL (Kooistra *et al*., 1999; Concannon, 2011). This gonadotropin exerts its effects through binding to its receptor (LHR), which is one of the seven transmembrane domain Gprotein- coupled receptor (GPCR) superfamily (Ascoli *et al*., 2002). It has been demonstrated in many species that at the ovarian tissue LHR is mainly expressed in theca cells, but it is also present in granulose cells of preovulatory follicles (Themmen and Huhtaniemi, 2000; Pakarainen *et al*., 2005). The interaction LH-LHR activates adenylate cyclase, phospholipase C and ion channels, which in turn control cellular cyclic AMP, inositol phosphates, Ca^{2+} and other secondary messengers (Gudermann *et al*., 1992; Pidoux *et al*., 2007). Through this pathway, the final differentiation of the granulosa cells and the enzymes responsible for androgen production in the theca cells are enhanced (Tsang *et al*., 1979; Oktay *et al*., 1998). However, alternative second messenger pathways, which may not include stimulation of cAMP

levels also exists (Bukovsky *et al*., 2003; Ascoli, 2005); but, none seems to be as effective as cAMP at mediating the induction of steroids synthesis (Hou *et al*., 2010).

In several species the LHR expression levels increase with follicle growth in response to follicle stimulating hormone (FSH), estradiol and paracrine factors, reaching maximum levels prior to ovulation (Menon *et al*., 2005). At this time, granulosa cells also express sufficient receptors for LH. Therefore, the activity of LH increases, supporting the growth and maturation of larger ovarian follicles and the oocyte as a result of specific LH granulosa cell receptors (Menon *et al*., 2004; Jeppesen *et al*., 2012). In dogs, the estrous cycle is longer than that of other mammals, with a non-seasonal anoestrus of about 3-12 months (De Gier *et al*., 2006). The LH pulses appear to occur in all stages of the oestrous cycle, including anoestrus (Kooistra *et al*., 1999). In fact, termination of the anoestrus involves selection of LH sensitive follicles, and this final follicles selection is caused by an increased frequency of high-amplitude LH pulses at the end of anoestrus (Concannon, 2009), since the responsiveness to gonadotropins increase circulating basal LH concentrations (Kumar and Sait, 2011). This period of increased LH pulsatility has been reported as an important determinant in the start of a new follicular phase (Concannon, 1993; De Gier *et al*., 2006).

It has even been suggested that changes in LH secretion may be more important than changes in FSH secretion in the initiation of a follicular phase leading to ovulation (Concannon, 1993). However, the endocrine mechanisms by which a cohort of follicles is recruited in anoestrus for further development in proestrus is unclear, as it is not known when this sensitivity to LH begins in those follicles (De los Reyes *et al*., 2017). Expression

of both LHR gene and LHR protein may be involved in the process of recruitment and selection of anestrous follicles, but unlike the evidences in rodents and other mammals, the canine LHR expression pattern is poorly understood (De los Reyes *et al*., 2017). Luteinization process begins in preovulatory follicles prior to ovulation in the bitch (Chastant-Maillard *et al*., 2011); therefore, canine follicular cells may increase receptors for LH before the end of proestrus (De los Reyes *et al*., 2017). An early acquisition of LH receptors on granulosa cells has been reported in dogs around the period of LH surge, by measuring the binding of radioactive labeled porcine gonadotrophins by autoradiography (Saint-Dizier *et al*., 2008).

2.1.9 Impact of multiple conceptuses per follicle on fecundity in the bitch

Due to the variation in ovulation rate among bitches (Andersen and Simpso, 1973; Nöthling and Volkmann, 1993; Nöthling *et al*., 2005), the relationship between the number of conceptuses and the number of corpora lutea may provide a better measurement of fertility in an experimental setting than the mere number of conceptuses (Nöthling and Volkmann, 1993). Follicles containing more than one oocyte, known as multi-ovular follicles (MOFs) occur in bitches (Telfer and Gosden, 1987; McDougall *et al*., 1997; Barber *et al*., 2001; Luvoni *et al*., 2005 Reynaud *et al*., 2005; Payan-Carreira and Pires, 2008; Reynaud *et al*., 2009). Ovariohysterectomy of the pregnant bitch enables the researcher to precisely and accurately determine the number of corpora lutea and post-implantation conceptuses (Nöthling and Volkmann, 1993; Nöthling 1995; Nöthling *et al*., 2006; Rota A *et al*., 2010; Steckler *et al*., 2013). However, the number of post-implantation conceptuses relative to the number of corpora lutea (implantation ratio)

reflects the net outcome of the number of follicles that ovulated, the number of oocytes that were fertilised and the number of early embryos that died (Ferguson *et al*., 1989). The higher the implantation ratio in a bitch is, the lower the effects of fertilisation failure and embryonic death (Steckler *et al*., 2017).

Multi-ovular follicles may have up to 17 oocytes although 2 or 3 are more common (Telfer and Gosden, 1987). They are more common in Indigenous breeds than in purebred bitches (Payan-Carreira and Pires, 2008), and their prevalence decreases with age of the bitch and with size of the follicle (Telfer and Gosden, 1987; McDougall *et al*., 1997). As many as 7% (Reynaud *et al*., 2009) to 40% (Payan-Carreira and Pires, 2008), of preantral follicles have been found to be MOFs (Steckler *et al*., 2017). Multi-ovular follicles may ovulate (Luvoni *et al*., 2005) because it was observed that the number of oocytes or embryos flushed from bitches was higher than the number of corpora lutea present (Bysted *et al*., 2001; Reynaud *et al*., 2005). However, the number of the oocytes released by ovulation from MOFs that are able to mature and be fertilized is not known (Chastant-Maillard *et al*., 2011) as the oocytes within a MOF can be at different developmental stages, and can be viable or degenerate (Barber *et al*., 2001). However, research suggests that only one oocyte of good quality is contained within a MOF (Payan-Carreira and Pires, 2008; Reynaud *et al*., 2009).

Uterine pathology, mating at the wrong time, sub-fertility of males (Johnston *et al*., 2001a) and various factors pertaining to artificial insemination such as semen quality, site of semen deposition, number of inseminations (Thomassen *et al*., 2001) and semen processing (Nöthling and Volkmann, 1993; Nöthling *et al*., 2005) may result in some

fertile oocytes released at ovulation not yielding conceptuses and thereby reducing the ratio between conceptuses and corpora lutea (Steckler *et al*., 2017). More than one conceptus may derive from the same follicle if a MOF releases more than one fertile oocyte at ovulation or if an embryo splits to yield monozygotic twins (Joone *et al*., 2016).

2.1.10 Gestation period and parturition in the bitch

The length of canine pregnancy apparently varies between 57 and 72 days because bitches show an extended period of receptivity and viability of spermatozoa in the female reproductive tract is prolonged (Concannon *et al*. 1983). Parturition occurs 65 ± 1 day after LH peak and 63 ± 1 day after ovulation (Concannon *et al*., 1983). The preovulatory LH surge can be detected through the measurement of the concomitant increase in serum progesterone concentrations, and this method provided a parturition prediction accuracy of 90% for 65 ± 2 days (Kim *et al*. 2007). Although LH could be measured directly, it is rather impractical because more than one daily samplings are necessary (Hase *et al*. 2000). In clinical settings, veterinarians have frequently to deal with near-term bitches whose history is limited to mating dates (Rota *et al*., 2015). At the end of pregnancy, some parameters can be monitored to predict parturition such as body temperature or progesterone concentration (Rota *et al*., 2015). Rectal and vaginal temperature may show a decline approximately 24 h before the onset of parturition (Concannon and Hansel 1977; Geiser *et al*. 2014), although it does not occur in all bitches (Veronesi *et al*. 2002), a significant decline in progesterone concentration occurs 24–40 h before parturition (Nett *et al*. 1975; Concannon *et al*. 1978; Veronesi *et al*. 2002; Brugger *et al*. 2011; Rota *et al*.,

2015), as a possible response to the rapid changes in circulating hormone concentrations (Concannon and Hansel 1977).

However, both the detection of LH peak and the ultrasonographic identification of ovulation may be unpractical, because of the necessity of daily determinations (Hase *et al*., 2000; Hayer *et al*., 1993). Nevertheless, LH peak and ovulation can be indirectly determined by progesterone rise (Beccaglia *et al*., 2016). It has been demonstrated that serum progesterone ranges between 2 and 3 ng/ml during LH peak and between 4 and 10 ng/ml during ovulation (Kutzler *et al*., 2003a). Progesterone concentration decreases below 2 ng/ml approximately 24 hr before parturition (Concannon *et al*., 1978), and this can be indirectly recognized by the drop in body temperature (Geiser *et al*., 2014; Johnston *et al*., 2001b). Recent studies demonstrated that in dogs, the imminence of parturition could be predicted by evaluating foetal flux and foetal heart rate by ultrasound (Gil *et al*. 2014; Giannico *et al*., 2015). For an accurate prediction of parturition date, the combination of different methods is desirable (Beccaglia *et al*., 2016). However, Concannon, (2000) reported that identification of the onset of dioestrus, through daily vaginal cytology after mating, has been used to predict the parturition. This occurs approximately 57 ± 1 days after the transitional smear from a cytological pattern characterized by a high number of keratinized cells to a pattern with predominance of basal and parabasal cells and leucocytes. It is common opinion that Caesarean sections can be safely performed when progesterone concentration declines below 2 ng/ml, that is, 24–40 h before natural parturition, (Concannon *et al*. 1977; Smith 2007). Progesterone concentration was reported to show diurnal variation in pregnant bitches from weeks 3 to 6, with morning values

higher than afternoon values (Steinetz *et al*. 1990); however, in the eighth and ninth week of pregnancy, morning and afternoon values resulted similar (Steinetz *et al.* 1990).

The gold standard to measure serum progesterone in bitches is radioimmunoassay (RIA), and previous works reporting peri-partum progesterone concentration in bitches have used this methodology (Nett *et al*. 1975; Concannon *et al*. 1978; Veronesi *et al*. 2002). The chemiluminescent (CLIA, Immulite) assay has also been validated for measuring progesterone in canine serum and has the advantage of being safe, fast and accurate (Kutzler *et al*. 2003a; Chapwanya *et al*. 2008). However, comparing RIA and Immulite serum progesterone values, the average Immulite measurement resulted in significantly higher concentrations than the RIA one, with an average difference of 0.69 ng/ml (Chapwanya *et al*. 2008).

2.2 Ultrasound and Ultrasonography

Ultrasonography is a non-invasive, quick and very easy diagnostic imaging modality (Pete *et al*., 1992). It is used to visualize the structure and architectural details of soft tissues and organs (Ulum *et al*., 2017). Its principle is based on the ability to reflect transmitted high-frequency sound waves by tissue (Kumari *et al*., 2016). Ultrasound waves are generated by the piezoelectric effect in a suitable medium such as lead zirconate (Kumari *et al*., 2016). Echoes produced by ultrasound depend on relative density of tissue (Pierson and Adams, 1995). A, B, and M modes are the three basic forms of ultrasound used in soft tissue imaging (Kumari *et al*., 2016). Diagnostic ultrasound frequencies range from 2 to 15 MHz and are inaudible to the human ear (Kumari *et al*., 2016). The image produced in sonogram is created using high-frequency sound wave which is displayed as real-time slices of

images similar to histological images (Pavlin and Foster, 1998). High-frequency sound wave of 20 MHz is commonly used in the medical services for dermatological examination (Gniadecka and Quistorff, 1996). This method is known as dermatosonography (Jemec *et al.*, 2000) while 30-100 MHz was used for eye examination (Pavlin and Foster, 1998). However, such high-frequency transducer is not available in most medical facilities. The most commonly available transducer in medical facilities are 3-15 MHz transducers which are used for obstetrical examination and imaging of other internal organs (Mantis, 2008).

The ultrasonogram is essentially an image of a thin slice of tissue, and therefore, enables serial examinations to monitor the progression of the condition, response to treatment and to practice scanning techniques (Hornbuckle and Kleine, 1980). However, one of the drawbacks of diagnostic ultrasound is that it has a steep learning curve and requires a great deal of skill and experience (Kumari *et al*., 2016).

The sound wave produced and captured by transducers consists of several parts namely Fresnel zone, focal zone and Fraunhoferzone (Noviana *et al.*, 2012; Mattoon and Nyland, 2014). Target organ examination is performed at focal zone to obtain an image with adequate resolution (Ulum *et al*., 2017). However, the limitation of 3-15 MHz transducers is the location of Fresnel zone more than 10 mm, hence the target organ could not be depicted clearly (Ulum *et al*., 2017). Fresnel zone is where complex diffractions patterns occur, located nearest to the transducer as the sound source. Focal zone is where the sound wave is most focused, producing an image with the best resolution. Fraunhofer zone is the part where sound wave diverges and the resolution of the image gradually decreases (Ulum *et al*., 2017). Ideally, the target organ of ultrasonography is directly positioned in the focus

zone by placing the transducer in the imaging area, especially for large organ or dense tissues. Focal zone position can be set to be close to the sound source in the transducer. However, visualizing small organ with direct technique was difficult because of its small size. Organs sized < 10 mm was automatically visualized in Fresnel zone; hence the image produced was not of diagnostic value (Ulum *et al*., 2017).

2.2.1 Historical overview of ultrasonography

World War I saw the first practical use of the piezoelectric effect in the development of sonar using a separate sound generator and detectors (Coltera, 2010). In 1880, the French Physicist; Pierre Currie discovered the Piezoelectric property of certain crystals which upon application of electricity resulted in the deformation and production of sound with a frequency greater than 20KHz known as Ultrasound (Vignoli and Saunders, 2011). Thereafter, in 1940, The American acoustical physicist; Floyd Firestone, devised the first ultrasonic imaging devise, the supersonic reflectoscope, to detect the internal flaws in metal casting. In 1941, the Austrian Neurologist; Karl Theo Dussik and a physicist; Friedriech are the first to use the ultrasound to image the human body (Siddharth, 2007; Levine, 2010). In the 1940s, Dr. George Ludwig was the first person to apply the ultrasonic energy to the human body for medical purposes at the Naval Medical Research Institute, Bethesda, Maryland (Anon, 2006; Anon, 2015). Douglass Howry with the help of Joseph Holmes, an American nephrologist, developed a *B-mode linear compound ultrasound scanner* to include superior transducers, amplifiers, and display imaging. Howry and his team introduced compound scanning in order to eliminate 'false' echoes. Compound scanning allowed Howry to differentiate between structures and tissues which ultimately produced better imaging and were then referred to as '*sonagrams'* (Anon,

2015). Today, the applications of ultrasound are diverse and include therapeutic purposes to stimulate tissue healing (Porter, 1991; Steiss, 2000), food sterilization (Piyasena *et al*., 2003) and vaccination of fish (Zhou *et al*., 2002).

2.2.2 General principles of ultrasonography

2.2.2.1 Resolution: Resolution is the ability of an ultrasound machine to distinguish echoes on the basis of space, time, and strength. (Kremkau, 2011) The better the resolution, the more likely an abnormality can be identified. As the frequency of an ultrasound wave increases, the resolution increases. There are three types of resolution found in ultrasound imaging; axial resolution, lateral resolution and elevation resolution (Kremkau, 2011).

Axial resolution is the resolution of two separate reflectors along the direction of the ultrasound beam (Kremkau, 2011). It is determined by the ability of the transducer to detect two reflecting echoes separately without an overlap in the returning echoes, therefore both echoes have to be separated by half the spatial pulse length (SPL) (Daniel, 2010). Spatial pulse length is the length of space in one pulse of ultrasound. When two reflectors are separated by a distance that is greater than half the SPL, the echoes from these two reflectors do not overlap as they return to the transducer and are interpreted as separate echoes (Daniel, 2010). The shorter the transmitted pulse and likewise its returning echo pulse, the better the axial resolution. The pulse length is determined by the number of cycles emitted multiplied by the wave-length (Daniel, 2010).

Lateral resolution is the ability to separate two adjacent reflectors perpendicular to the beam direction in (Kremkau, 2011). This was determined by the width of the ultrasound

beam which must be narrower than the space separating the reflectors (Daniel, 2010). The width of an ultrasound beam decreases with increasing frequency. In a focused ultrasound beam, where the width of the beam is restricted, the lateral resolution is best at the focal point of the ultrasound beam because this was the narrowest part of the beam (Kremkau, 2011).

*2.2.2.2 Focusing:*Focusing the beam results in reduced beam diameter and improved lateral resolution (Powis, 1986). Focusing of the beam can occur only in the near field of the ultrasound beam (Kremkau, 1993). Beam focusing characteristics are variable for each transducer and frequency and should be obtained from the manufacturer at the time ultrasound equipment is purchased. The focal distance and focal zone can be verified with an ultrassound test object. A number of devices are available commercially for testing imaging performance. Focusing can be performed dynamically or manually (Kremkau, 2011).

Table 2.1:Depth and axial resolutions obtained by different transducer frequencies

Frequency (MHz)	Image depth (cm)	Axial Resolution (mm)
2.0	20	0.77
3.5	17	0.44
5.0	12	0.31
7.5	8	0.20
10.0	6	0.15

Source: (Nyland and Matton, 1995)

2.2.3 Transducers

A transducer is a very important part of an ultrasound imaging system (Scampi, 2017). A typical ultrasound machine uses a wide variety of transducers optimized for specific diagnostic applications (Scampi, 2017). Each transducer is comprised of an array of piezoelectric materials that send focused energy into the body and receive the resulting reflections. Each transducer is connected to the ultrasound imaging system with fine coaxial cables (Scampi, 2017). Typical transducers have up to 32 to as many as 512 piezoelectric elements that operate at frequencies from 1MHz to 15MHz (Scampi, 2017). Most ultrasound systems provide two to as many as four switchable transducer connections to allow the clinician to easily switch among the various transducers for each examination type (Scampi, 2017).

2.2.4 Types of Transducers

2.2.4.1 Array transducers: The array transducers are either linear or curved in shape and their piezoelectric crystals are arranged in a line along the surface of the transducer (Patrick, 2010). They have the advantage of allowing a wide superficial field of view. The linear array is particularly important in viewing superficial tissues. They are used for the examination of the musculoskeletal system, abdominal organs, eyes, body wall and non-cardiac thoracic structures (Patrick, 2010).

2.2.4.2 Phased array transducers: Phased array (sector) transducers are composed of only a small number of crystals in rectangular format that are electronically excited as a group with small time difference (phasing), which results in the sound pulse being sent out in a specific direction that is constantly changing (Patrick, 2010).

They have the advantage of working with small foot prints and creating pie-shape images, which allow deep structures to be view clearly (Patrick, 2010). These transducers have a limited near field visibility compared with the linear array or curvilinear array transducer and are not a good choice for imaging superficial structures (Powis, 1993; Patrick, 2010). Phased array transducers are used for echocardiography and for scanning structures that are deeply located such as the abdominal or thoracic structures in large breeds of dogs (Patrick, 2010).

2.2.5 Transducer frequencies

The ultrasound wave frequency produced by the transducer is a function of the crystals within it (Patrick, 2010). A wide range of transducer frequencies is currently available from low frequency transducers imaging at 2.0 MHz to high frequency transducers imaging at 10.0 MHz (Patrick, 2010). Ultra high frequency transducers exist for specialty imaging. The lower the transducer frequency the deeper the penetration, but less resolution (Patrick, 2010). Routinely, 7.5 MHz penetrates a depth of 4-6 cm, 5 MHz penetrates 10-12 cm, 3.5MHz penetrates 15-20 cm and 2.5 MHZ penetrates 25-30 cm. To reach superficial structures a standoff pad can be used to transfer the focal zone to a higher level (Allen and Stone, 1990). The selection of the appropriate transducer for an examination depends upon the structure being evaluated, and the depth of the area from the transducer surface and the acoustic properties of the intervening tissues (Powis, 1986).

2.2.6 Ultrasound image formation

The formation of image in ultrasonography is based on the principle of pulse echo (Hangiandreou, 2003; Kremkau, 2011). As pulse transmits through the patient, sound waves are reflected back to the transducer from each tissue interface. In between, the

transducer is dampened to stop the piezoelectric crystal from vibrating. The electric signals generated from the returning echoes are amplified to form the final image (Kremkau, 2011). Two modes of echo display are commonly used in ultrasonography: brightness mode (B-mode, B scan, or gray scale) and motion mode (M-mode). B-mode is used commonly in both abdominal and cardiac imaging while M-mode is used only for echocardiography (Kremkau, 2011).

2.2.7 Modes of sonography

2.2.7.1 A-mode: A-mode is the simplest type of ultrasound (Jauhiainen, 2009). A single transducer scans a line through the body with the echoes plotted on screen as a function of depth. Therapeutic ultrasound aimed at a specific tumor or calculus is in A-mode to allow for pinpoint accurate focus of the destructive wave energy (Jauhiainen, 2009).

2.2.7.2 B-Mode: In B-mode ultrasound, a linear array of transducers simultaneously scans a plane through the body that can be viewed as a two-dimensional image on screen (Jauhiainen, 2009). B-mode displays a static image of a section of a tissue (Daniel, 2010). They are made up of collection of dots that corresponds to the amplitude and strength of the returning echo (Kremkau, 2011). These dots are displayed on a black background, and the brightness of the dot is highest (whitest) for the strongest returning echoes (Kremkau, 2011). The greater the amplitude the brighter the dot on the image. B-mode is commonly used in both abdominal and cardiac imaging (Kremkau, 2011).

2.2.7.3 M-mode: M stands for motion. In M-mode a rapid sequence of B-mode scans whose images follow each other in sequence on screen enables doctors to see and measure range of motion, as the organ boundaries that produce reflections move relative to the

probe (Jauhiainen, 2009). M-mode is a time motion displayed on the ultrasound wave along a chosen ultrasound line (Anon, 2017). It displays a static image of a section of a tissue. The echo generated from a single ultrasound wave passes through a moving structure and are acquired and displayed as a function of time on the horizontal axis. The motion pattern allows functional evaluation of moving structures such as the heart (Daniel, 2010). M-mode provides a monodimensional view of the heart and thus used for echocardiography (Kremkau, 2011). It has an advantage of very high sampling rate, which results in a high time resolution so that even very rapid motions can be recorded, displayed, and measured (Anon, 2017).

2.2.7.4 Doppler mode: This mode makes use of the Doppler effect in measuring and visualizing the flow of blood in the body (Jauhiainen, 2009).

2.2.8 Ultrasound artefacts

Ultrasound artefacts are often part of the images, and may lead to misinterpretations (Kirberger, 1995; Feldman *et al*., 2009; Hindi *et al*., 2013). They may be mistaken for lesions or may obscure the area of interest during ultrasound scanning (Hindi *et al*., 2013). For instance, artefacts commonly encountered when imaging the urinary tract include acoustic shadowing, reverberation artefact, distal acoustic enhancement, slice thickness artefact, grating or side-lobe artefact, edge shadowing (refraction) artefact, and twinkle artefact (Douglass, 1993; Douglass and Kremkau, 1993; Kirberger, 1995; Barthez *et al*., 1997; Louvet, 2006; Sutherland-Smith, 2008; Barrett, 2011).

The sound interaction with biological tissues is complex and may be responsible for many explained and unexplained artefacts. Additionally, the understanding of physical properties

of artefacts has been studied *in vitro* by several authors (Barthez *et al*., 1997; Heng and Widmer 2010).

2.2.8.1 Acoustic shadows: Acoustic shadows are areas of decreased echogenicity below a dense object with high reflectivity (Park *et al*., 1981; Penninck, 2002). It occurs if the primary beam is almost completely reflected or absorbed**.** An insufficient quantity of echoes returned from the location distal to the strong reflector makes these regions appear anechoic (black). This type of artefact is useful because it gives information about the composition of the structure causing the shadowing (Heng and Wigner, 2010; Kremkau, 2011). Bone, air, foreign bodies and calcification stops the transmission of sound waves producing a 'sonic shadow' which is a dark region distal to the echogenic obstructing region. Acoustic shadowing artefacts are minimized with spatial compounding (Heng and Wigner, 2010; Kremkau, 2011). This may allow a lesion hidden by a shadowing artefact to be identified. Conversely, the composition of a structure may be misinterpreted because it is not shadowing (Hangiandreou, 2003).

According to Kirberger (1995), acoustic shadowing occurs because of mineral or air interfaces causing loss of echo intensity deep to the interface because of absorption and/or reflection. Mineral interfaces such as uroliths typically cause strong distal hypoechoic to anechoic shadows. Air interfaces typically cause echogenic ("dirty") distal shadows because of concurrent reverberation artefact. This artefact is seen when gas is present within the bladder lumen or wall (Kirberger, 1995).

2.2.8.2 Acoustic enhancement/ distal acoustic enhancement: This is due to passage of sound through an area of decreased attenuation resulting in higher echo intensity from

tissues deep to the area compared with surrounding tissues at the same depth. This artefact is typically seen with fluid-filled structures such as the urinary bladder (Kirberger, 1995). This is an area of increased echogenicity behind structures of low attenuation (Kirberger, 1995; Penninck, 2002; Kremkau, 2011). It mostly occurs at sites distal to cysts, gallbladder and urinary bladder and appears as a hyper intense (hyperechoic) signal (Kirberger, 1995). The attenuation of ultrasound wave in fluid is much lower compared to other tissues; therefore tissues distal to the fluid are enhanced. Artificial enhancement may also be found distal to a homogenous solid tumor surrounded by adipose fat (Kremkau, 2011).

2.2.8.3 Reverberation artefacts: They occur when the sound waves encounter an area of high reflectivity such as bowel gas and the sound is reflected towards the transducer. They are produced from multiple reflections of sound wave, if the acoustic impedances of tissue layers are too much different and the detected echo does not run the shortest sound path because it bounces back and forth between the object and transducer (Park *et al*., 1981; Feldman *et al*., 2009). They appear as multiple hyperechoic foci that occur at regular intervals.

2.2.8.4 Comet tail artefacts: This is another form of reverberation artefact that appears as a series of short, very closely spaced successive echoes that are typically decrease in intensity and width in depth. They are caused by two closely spaced, discrete highly reflective surfaces (Feldman *et al.,* 2009; Kremkau, 2011). When gas bubbles form thin layers separated by liquid just as in the digestive tract, the waves rebound between the layers, resulting in many echoes that return to the probe at regular intervals, making a trail

of echoes in the form of a shadow looking like a comet's tail (Feldman *et al.,* 2009; Kremkau, 2011).

This is also a variation of reverberation artefact that appears as a series of short parallel reflective lines that extends behind a gas collection. It occurs when ultrasound wave reverberates within fluid trapped between collections of air bubbles (Feldman *et al*., 1995; Kirberger, 1995).

2.2.8.6 Mirror-image artefacts: This type of artefact occurs when a highly reflective and obliquely oriented surface reflect sound beam distally instead of returning it to the transducer (Daniel, 2010). It appears as a duplication of a normal structure on the opposite side of a strong reflector (Kremkau, 2011). This goes against the assumption that echoes return to the transducer after a single reflection and that sound wave travel in a straight line This is most commonly encountered when the liver is imaged with the diaphragm/lung interface is acting as a strong reflective surface (Feldman *et al*, 2009).

2.2.8.7 Side-lobe, grating-lobe artefacts and slice thickness: They cause the appearance of echoic material within anechoic areas. These artefacts can give the false impression of sediment within the urinary bladder (Barthez *et al*., 1997). Side lobes and grating lobes are different types of secondary lobes present on the side of the primary sound beam. Side lobes are present in all transducers and originate from additional mode vibrations of the piezoelectric crystal (Barthez *et al*., 1997). They are usually of low intensity and can create spurious echoes in the near field. Grating lobes are associated with the geometric construction of linear probes (Barthez *et al*., 1997). The artefacts created by secondary lobes result in misplacement of reflected echoes. The side or grating lobes are weaker than

the primary sound beam. These lobes must encounter a highly reflective surface and be of sufficient intensity to be noticed. In clinical situations, secondary lobe artefacts are difficult to differentiate from volume-averaging artefacts (Barthez *et al*., 1997).

Slice thickness artefacts occurs when the ultrasound beam is wider than the diameter of the cystic structure being scanned and include normal structures which lie immediately close to the cystic structure, the echoes from the tissues are displayed within the cystic structure mimicking the presence of a pseudo-sludge (Daniel, 2010). This does not occur when the entire beam width includes the cystic structure alone. Thus slice thickness artefact are avoided if the beam width is narrowed, such as in the focal zone of the beam. The surface of a pseudosludge is curved while the surface of a real sludge is flat (Penninck, 2002).

2.2.8.8 Refraction: This occurs when a sound wave changes in direction on passing from one medium to the other (Daniel, 2010). It occurs when sound waves travel from one tissue of different acoustic velocities, its frequency remains the same but its wavelength changes (Daniel, 2010). As the sound wave moves to the new medium, it deviates resulting in display of organs in incorrect locations, usually to the side of their actual location (Feldman *et al*., 2009; Kremkau, 2011). Refraction artefacts may lead to measurement errors because organs may appear wider than normal (Kremkau, 2011).

2.2.8.9: Edge-shadowing artefacts: This is caused by refraction of the ultrasound beam. It occurs at the margin of curved structures and results in a dark line or shadow extending distally. If this artefact involves a curved fluid-filled structure (bladder) surrounded by fluid (abdominal effusion), it will give the impression of a defect in the bladder wall (Douglass and Kremkau, 1993). They are a form of refraction artefacts that are produced

when sound wave deviates as they encounter a curved surface tangentially (Kirberger, 1995). As a result of the sound waves that have been deviated by the curved surfaces, areas of anechoic regions are seen distal to the curved surface. This type of artefacts is most commonly encountered when the kidneys, urinary bladder or gallbladder are imaged and can be eliminated with spatial compounding (Heng and Widmer, 2010).

2.2.8.10 Twinkling artefact: Twinkling artefact is a colour Doppler artefact that occurs behind strongly reflective interfaces such as those produced by urinary tract stones or parenchymal calcifications (Louvet, 2006). It appears as a quickly fluctuating mixture of Doppler signals with an associated spectrum of noisy appearance and can be used to differentiate mineral structures from other hyperechoic material (Louvet, 2006).

2.3 Doppler Ultrasonography

Doppler ultrasonography is a non-invasive real time pulse-wave technique used for the study of the reproductive system hemodynamics (Ferreira *et al*., 2011). This technology allowed re-evaluating previously conceptions considered definitive regarding the physiology of reproduction, and it is effective in the evaluation of animals' reproductive tract for the diagnostic, monitoring, and predictive purposes (Ginther, 2007).

Colour-Doppler ultrasonic imaging is based on the principle that proposes the change in frequency of a wave for an observer moving relative to the source of the respective wave. The frequency is constant when the wave source and observer are stationary (Ferreira *et al*., 2011). However, if there is a motion toward or away from each other, the returning echoes frequency increase and decrease, respectively (Ginther and Utt, 2004). This principle is used for different applications as temperature, velocity and vibration

measurements. In Doppler sonography, the wave source and stationary object are the red blood cells and the transducer respectively (Ferreira *et al*., 2011). During an ultrasonic Doppler examination it is possible to perform a qualitative and/or quantitative evaluation of specific vessels and tissues. The direction and velocity of red-blood cells can be represented by different types and intensities of colours pixels or as a velocity spectral graphic (Ferreira *et al*., 2011).

2.3.1 Coloured ultrasonic imaging

There are two specific approaches for coloured ultrasonic imaging evaluation: Colour-flow and Spectral modes (Ferreira *et al*., 2011). Colour-flow mode uses Colour-Doppler signals superimposed on a B mode image of a structure to estimate its vascularity, while pulsed-Doppler spectral analysis of blood velocities of a specific artery is done on Spectral mode as shown in Plate 2.1. Both methods are based on Doppler-shift frequencies, also called Doppler frequency (Ginther and Utt, 2004). Doppler frequency is the difference between the frequency of received echoes and frequency transmitted by the transducer (Ferreira *et al*., 2011). The transducer transmitted frequency is constant while the frequency of the returning echoes varies according to the Doppler effect principle. Therefore, when the wave source (red blood cells) is stationary or moving parallel to the transducer there is no difference between transmitted and returning frequencies and coloured Doppler signals are not detected (Ferreira *et al*., 2011). If the blood flow moves toward the transducer, the returning frequency is greater than the transmitted frequency resulting in a positive Doppler frequency. A negative Doppler signal is produced when the returning frequency is

lower than the transmitted frequency or, in other words, when the blood red cells move away to the transducer (Zagzebski, 2005).

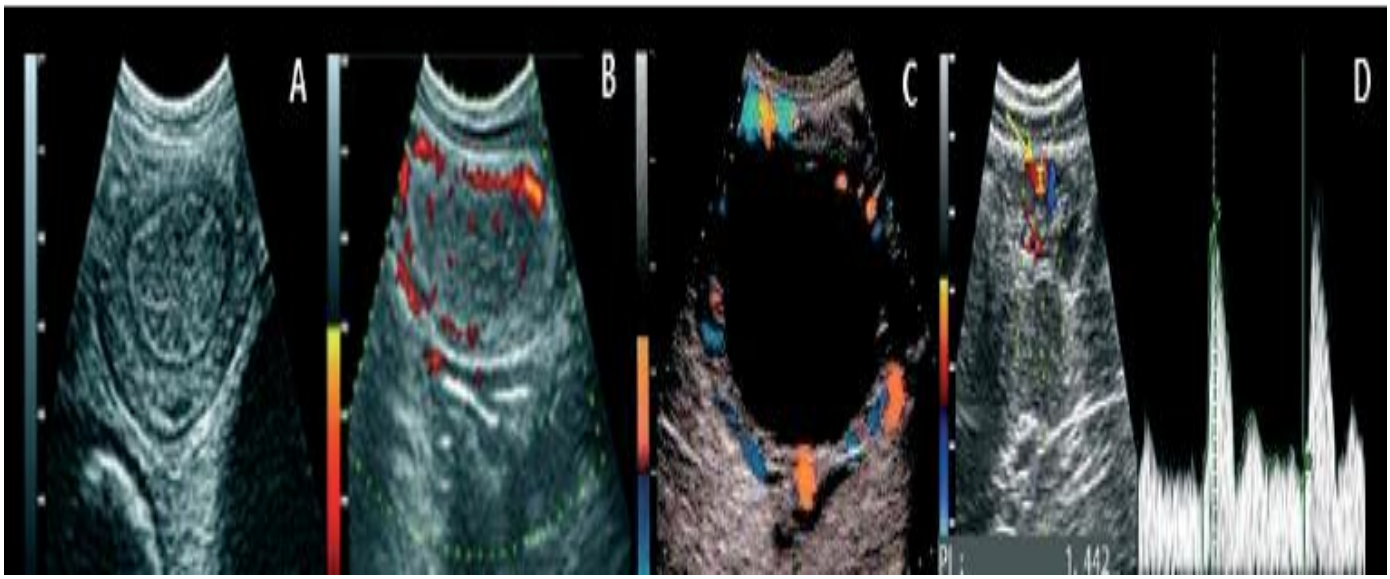


Plate 2.1 Ultrasonic images collection of female reproductive tract in B, Power, Colour and Spectral modes (A, B, C and D, respectively). (A and B) Cross section image, using B-mode and Power-flow Doppler, of a non-pregnant uterine horn in dioestrus. (C) Colour Doppler evaluation of a pre-ovulatory follicle. (B and C) Presence of coloured pixels within uterine tissue (endometrium and myometrium) and follicular wall indicate the red blood cells displacement and have been used to estimate the tissue vascularity. (D) Spectral graph consisting of maximum speeds values of blood flow in mesometrial artery. Source: Ferreira *et al*., (2011).

2.3.1.1 Spectral mode: In the spectral mode, blood flow velocity variations are represented as a graphic wave form called spectrum (Plate 2.2). The spectrum provides maximum velocities values as Peak systolic (PSV), End diastolic (EDV) and Time-average maximum (TAMV) velocities. Peak systolic Velocity is the maximum point along the length of the spectrum, while EDV is the ending point of the cardiac cycle (Ferreira *et al*., 2011). Time-average maximum is the maximum velocity values average. Additionally, the position of the spectrum in relation to the baseline of the monitor indicates the blood flow direction. By convection, wave forms above and under the baseline indicate, respectively, blood-red cells moving toward and away from the transducer (Ginther, 2007; Ferreira *et al*., 2011).

An alternative used in early studies is to calculate the Doppler index (Silva *et al*., 2005; Silva and Ginther, 2006; Ferreira *et al*., 2008; Ferreira *et al*., *et al*., 2010). They are good indicators of vascular perfusion of reproductive organs and are independent of the Doppler angle (Ginther and Utt, 2004). Resistance and Pulsatility indices (RI and PI, respectively) are calculated using the ratio of the three velocities measurements (PSV, EDV and TAMV) and both indices have a negatively correlation with the vascular perfusion of the tissue downstream from the sample gate (Ginther, 2007).

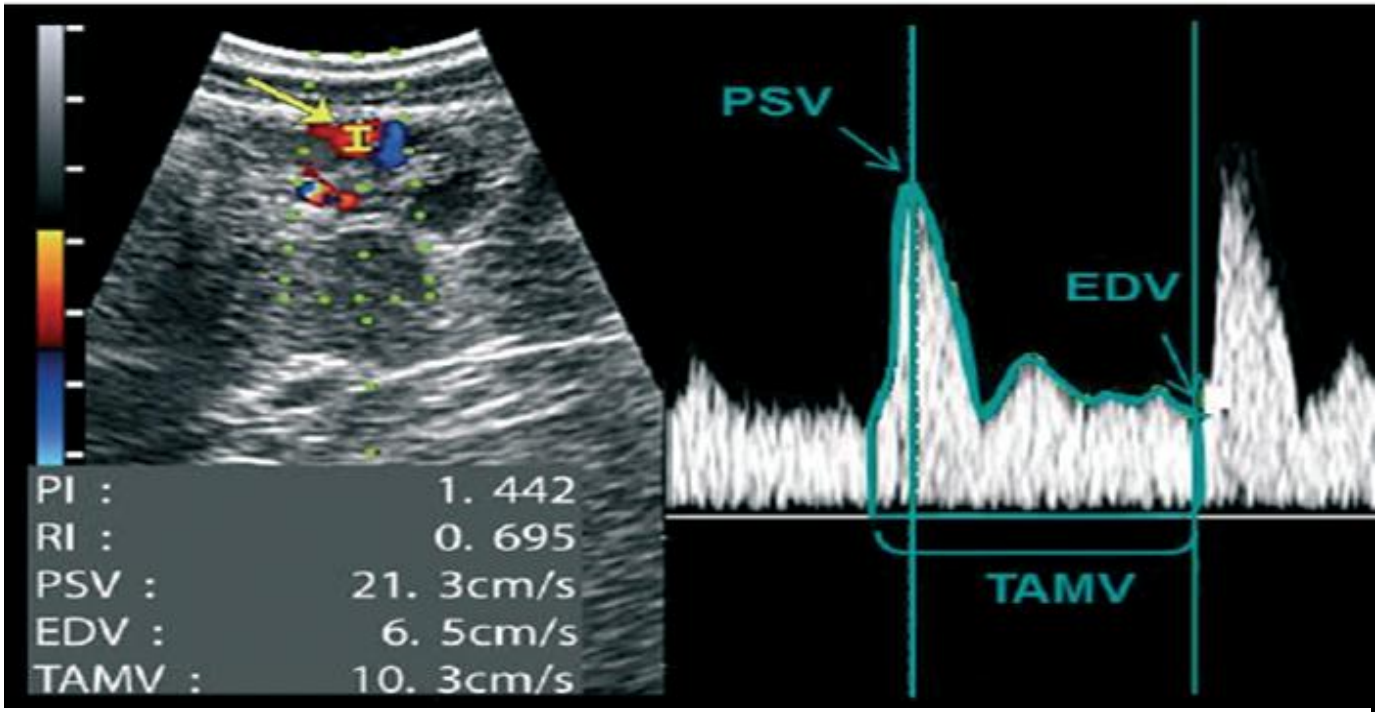


Plate 2.2 Spectral Doppler evaluation of mesometrial attachment artery. The gate (yellow) is placed on an artery of the mesometrial attachment (red) to capture velocities data of uterine blood flow and, posteriorly, produce a spectral graph. Peak systolic (PSV) and end diastolic (EDV) velocities are, respectively, the maximum and ending blood flow velocities of a cardiac cycle. TAMV is the average of an espectral maximum blood flow velocities. Doppler device automatically calculates Pulsatility and Resistance indices (RI and PI, respectively). Spectral graph located above the baseline indicates erythrocytes moving toward to the transducer. Source: Ferreira *et al*., (2011).

2.3.1.2 Colour-flow mode: Colour-flow mode is a simple, rapid and functional method of evaluation, which has been applied for farm practical purposes (Ginther, 2007). Colour-flow Doppler provides an immediately qualitative evaluation of the blood flow of different organs and tissues. The vascularity of a structure is estimated subjectively considering the extension of a tissue with coloured pixels during a continuous real time examination (Ferreira *et al*., 2011).

The current Doppler equipment offers two different modes of colour-flow imaging: Colour-flow and Power-flow modes (Ferreira *et al*., 2011). The classic colour-flow mode uses two distinct colours, usually variations of red and blue colours, to represent the vascular blood perfusion of a structure. Also, coloured pixels indicate the blood red cells direction in relation to the transducer. By convection, red coloured spots indicate blood flow moving toward the transducer, while blue coloured spots represent blood-red cells moving away from the probe. Additionally, the intensity of the coloured pixels suggests the velocity of blood flow ranging from dark to bright tonalities for slower and faster velocities respectively (Ferreira *et al*., 2011).

Power-flow imaging has been used to evaluate uterine and ovarian vascularity of mares and cattle (Silva and Ginther, 2006; Ferreira *et al*., 2008; Ginther *et al*., 2008; Ferreira *et al*., *et al*., 2010). In power-Doppler mode, the blood flow movement is graduated using a single colour and the colour pixels intensity vary according to the power of the Doppler signals (number of blood red cells moving at a specific velocity). Power mode Doppler could improve the evaluation of vessels with small diameters and slow blood flow, as are present in the uterus (mesometrium attachment, endometrium and myometrium) and

ovaries (mesovarium, corpus luteum and follicular wall) of mares (Ferreira *et al*., 2008; 2010; 2011).

2.3.2 Evaluation of the reproductive tract of bitches by Doppler ultrasonography

The use of colour and power Doppler ultrasonography has become one of the best available and reliable techniques for the diagnoses and studies of reproductive haemodynamics (Ginther, 2007; Ferreira *et al*., 2008). Considering the presence of a substantial number of small vessels with slow blood flow that do not appear on a conventional colour-flow image, it suggested the use of power-flow mode for the vascular perfusion evaluation of follicles, corpus luteum and uterus (Ferreira *et al*., 2008; Ginther *et al*., 2008; Siddiqui *et al*., 2010).

The evaluation of uterine vascular perfusion using colour-flow imaging must be done considering all uterine layers (Ferreira *et al*., 2008; Ferreira *et al*., 2010). The vascularity of the uterus is estimated subjectively using the percentage of uterine tissue with colour signals Doppler during a real time cross-sectioning examination of the uterus in a continuous span of 1 minute. Multiple cross sections must be viewed as consequence of animal and uterine movements (Silva *et al*., 2005).

The mesometrium attachment is an important reference point in equine trans-rectal Doppler ultrasound for indicating the area of entry of the blood vessels into the uterus (Ginther, 2007). According to Silva *et al*. (2005), colour Doppler signals within the endometrium are commonly inadequate for the production of spectral waveforms. However, early studies have reported the successful use of mesometrial arteries as an alternative assessment for the spectral evaluation of uterine blood flow velocities of

pregnant and non-pregnant mares (Silva *et al*., 2005; Silva and Ginther, 2006; Ferreira *et al*., *et al*., 2010).

Luteal vascularity is estimated while viewing, in a slow and continuous motion, of the entire CL real-time scan (Ginther *et al*., 2008). Colour spots at the periphery and within the CL must be included in the luteal vascular perfusion estimation. Spectral mode from CL is considered too time consuming hence, it is not used for objective evaluation (Ginther *et al*., 2007). An alternative to validate the subjective examination of CL is determining the number of coloured pixels in a captured selected image representing the largest cross-section of the CL with distinctive coloured areas as described by Ginther and Utt, (2004). Vascular blood perfusion of follicles is estimated considering the coloured Doppler signals from the vessels of the follicular wall (Siddiqui *et al*., 2010). The entire follicle must be scanned several times in a slow and continuous motion (Ferreira *et al*., 2011).

2.4 Contrast-enhanced Ultrasonography

It is well known that the presence of gas bubbles *in vivo* and particularly in the bloodstream can be highly undesirable as this could lead to some pathologic conditions such as air emboli (Chappell and Payne, 2006). However, there are numerous numbers of biomedical applications of gas bubbles particularly in the context of diagnostic and therapeutic ultrasound (Stride and Coussio, 2009). Gas bubbles could be formed *in vivo* by injection into the vein in form of suspension of stabilized micro bubbles or liquid droplets, which subsequently vaporize to form bubbles. These types of micro bubbles produced have been in clinical use for more than two decades as contrast agents for ultrasound imaging (Schlief, 1991; Cosgrove, 2006). Pressure reduction in a given region could also be used to

generate gas bubbles (Stride and Coussio, 2009). Since the solubility of a gas in a liquid falls with pressure, reducing the pressure would drive the gas out of solution (Stride and Coussio, 2009). Similarly, if the pressure falls below the liquid vapour pressure, vapour-filled cavities or bubbles would also be formed. Further reductions in pressure would promote further diffusion of gas and/or vapour into existing bubbles and also growth of the bubbles according to the appropriate gas law (Stride and Coussio, 2009). More so, bubbles can also be formed as a result of an increase in temperature (Stride and Coussio, 2009). It is known that the solubility of most gases falls with increasing liquid temperature while the vapour pressure increases, in both cases promoting the formation and growth of gas- and/or vapour-filled cavities (Stride and Coussio, 2009). The formation and growth of bubbles in tissue or *in vivo* as a result of ultrasound exposure normally involves a combination of changes in both pressure and temperature (Stride and Coussio, 2009).

2.4.1 The use of bubbles in ultrasound imaging and therapy

Ultrasound is the propagation of a pressure disturbance through a medium at a particular frequency, and the pressure at a given location would therefore fluctuate at that frequency (Stride and Coussio, 2009). Similarly, part of the energy carried by the propagating wave was converted into heat by viscous absorption, resulting in a local temperature rise. The formation of and subsequent interaction between bubbles and ultrasound are the key to their exploitation in diagnostic and therapeutic applications (Stride and Coussio, 2009).

Carbon dioxide micro bubbles were used as ultrasound contrast (Kitano *et al*., 2011). It required an angiographic technique that was relatively invasive because the carbon dioxide micro bubbles must be selectively infused into the celiac artery or superior mesenteric

artery (Kitano *et al*., 2011). In a preliminary study using the intravenous ultrasound contrast agent, Albunex® (Shionogi, Osaka, Japan), air-filled micro bubbles stabilized with a thin shell of human albumin and showed that ductal adenocarcinomas were less enhanced than the normal pancreatic parenchyma in contrast enhanced Echo Ultrasound Scan (EUS) after the intravenous infusion of the contrast (Hirooka *et al*., 1997).

There are several reports on the utility of contrast-enhanced EUS with the Doppler mode for diagnosing lesions in esophagus, stomach, gallbladder, pancreas and lymph nodes (Reddy *et al*., 2011). Particularly, power Doppler EUS with or without contrast agents depicted most adenocarcinomas in the pancreas as a hypovascular tumor (Kitano *et al*., 2011). The contrast-enhanced EUS using this carbon dioxide gas depicts ductal carcinomas as hypovasculsr masses whereas it depicts chronic pancreatitis and neuroendocrine tumors as isovascular and hypervascular masses, respectively (Kato *et al*., 1995). Several limitations in contrast enhanced Doppler ultrasonography include blooming artefacts, poor spatial resolution, low sensitivity to slow flow and high sensitivity to motion artefacts (Kudo, 2003; Whittingham, 2005)

2.4.2 The basic principles of micro bubbles as echo contrast agents

The micro bubbles act as echo-enhancers by basically the same mechanism as that determining echo-scattering in all the other cases of diagnostic ultrasound due to the fact that the backscattering echo intensity is proportional to the change in acoustic impedance between the blood and the gas making the bubbles (Calliada *et al*., 1998). The different acoustic impedance at this interface is very high and in fact all of the incident sound is reflected, even though not all of it travels back to the transducer (Calliada *et al*., 1998).

Reflectivity is proportional to the fourth power of a particle diameter and directly proportional to the concentration of the particles themselves (Calliada *et al*., 1998).

2.4.3 Types of micro bubble agent

In the late 1960s, Dr Claude Joyner found that micro bubbles could be used as ultrasound contrast agents while conducting a study on cardiac output by periodically injecting indocyanine green dye into the patient's left ventricle and simultaneously performing an M-mode echocardiogram (Feigenbaum *et al*., 1970). He observed that each injection of dye produced a temporary increase in the ultrasound echo from the ventricle. Initially it was thought that the contrast enhancement was due to the nature of the dye, but further investigation by Gramiak and Shah (1968) and Kremkau *et al*. (1970) showed that formation of gas bubbles at the catheter tip was responsible for the observed contrast enhancement. Since then, series of the contrast agents were developed for use in ultrasonography for diagnostic and therapeutic use.

*2.4.3.1. AlbunexH (Mallinckrodt Inc., Hazelwood, MO, USA**):*** This consists of air micro bubbles coated with a thin stabilizing layer of cross-linked human serum albumin. Although Albunex micro bubbles were stable compared to uncoated bubbles, they were unable to provide prolonged contrast enhancement *in vivo* hence, newer agents containing higher-molecular-weight gases were consequently developed (Stride and Coussio, 2009).

*2.4.3.2. OptisonTM (GE Healthcare Inc., Princeton, NJ, USA)**:***It contains perfluoropropane with a relative molecular mass approximately six times that of air (188 as opposed to 29), resulting in lower diffusion rate of the gas across the bubble wall (Stride and Coussio, 2009).

*2.4.3.3. EchovistH and LevovistH (Schering AG, Berlin, Germany):*They contain suspensions of galactose microcrystals, which dissolved in the blood following injection, thereby releasing air micro bubbles from defects on the crystal surfaces (Stride and Coussio, 2009). Levovist also contained palmitic acid to provide additional stability. Levovist micro bubbles were found to be more echogenic than either Albunex or Optison, but considerably less stable because of higher diffusion rate of their surfactant coatings (Stride and Coussio, 2009). Hence, Levovist was soon superseded by agents like SonoVueH (Bracco International BV, The Netherlands), DefinityH (Bristol- Myers Squibb Medical Imaging Inc., USA) and SonozoidTM (GE Healthcare Inc., Princeton, NJ, USA) containing bubbles stabilised by phospholipid monolayers which had a better compromise between longevity and echogenicity (Stride and Coussio, 2009).

*2.4.3.4. EchoGenH (Sonus Pharmaceuticals Inc., Bothell, USA):*This consists of surfactant-coated droplets of perfluoropentane that have a boiling point coinciding with normal body temperature (37 ºC) (Rapoport *et al*., 2007).

2.4.3.5. Echogenic liposomes: This consists of phospholipid bilayers encapsulating a mixture of liquid and gas (Tiukinhoy *et al*., 2004; Huang, 2008). These are also more stable than monolayer-coated micro bubbles and are particularly attractive for drug delivery applications, as larger quantities of either aqueous or non-aqueous material can be encapsulated. Larger doses of echogenic liposomes are required to obtain equivalent levels of contrast enhancement during imaging, on account of their lower gas content per particle, but such high concentrations are well tolerated physiologically (Hamilton *et al*., 2002). Similarly, specific pulse regimes are required to initiate drug release (Smith *et al*., 2007).

In terms of scattering efficiency, proportion of the incident field is scattered rather than absorbed (Coussios *et al*., 2004). However, it has been reported that the acoustic properties of echogenic liposomes may be superior to those of micro bubbles (Coussios *et al*., 2004).

2.4.4 Use of micro bubbles as quantitative imaging

In general, micro bubbles would remain in the vascular system and potentially therefore can be used as a means of quantifying tissue perfusion and other physiologically relevant parameters such as relative vascular volume and flow velocity (Stride and Coussio, 2009). These measurements are particularly significant for examination of myocardial function, kidney, and tumour vasculature (Stride and Coussio, 2009). Flash replenishment imaging is the most commonly employed technique whereby the micro bubbles in the image plane are initially destroyed by a high-amplitude ultrasound 'release burst', and the rate at which the image plane is replenished with bubbles is then monitored in real time using low amplitude pulses. Fitting the results to an appropriate flow model provides estimates for the blood volume and tissue perfusion (Schlosser *et al*., 2001; Metoki *et al*., 2006).

2.4.5 Using micro bubbles to target tissues and molecular imaging

Some commercial contrast agents such as Levovist have been found to have a tissue (liver and spleen) specific late phase (Albrecht *et al*., 2000). Targeted imaging and therapy of tissues requires some form of functionalization of the microbubble surface. Micro bubbles coated with material carrying a charge have been shown to preferentially locate inflamed tissues (Lindner, 2002), but a more effective method is to attach ligands to the bubble surface that would bind to receptors on particular types of cell (Lindner, 2002). Contrast agents has been used as targetings to activated leucocytes by incorporating

phosphatidylserine in the microbubble coating (Lindner *et al*., 2000), angiogenic markers (Leong-Poi *et al*., 2003; Emoto *et al*., 2007) and antibodies attachments (such as anti-P-selectin monoclonal antibody, anti- ICAM antibody, anti-VCAM antibody) to detect receptors that is expressed during inflammation (Song *et al*., 2001; Lu *et al*., 2004; Takalkar *et al*., 2004).

2.4.6 Therapeutic applications of contrast agents

2.4.6.1 Bubbles as delivery vehicles: There has been increasing interest in the use of micro bubbles for drug and gene delivery. Micro bubbles represent excellent vehicles for this type of application as drugs or DNA can be incorporated into the microbubble coating, traced through the body using low-intensity ultrasound, and then released by destroying the micro bubbles with high-intensity ultrasound at a target site such as a tumour (Stride and Coussio, 2009). The ability of ultrasound to be tightly focused into small tissue volumes ($5mm^3$) provides a physical means of localizing microbubble activity (Stride and Coussio, 2009). With some polymeric micro bubbles it is possible to dissolve or disperse the drug directly in the coating, provided that it is of sufficient thickness for the required dose (Unger *et al*., 1998; Lentacker *et al*., 2006). The drug is released as the coating breaks down when the microbubble oscillates at sufficient amplitude (Stride and Coussio, 2009).

2.4.6.2 Microjetting: In addition to providing a means of encapsulating therapeutic material, there are a number of physical phenomena produced by microbubble oscillations which may contribute to the therapeutic effect by enhancing the rate of uptake of drugs/DNA by cells, and/or increasing the rate of erosion or denaturing of diseased tissue (Stride and Coussio, 2009). The formation of microjets is an example of one of these. It

has been shown *in vitro* that a microjet can easily puncture a cell membrane and this effect has been observed extensively with contrast agent micro bubbles near cell monolayers (Prentice *et al*., 2005). It has been suggested that microjetting could potentially be the cause of the enhanced cell uptake generated by micro bubbles (Prentice *et al*., 2005).

2.4.6.3 Microstreaming: When a sound wave propagates through a liquid, steady currents are set up in the direction of the beam as a result of momentum transfer from the wave to the liquid (Lighthill, 1978; Hill *et al*., 2004). This acoustic streaming also occurs, albeit on a much smaller scale, around micro bubbles undergoing stable oscillations (Elder *et al*., 1954; Elder and Nyborg, 1956). These eddying flows may, in turn, impose shear stresses on nearby surfaces, such as cell membranes, and it is thought that this may promote the uptake of therapeutic components (Marmottant and Hilgenfeldt, 2003; Marmottant *et al*., *et al*., 2008). Microstreaming has also been shown to cause significant damage to cells at higher amplitudes of oscillation (Clarke *et al*., 1970; Rooney, 1970; Ward *et al*., 1999). In addition, microstreaming would contribute to circulating therapeutic agents in the target region, which is also likely to be important in the context of both sonothrombolysis and drug uptake (Prokop *et al*., 2007).

*2.4.6.4 Heating enhancement:*The absorption of energy as ultrasound propagates through a medium also produces a heating effect. In most materials, including tissue, the rate of absorption increases with frequency and thus the presence of bubbles can enhance this heating effect owing to their ability to generate higher harmonics of the excitation frequency (Hill *et al*., 2004). Bubbles also dissipate energy as heat, both as a result of viscous friction in the surrounding liquid and any coating material, and via conduction

during compression (Stride and Coussio, 2009). The relative significance of each of these dissipation mechanisms depends on the size of the bubble, the physical properties of the surrounding liquid, and the frequency and intensity of the ultrasound field (Hilgenfeldt *et al*., 2000; Coussios *et al*., 2008). Similarly, the magnitude of the temperature rise generated also depend on these parameters, as well as on the concentration of bubbles present, the pulse repetition frequency/ duty cycle and proximity to blood vessels (Coussios *et al*., 2007; Hariharan *et al*., 2007).

Large temperature rises are clearly beneficial for tissue ablation, certain types of drug and gene delivery where thermal activation is required (Plathow *et al*., 2005; Liu *et al*., 2006). In diagnostic imaging and where therapeutic agents are temperature sensitive, however, significant heating is normally undesirable, and the relevance of safety indices (e.g. thermal index) for applications employing micro bubbles require further investigation.

2.4.6.5 Chemical effects: The large and rapid reduction in volume experienced by a bubble undergoing inertial collapse can produce a significant rise in pressure and temperature (Kim *et al*., 2000a). These extreme conditions are confined to the centre of the bubble (Unger *et al*., 1998), but highly reactive chemical species may be produced within this space (Suslick, 1990). Of particular interest in the context of medical ultrasound is the potential for the formation of free radicals and toxic chemicals such as hydrogen peroxide (H_2O_2), which may be harmful to cells. For example, Juffermans *et al*. (2008) have shown that rat cardiomyoblast cells experience a calcium ion (Ca^{2+}) influx upon exposure to low-intensity ultrasound with SonoVue, causing localized hyperpolarisation of the cell

membrane, which may promote molecular uptake and this may also be related to the generation of H_2O_2 despite the much lower ultrasound intensities.

Another application undergoing exciting developments is sonothrombolysis, where micro bubbles have been shown to increase markedly the effectiveness of tissue plasminogen activator (t-PA) and the rate of clot lysis *in vitro* and *in vivo* (Culp *et al*., 2004; Molina *et al*., 2006; Datta *et al*., 2008). More so, there is considerable evidence that micro bubbles increase the permeability of not only individual cell membranes but also the endothelium (van Wamel *et al*., 2006; Iwanaga *et al*., 2007) including temporary opening of the blood–brain barrier (Meairs and Alonso, 2007).

2.5 Contrast Harmonic Imaging

This detects signals from micro bubbles and filters signals originating from tissue, by selectively detecting the harmonic components (Kudo, 2003; Whittingham, 2005). This technology can detect signals from micro bubbles in vessels with very slow flow without Doppler-related artefacts and is used to characterize tumor vascularity in liver, pancreas, gallbladder and the gastrointestinal tract during transabdominal ultrasonography (Kitano *et al*., 2004; Fukuta *et al*., 2005; Inoue *et al*., 2007 Kersting 2009). Until recently, a contrast harmonic imaging technique was not available for EUS because the transducer of the echoendoscope was too small to produce enough acoustic power for contrast harmonic imaging using the first generation ultrasound contrast agents like Levovist® (Schering AG, Berlin, Germany) (Kitano *et al*., 2011). Second generation ultrasound contrast agents like SonoVue® (Bracco Imaging, Milan, Italy) and Sonazoid® (Daiichi-Sankyo, Tokyo, Japan; GE Healthcare, Milwaukee, WI, USA) produce harmonic signals at lower acoustic powers

(Kudo, 2003; Whittingham, 2005) and are therefore suitable for EUS imaging at low acoustic powers (Kitano *et al.*, 2011).

2.5.1 Principle of contrast harmonic imaging

Ultrasound contrast agents are micro bubbles of gas covered by a shell of biocompatible material such as a lipid (Kudo, 2003; Whittingham, 2005). When exposed to the ultrasound beams, the micro bubbles are disrupted or resonated, which releases a large amount of harmonic signals (Kudo, 2003; Whittingham, 2005). When the tissue and the micro bubbles receive transmitted ultrasound waves, both produce harmonic components that are integer multiples of the fundamental frequency, but the harmonic content from the micro bubbles is higher than that from the tissues (Kitano *et al.*, 2011). Selective depiction of the second harmonic component visualizes the signals from the micro bubbles more strongly than those from the tissue. In addition, signals from the contrast agents vary greatly in phase with no relation to its motion when ultrasound pulses are transmitted multiple times successively (Kitano *et al*., 2011). However, signals are hardly changed between multiple transmissions in the part without the contrast agent (Kitano *et al*., 2011). The extended pure harmonic detection mode equipped with the EUS system specific for contrast harmonic imaging receives not only the second harmonic components but also signals produced by the relative phase shifts of the received signals by transmitting and receiving ultrasound beams multiple times (Kitano *et al*.,2008a and b). This processing is capable of enhancing the imaging of signals from contrast agents (Kitano *et al*.,2008a and b).

2.5.2 Second harmonic imaging

The micro bubbles reached by an ultrasound signal resonate with a specific frequency depending on microbubble diameter (Calliada *et al*., 1998). However, the main resonance frequency is not the only resonance frequency of the bubble itself and multiple frequencies of the fundamental one are emitted, just like in a musical instrument (Calliada *et al*., 1998). These harmonic frequencies have decreasing intensity, but the second frequency, known as the second harmonic, is still strong enough to be used for diagnostic purposes (Calliada *et al*., 1998). The theoretical advantage of the harmonic over the fundamental frequency is that only contrast agent micro bubbles resonate with harmonic frequencies, while adjacent tissues do not resonate, or else their harmonic resonation is very little (Calliada *et al*., 1998). Thus, using a unit especially set to produce ultrasounds at a given frequency (3.5 MHz) and receive an ultrasound signal twice as powerful (7 MHz). Moreover, second harmonic imaging permits to show extremely small vessels (down to 40µ) with very slow flow, which was missed with a conventional method (Calliada *et al*., 1998).

2.5.3 Ultrasound tissue harmonic imaging (THI)

In THI nonlinear distortion of the transmitted frequencies within the body is useful for producing a shaper, higher-contrast ultrasound image than that of the fundamental frequency (Ward *et al*., 1997; Hamilton and Blackstock, 1998; Tranquart *et al*., 1999; Desser and Jeffrey, 2001). This is due to its improved conspicuity and border definition hence; it is widely used for detecting subtle lesions (as in thyroid and breast) and visualizing technically challenging patients with high body mass index (Kornbluth, 1998; Shapiro *et al*., 1998; Szopinski *et al*., 2003a; Szopinski *et al*., 2003b). However, compared

to conventional ultrasound imaging, THI suffers from the low signal-to-noise ratio (SNR), resulting in limited penetration depth (Rosenthal *et al*., 2001; Hohl *et al*., 2004; Song *et al*., 2010).

The SNR in THI can be substantially increased by utilizing the recently-developed coded excitation techniques, i.e., coded tissue harmonic imaging (CTHI) (O'Donnell, 1992; Li, 1999; Phillips *et al*., 2001). In CTHI, similar to conventional coded excitation, specially-encoded ultrasound signals (e.g., Barker, Golay and chirp) are transmitted, and then backscattered receive signals containing fundamental and harmonic frequencies are selectively decoded via pulse compression (Takeuchi, 1996; Chiao and Hao, 2005; Song *et al*., 2010). In THI, various methods, such as band pass filtering (BF), power modulation (PM) and pulse inversion (PI), have been proposed to separate the second harmonic signal from the receive signal (Takeuchi, 1996; Averkiou and Hamilton, 1997; Jiang *et al*., 1998; Hwang and Simpson, 1999; Chiao and Hao, 2005). It has been known that CTHI, compared to conventional THI, requires higher rejection of the fundamental and third harmonic components since they may introduce range side lobe artefacts due to incomplete decoding during pulse compression (Kim *et al*., 2000b; Arshadi *et al*., 2007).

2.5.4 Future perspective of contrast-enhanced harmonic EUS

The technique of contrast-enhanced EUS has been used for the evaluation of vascularity (Kitano *et al*., 2011). However, some studies showed this technique can be applied for molecular imaging in the future (Palmowski *et al*.,2008; Willmann *et al*., 2008). The feasibility of new technologies using contrast-enhanced ultrasound with micro bubbles

targeted to vascular endothelial growth factor receptor type 2 are currently being tested (Kitano *et al*., 2011).

2.6 Other Imaging Modalities

There have been significant advances in recent years in the use of diagnostic imaging to evaluate pregnant bitches (Garcia *et al*., 2015). Ultrasound is the modality of choice for pregnancy diagnosis and assessment of foetal viability in small animals (Davidson and Baker, 2009). However, ultrasound has poor accuracy in determining the number of intrauterine foetuses (Root and Spaulding, 1994). Abdominal radiography provides a more accurate assessment of foetal numbers and is thus still the main technique used for counting foetuses (Rendano, 1983; Rendano *et al*., 1984; Toal *et al*., 1986; Lopate,2008; Garcia *et al*., 2015). Once foetal skulls have mineralized they can be counted on radiographs and provide a precise method of determining foetal numbers (Toal *et al*., 1986; Gradial *et al*., 2000). Studies have shown that the best time to perform radiographic examination to assess the number of foetuses in bitch is between 54 and 57 days of gestation when clear visualization of the foetal skull is possible using digital radiography (Meyer-Lindenberg *et al*., 2008; Garcia *et al*., 2015).

Ultrasonography is an important technology for understanding the status of the female reproductive organs (Maeda *et al*., 2016). However, ultrasonography has disadvantages in that imaging accuracy varies with the proficiency of the examiner and not everyone can observe the activity of the ovaries (Leonhardt *et al*., 2014). Moreover, the observable field of ultrasonography is narrow, and it is possible that only limited information is obtained (Leonhardt *et al*., 2014).

2.6.1 Advanced diagnostic imaging (Computed Tomography, Magnetic Resonance Imaging)

With increasing availability, the use of cross-sectional imaging modalities (computed tomography [CT]; magnetic resonance imaging [MRI]) for diagnostic imaging of the abdomen including the lower urinary tract in small animals is likely to increase (Hecht, 2015). Initial applications described include the use of CT excretory urography to evaluate the ureterovesicular junction in patients with suspected ureteral ectopia and the use of either CT or MRI to evaluate intrapelvic lesions (Rozear and Tidwell, 2003; Samii *et al*., 2004; MacLeod and Wisner, 2011; Schwarz, 2011). Both CT and MRI allow excellent anatomic delineation and are useful in assessing origin and extent of intrapelvic masses, identifying lesions associated with the pelvic urethra such as wall thickening or calculi, and evaluating surrounding structures including the spine and regional lymph nodes for the presence of additional lesions like metastatic disease (Hecht, 2015). CT has proven superior to ultrasound in accurately measuring the size of bladder wall masses (Naughton *et al*., 2012). CT and MRI are useful in planning a surgical approach for intrapelvic lesions and may be needed if radiation therapy planning is to be pursued for pelvic canal neoplasia (Hecht, 2015).

Magnetic resonance imaging (MRI) uses the phenomenon of nuclear magnetic resonance, and information taken *in vivo* is converted into images (Maeda *et al*., 2016). Magnetic resonance imaging renders images of soft tissue with excellent contrast (Maeda *et al*., 2016). It has been used for observing normal ovaries and diagnoses of tumors (Nothling *et al*., 2006; Stephenson *et al*., 2012), and can produce images that are not affected by the skill of the examiner and allows observation not only of the ovaries but also of the female

reproductive organs overall (Leonhardt *et al*., 2014; Maeda *et al*., 2016). In MRI examination the ovarian follicles were distinctly seen as hyper intense circular structural images of about 10 mm within the abdominal cavity (Maeda *et al*., 2016). While the ovarian position in the abdominal cavity were confirmed. In addition, it was also possible to estimate the general size of the uterus (Maeda *et al*., 2016).

However, MRI has disadvantages in observing the ovaries, one being that the ovaries cannot be observed in real time (Leonhardt *et al*., 2014), and another is that with the open type MRI system the resolution is not particularly high, hence the detailed ovarian structure cannot be observed (Maeda *et al*., 2016). However, it has been reported that even mouse ovarian follicles can be observed using 11.7-T MRI (Stephenson *et al*., 2012).

2.7 Application of Ultrasound in Pregnant Bitches

2.7.1 Doppler ultrasound scanning evaluation of uterine blood in bitches

In dogs, ultrasonographic evaluation of the uterus provides valuable information to diagnose uterine diseases (Batista *et al*., 2016). The thickness of the uterine walls and the presence of luminal contents can be determined, allowing differentiation between pregnancy and pathologic conditions (Bigliardi *et al*., 2004; Davidson and Baker, 2009). The echogenicity of the uterine contents suggests cellularity, whereas, it is difficult to differentiate the content using ultrasound (Nyland and Mattoon 2002; Bigliardi *et al*., 2004; Davidson and Baker, 2009).

Doppler ultrasonography is a non-invasive technique that provides information about the organs perfusion (Fielder and Baker, 1969). This technique has been found to be a useful tool to diagnose numerous gynaecologic lesions in several species (Emoto *et al*., 2002;

Özbay and Deveci, 2011; Heppelmann *et al*., 2013). In women and cows, uterine pathologic conditions may cause a blood flow increase that can be detected by Doppler ultrasound (Batista *et al*., 2016). In dogs, this technique has been used to evaluate uterine arteries blood flow during oestrus, normal and abnormal pregnancy, and puerperium (Blanco *et al*., 2011; Batista *et al*., 2013; Freeman *et al*., 2013). Although the characterization of uterine blood flows by Doppler ultrasound probably contribute to the precocious diagnosis of cystic endometrial hyperplasia and pyometra (Batista *et al*., 2016).

Although normal bitches presented a significantly higher resistance Doppler wave form of the uterine arteries, studies revealed that Doppler ultrasonography can accurately reflect hemodynamic changes that are present in bitch patients with different uterine pathologic conditions (Batista *et al*., 2016). In pathological condition such as cystic endometrial hyperplasia and pyometra, uterine blood flow was higher than in normal animals. Uterine infection in women was also associated with high uterine blood flow (Alatas *et al*., 1996; Özbay and Deveci, 2011). Resistivity index (RI) is higher in normal diestrous bitches than in pathologic conditions such as cystic endometrial hyperplasia and pyometra (Batista *et al*., 2016) whereas England *et al*. (2012) reported that it was lower in normal diestrous bitches.

During an inflammatory response, there is vasodilation leading to increased blood flow and angiogenesis are present (Özbay and Deveci, 2011). Hence uterine inflammation associated with an infectious process may lead to a diminution of RI values of uterine arteries (Özbay and Deveci, 2011). Furthermore, a previous study in bitches with pyometra reported an increase in the intrauterine concentration of prostaglandin E, which may also

lead to an increase in uterine perfusion (Heap and Poyster, 1975; Still and Greiss, 1978). In addition to prostaglandins, nitric oxide, a potent vasodilator, is also released locally in several mammalian species during endometrial inflammation (Rosselli *et al*., 1998; Fang *et al*., 1999). The low vascular resistance detected by Doppler ultrasonography in pathologic condition can be used to support the diagnosis of canine pyometra (Batista *et al*., 2016).

Doppler ultrasound has been found to be a useful tool to evaluate response to medication (Tinkanen and Kujansuu, 1993). The possibility to follow up the evolution of treated patients could improve the clinical outcome, thus avoiding unnecessary surgery and preserving the reproductive tract in valuable bitches (Batista *et al*., 2016).

2.7.2 Indication for ultrasonography during parturition

Ultrasonographic foetometry has great potential, but the huge morphological and size variability in dogs would require breed-specific foetal growth curves to increase the accuracy of this method (Kutzler *et al*. 2003b; Socha and Janowski 2014). At presentation, in the absence of previous monitoring, ultrasound examination can help the clinician to date pregnancy through measuring some foetal parts such as the biparietal (BP) diameter in the second half of pregnancy (Lopate 2008). This method is relatively simple but breed-specific foetal growth curves are necessary to increase its accuracy (Kutzler *et al*. 2003b; Socha and Janowski 2014).

Even when using a specific equation for a single breed, some variables, such as smaller than average litter size, can greatly reduce the accuracy of the prediction (Groppetti *et al*. 2015b). In small and medium size breeds, approximately 70% of parturition dates were predicted within 1 day and 85% within 2 days using BP (Beccaglia and Luvoni 2006).

Neither of the two methods alone, that is progesterone concentration and ultrasound foetometry appears to be accurate enough to effectively help the clinician, it could be worth to test the association of the two determinations in order to assess whether the diagnostic efficacy becomes higher than with a single one (Rota *et al*., 2015).

2.7.3 Appearance of embryonic/foetal structures using ultrasonographic or radiographic examination

Determination of gestational age has been attempted by both ultrasonographic and radiographic examination through the identification of the degrees of foetal mineralization and the first appearance of different foetal skeletal structures (Haney *et al*., 2003; Rendano, 1983; Rendano *et al*., 1984). The mineralization of structures occurs earlier in cats than in dogs (Haney *et al*., 2003; Lopate, 2008) in which the bone structures characterized by the narrowest period of mineralization are the humerus at 20 ± 1 day (range between 20 and 24 days) and femur at 21 ± 1 day (range between 19 and 23 days) prior to parturition (Boyd, 1971). Some other bones, like fibula, calcaneus and phalanges, mineralize only after birth (Beccaglia *et al*., 2016).

A single lateral radiograph of the abdomen is usually sufficient for pregnancy diagnosis and foetal counting, but a ventro-dorsal radiograph is more suitable to evaluate gestational age although with the aforementioned limitations (Rendano, 1983; Rendano *et al*., 1984., Concannon, 2000;).

2.7.4 Ultrasonographic measurement of extra-foetal or foetal structures

Ultrasonographic measurement of extra-foetal and foetal structures is a common and accurate method for the prediction of delivery date during pregnancy even when the time

of ovulation is unknown (Beccaglia *et al*., 2016). During the first half of gestation (between 19 and 37 days after LH peak), measurements of gestational sac diameter (inner chorionic cavity, ICC or outer uterine diameter, OUD) or foetal crown-rump length (CRL) can easily be obtained. In the second half of pregnancy (after 37 days), foetal measurements of the biparietal diameter (BP) and body diameter (BD) can be used to predict the delivery date (Shille and Gontarek, 1985., England *et al*., 1990; Luvoni and Grioni, 2000; Luvoni and Beccaglia, 2006; Lopate, 2008; Michel *et al*., 2011).

From days 35 to 58, the deep portion of the foetal diencephalo-telencephalic vesicle (DPTV) can be recognized as a symmetric anechoic area viewed on sagittal midline of the foetal head and its measurement could be useful to determine the gestational age (Beccaglia *et al*., 2008; Beccaglia and Luvoni, 2004). An accurate gestational timing in the first and second half of pregnancy is provided by ICC and BP parameters, respectively (Kutzler *et al*., 2003b; Luvoni and Beccaglia, 2006; Lopate, 2008; Michel *et al*., 2011).

2.7.5 Ultrasonographic evaluation of heart rate or foetal flux

In human medicine, it is known that a variation in foetal heart rate (FHR) is connected to antepartum uterine contraction (Nageotte, 2015). In the canine species, it has been reported that FHR should be greater than 220 beats per minute (bpm), with FHR between 180 and 220 bpm indicate moderate foetal distress and values less than 180 bpm indicate severe foetal distress (Zone and Wanke, 2001). It has been proved that when umbilical artery resistivity index (RI) is less than 0.7 in all foetuses, the delivery would occur within 12 hr. As foetal distress could affect this value, the FHR variations should be measured at the same time (Giannico *et al*., 2015; Beccaglia *et al*., 2016).

2.7.6 Use of B-mode ultrasonography for foetal sex determination in dogs

Gestational ultrasonography is used routinely in prenatal monitoring in women and in some animal species (Curran, 1992; Meagher and Davison, 1996; Lopate 2008; Davidson and Baker, 2009; Lamm and Makloski, 2012). It is used to confirm pregnancy, identify congenital foetal defects, assess placental morphology and blood flow, evaluate uterine fluid and endometrial edema or inflammation, identify the presence of cystic endometrial hyperplastic changes, evaluate foetal viability and development, and aid in the identification of foetal distress (Aissi and Slimani, 2008; Lopate 2008; Davidson and Baker, 2009; Lamm and Makloski, 2012). In veterinary medicine, it has been shown that ultrasonographic determination of intrauterine sex is possible in cats, cows, buffaloes, mares, and sheep (Bürstel *et al*., 2002; Mar *et al*., 2002; Holder, 2003; Tainturier and Tainturier, 2004; Zambelli and Prati, 2006; Yotov *et al*., 2011) and this has economic relevance (Gil *et al*., 2015).

Sexual differentiation in canine foetuses starts from the 30th day of gestation and continues until about the 50^{th} day (Pretzer, 2008; Evans and Lahunta, 2013). Examination of the first pregnant bitch every 4 days showed that between 55 and 58 days of gestation, foetuses were larger and foetal organogenesis was complete, making this the ideal age for identification of the genitalia (Evans and Lahunta, 2013; Gil *et al*., 2015).

Ultrasonographic determination of the sex of the female fetus in bitch is similar to that of human in which two or four parallel lines representing the major and minor labia can be identified (Elejalde *et al*., 1985; Whitlow *et al*., 1999; Gil *et al*., 2015). Whereas, the testicles are not seen in male canine foetuses as these migrate into the scrotum after birth

(Baumans *et al*., 1981; Achiron *et al*., 1998). Although the penis develops in the foetal stage, it is not readily visible in gestational ultrasonography in dogs (Gil *et al*., 2015). Again, this characteristic differs from that found in human foetuses because the human foetal penis can be viewed more easily and the testes migrate to scrotum after the 25th week of gestation (Curran *et al*., 1989).

2.7.7 Bidimensional and Doppler ultrasonographic evaluation of postpartum uterine involution

Puerperium is a physiological period after parturition in which uterine involution occurs (Blanco *et al*., 2015). Complete uterine involution is essential for the establishment of future gestations, as failure of normal uterine regression could lead to several diseases such as postpartum metritis and retained placenta in many mammalian species (Hirt *et al*., 2000; Magata *et al*., 2013). Ultrasonographic evaluation of the postpartum uterus is useful for monitoring its normal involution and for the early diagnosis of disorders (Blanco *et al*., 2015). In bitches, postpartum metritis is differentiated from the normal postpartum uterine enlargement by the failure of normal progressive decrease in uterine lumen contents and horn width (Davidson and Baker, 2009). The uterine wall can also be thickened or irregular in these cases (Grundy and Davidson, 2004).

Doppler ultrasound has been used to assess postpartum uterine involution in women, cows, mares, and bitches (Krueger *et al*., 2009; Mulic-Lutvica *et al*., 2009; Mortensen *et al*., 2011; Batista *et al*., 2013). A decrease in the blood flow of uterine arteries throughout puerperium has been reported in these species. However, Doppler uterine blood flow measurements are altered by puerperal uterine diseases in women and cows, contributing

to the diagnosis of postpartum disorders (Mulic-Lutvica *et al*., 2009; Heppelmann *et al*., 2013; Magata *et al*., 2013). A marked decrease in uterine blood flow during the first postpartum week has also been described in bovine and canine puerperium (Krueger *et al*., 2009; Batista *et al*., 2013). However, it has been reported that clinical evidence of involution disappeared before the ultrasonographic evidence of involution in women, cows, mares, and bitches (Krueger *et al*., 2009; Mulic-Lutvica *et al*., 2009; Mortensen *et al*., 2011; Batista *et al*., 2013).

Queen seems to have more rapid postpartum uterine involution than bitch (Walter and Schönkypl, 2006). Study revealed that canine and feline uterine ultrasonographic appearance became indistinguishable from that of anoestrus on Day 52and 18 respectively after parturition (Batista *et al*., 2013). The rapid involution in the feline is due to histologic differences between both species (Dall'Aglio *et al*., 2012a; Dall'Aglio *et al*., 2012b). Postpartum uterine diseases are less frequent in cats than in dogs due to the rapidity of feline uterine involution. More so, uterine wall layers after parturition also seem to differ between dogs and cats (Blanco *et al*., 2015). Up to five ultrasonographically distinct layers have been identified in the postpartum canine uterine wall (Yeager and Concannon, 1990), whereas only three layers were observed in feline (Ferretti *et al*., 2000; Blanco *et al*., 2015). Moreover, uterine wall thickness is markedly thinner in cats than in dogs (Chatdarong *et al*., 2005; Orfanou *et al*., 2009).

2.8 Oestrus Induction in Canine Patients

Both LH and FSH appear to be follicotrophic in the dog, so that administration of either LH or FSH induces oestrus. However, protocols mimicking the natural, gradual increase in

LH and FSH in canine patients have not been successful (Kutzler, 2005). An estrogen peak approximately 30 days before the onset of oestrus is believed to be required to prime the hypothalamuspituitary- ovarian axis, causing release of LH in a pulsating fashion (Shille *et al*., 1984; Jeffcoate, 1993). Successful induction of fertile oestrus in bitches has been accomplished with diethylstilbestrol (DES) in various doses with or without FSH and LH (Bouchard *et al*., 1993; Concannon *et al*., 1997). Other gonadotropins have been evaluated with limited success (Arnold *et al*., 1989; Van Haaften *et al*., 1989; Verstegen *et al*., 1997; Wanke *et al*., 1997). Pregnant mare serum gonadotropin (PMSG) has been effective, but is associated with premature luteal failure, shortened dioestrus, and pregnancy loss (Jones *et al*., 1973; Nakao *et al*., 1985; Weilenmann *et al*., 1993). Protocols using estrogens for oestrus induction in bitches typically also include FSH or PMSG for folliculogenesis and human chorionic gonadotropin (HCG) or LH for induction of ovulation (Kutzler, 2007).

Prolactin inhibition also plays a role in interestrous intervals and is believed to have effects on gonadotropin release and ovarian response to gonadotropins (Wiebe and Howard, 2009). Suppression of prolactin release by dopamine agonists (bromocriptine, cabergoline) shortens the duration of anoestrus and induces oestrus in animals with prolonged anoestrus (Arbeiter *et al*., 1988; Jochle *et al*., 1989). The dopamine agonist, bromocriptine, was the first prolactin inhibitor studied in dogs for oestrus induction, but resulted in significant vomiting in dogs (Van Haaften *et al*., 1989; Zoldag *et al*., 2001).

A newer agent, cabergoline (Dostinex®), has been shown to induce oestrus in most bitches (0.005 mg/kg orally daily, until 3-8 days after onset of proestrus or day 40) with fewer side effects (Kutzler *et al*., 2002). Cabergoline is only available as a 0.5-mg tablet that is

inactivated over time in aqueous solutions containing water (Wiebe and Howard, 2009). For accurate dosing of small animals, tablets can either be compounded into the appropriate- strength capsules or a portion of the tablet can be crushed and diluted with fluid just before dosing (Wiebe and Howard, 2009). McLean and co-workers also report that cabergoline is stable for 28 days if compounded in acidic fluids (1% acetic acid solution) (McLean *et al*., 2007).

GnRHs have also been shown to be very effective if given in pulsated fashion (0.2-0.4 µg/kg every 90-minute intervals intravenously [IV] or subcutaneously [SC] for 3-9 days) to mimic the natural cycle, resulting in LH peaks at the end of proestrus. Subcutaneous administration has been noted to be more practical, but availability of the canine analog is problematic (Vanderlip *et al*., 1987; Cain *et al*., 1988). However, protocols using native GnRH or its agonists via this technique are not practical for clinic purposes because of the expense, time commitment, and lack of availability of analogs (Wiebe and Howard, 2009). GnRH analogs (lutrelin, deslorelin, leuprolide) can also be administered as long-acting dosage forms via subcutaneous injections, mini pumps, or implants. Both expense and side effects may limit the use of these products. Side effects have included premature luteal failure, shortened dioestrus, and pregnancy loss (Concannon, 1989; Kutzler *et al*., 2001). Long-term use is also associated with pituitary overstimulation, down regulation of GnRH receptors, suppression of LH, decreased progesterone secretion, and decreased luteal responsiveness to LH (Kutzler, 2007).

CHAPTER T HREE

3.0 MATERIALS AND METHODS

3.1. Study area

The study was conducted at the Department of Veterinary Surgery and Radiology, Faculty of Veterinary MedicineAhmadu Bello University, Zaria Kaduna Sate. The dogs were housed in the homes of the hunters in Samaru, Zaria which lies between latitude 10 and 11^0North and longitude 7 and 8^0 East, and situated in the Northern boundary of the sub-humid zone.

3.2. Research considerations

Strict adherence to ehical guidelines such as animal welfare and humane practices was observed with the barest minimal stress inflicted on the animal during the course of handling and insonation as approved by the Ahmadu Bello University Committee on Animal Use and Care with clearance number **ABUCAUC/2016/030.**

3.3. Materials

3.3.1 Experimental animals

Nine (9) matured female Nigerian Indigenous dogs that had whelped at least once were used for the study. They were acclimatized for two weeks, during which period they were vaccinated with antirabies and Distemper, Hepatitis, Leptospirosis, Parainfluenza and Parvoviral enteritis (DHLPP) vacines. They were routinely dewormed using membendazole at 7.5 mg/kg body weight orally daily for 3 consecutive days and with praziquantel at the rate of 5.0 mg/kg body weight once orally. They were bathed with Amitrax® against ectopasites. Other Veterinary services were rendered when indicated. All

dogs were clinically examined and confirmed free from any obvious reproductive and systemic disorders. They were fed once a day and water was provided *ad libitum.*

3.3.2 Consumables

The consumables used in the study included, thermal printing paper, aquasonic gel, razor blades, soap and tissue papers. Others were cabergoline (Dostinex®, By Pfizer), methylated spirit, vaccines, dewormers, syringes, sample bottles and antibiotics (Oxytetracycline LA).

3.3.3 Equipment

Ultrasound machine (Sonostar C5 (R) LaptopUltrasound machine by Xuzhou Palmary Electronics Co. Ltd China) with a 6.5MHz transcutaneous probe, a Sony videographic(R) thermal printer, Ultrasound bed. Other equipment used in this study included an electricity generator (Tiger 1.5HP) and a deep freezer (Skyrun 100L).

3.4 Methodology

3.4.1 Experimental Design

The overview of the Experimental design is as shown in the scheme (Fig 3.1) below. The study was divided into four (4) phases during which blood sampling accompanied every other ultrasound examination of the bitches in the various groups. We hope to achieve all outlined and stated objective of this study following this design. The phases are: Phase I (Acclimatization), Phase II (Induction), Phase III (Gestation), and Phase IV (Postpartum).

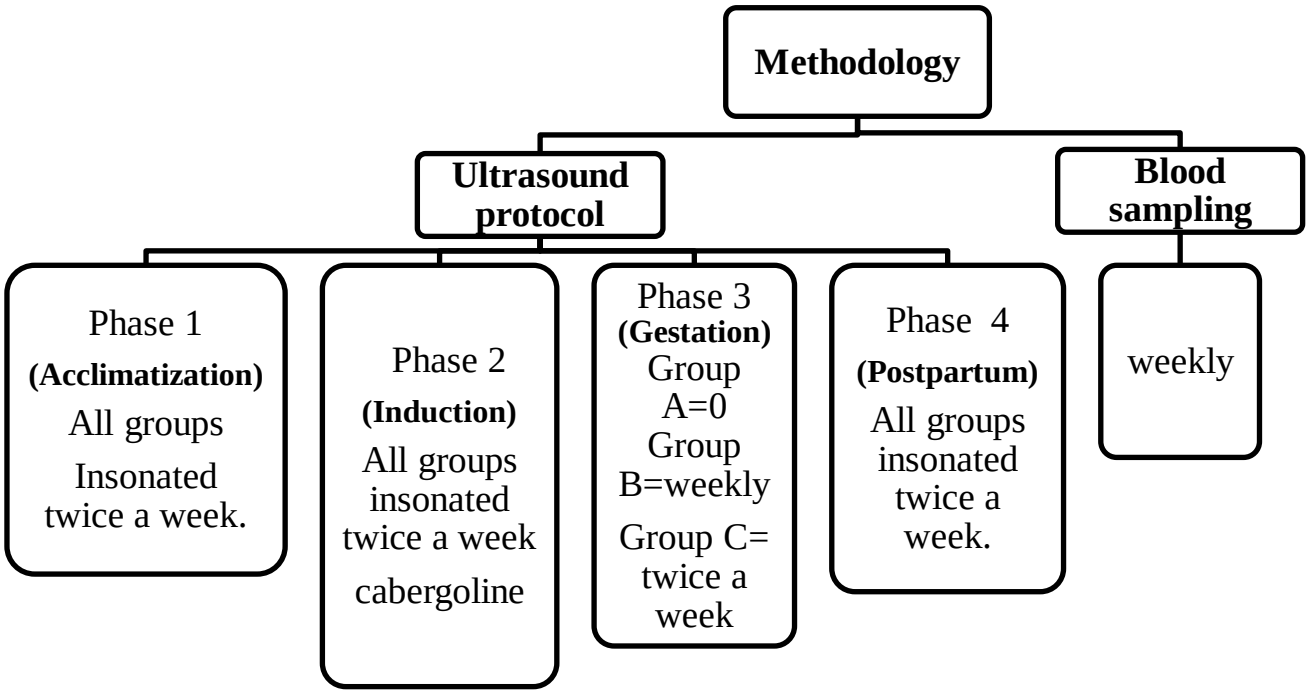


Fig.3.1 shows the overview of the experimental design

3.4.1.1. Phase I (Acclimatization)

This was the 1st phase of the study; it lasted one month and the following activities were carried out:

*a. Sonography***:** During this phase all the bitches had their ventral abdominal areas liberally shaved from the level of the xyphoid cartilage to pubic area. The shaved are were scrubbed, antiseptic applied with the bitch restrained on dorsal recumbency on the examination table. Aquasonic gel applied to the shaved area. Each bitch was then examined sonographically to visualize the internal genitalia of the bitch. This was done to map out the normal sonographic appearance of the reproductive tract. The examination was repeated twice a week for the duration of the phase.

*b. Blood sampling***:** During this phase the bitches were well restrained and blood collected aseptically via cephalic/recurrent tarsal venepuncture. Six millilitre of blood was collected at every sampling period. The first 3 mL were collected in EDTA bottle and was used for haematology and erythrocyte osmotic fragility test, while the remaining 3 mL were placed in anticoagulant free bottles for biochemical analysis. This was done once a week for the duration of the phase.

3.4.1.2 Phase II (Induction and breeding)

This was the 2nd phase of the study; it lasted three weeks and the following activities were carried out:

*a. Sonography***:** During this phase all the bitches had their ventral abdominal areas liberally shaved from the level of the xyphoid cartilage to pubic area. The shaved area was scrubbed, antiseptic applied with the bitch restrained on the examination table. Aquasonic gel was then applied to the shaved area. Each bitch was then examined sonographically to

visualize the internal genitalia. This was to map out the normal sonographic changes of the reproductive tract during the oestrus cycle. The examination was then repeated on each bitch in the study groups twice a week for the duration of the phase.

*b. Blood sampling***:**During this phase the bitches were well restrained and blood collected aseptically via cephalic/recurrent tarsal venepuncture. Six millilitre of blood was collected at every sampling period. The first 3 mL was collected in EDTA bottle and was used for haematology and erythrocyte osmotic fragility test, while the remaining 3 mL was placed in anticoagulant free bottles for biochemical analysis. This was done once a week for the duration of the phase.

c. Induction**:** Each bitch was treated with cabergoline at 5 µg /kg per os for 20 days. This was to induce fertile oestrus in Nigerian indigenous bitches. Five tablets (2500 µg) were placed in a pharmacist's mortar and gently pounded until it had become a powder. Thereafter Vitamin C was added and gently mixed until 100ml of the Vitamin C had been used to dissolve the cabergoline. The solution then contained 25 µg/ml of a stable mixture which was administered to the bitches. They bitches swere observed for signs of oestrus during the treatment period.

*d. Breeding***:**The bitches were bred by natural mating, that was done by taking the bitches to kennels containing adult males in the ratio of three females per male This was modified from the ratio as described by Ajala *et al* 2012 (six: one). This modification was undertaken to accommodate possible decreased fertility, since the oestrus was induced. Breeding was done when signs that the bitches are coming on heat was noticed physically. They were then left with the males for two weeks.

When pregnancy was confirmed by ultrasonography, the bitches were divided into three groups (A,B and C) of three dogs each. Group A dogs had no insonation , Group B had insonation once a week and Group C dogs had insonation twice a week until whelping.

2.4.1.3 Phase III (Gestation)

This was the 3rd phase of the study; it lasted nine weeks and the following activities are expected to be carried out.

*a. Sonography***:** During this phase all the bitches had their ventral abdominal areas liberally shaved from the level of the xyphoid cartilage to pubic area. The shaved area was and scrubbed, antiseptic applied with the bitch adequately restrained on the examination table on dorsal recumbency and aquasonic gel applied to the shaved area. Each bitch was then examined sonographically to visualize the internal genitalia and establish pregnancy. This phase is also designed to map out normal sonographic changes of the female reproductive tract of the bitches during the gestation. The examination was carried out once on all the bitches, and then repeated on each bitch in group B once a week and group C twice a week for the duration of the phase.

*b. Blood sampling***:** During this phase the bitches were restrained and blood collected aseptically via cephalic/recurrent tarsal venepuncture. Six millilitre of blood was collected at every sampling period. The first 3 mL was collected in EDTA bottle and was used for haematology and erythrocyte osmotic fragility test, while the remaining 3 mL was placed in anticoagulant free bottles for biochemical analysis. This was done once a week for the duration of the phase.

Post whelping, the average litter size and other physical parameters were recorded for each group and blood taken from each puppyand evaluated. The average post natal foetal daeth were also recorded.

3.4.1.4 Phase IV (Postpartum)

This was the 4th and last phase of the study; it lasted eight weeks and the following activities were carried out:

*a. Sonography***:** During this phase all the bitches had their ventral abdominal areas liberally shaved from the level of the xyphoid cartilage to pubic area. The shaved area was scrubbed antiseptic applied with the bitch restrained on the examination table. Aquasonic gel was then applied to the shaved area. Each bitch was then examined sonographically to visualize the internal genitalia. This was to map out the normal sonographic changes of the reproductive tract during the oestus cycle. The examination was then repeated on each bitch in the study groups twice a week for the duration of the phase.

*b. Blood sampling***:** During this phase the bitches were restrained and blood collected aseptically via cephalic/recurrent tarsal venipuncture. Six millilitre of blood was collected at every sampling period. The first 3 mL were collected in EDTA bottle and were used for haematology and erythrocyte osmotic fragility test, while the remaining 3 mL would be placed in anticoagulant free bottles for biochemical analysis. This was done twice a week for the duration of the phase.

Ultrasonography of all the bitches was done postpartu twice a week for eight weeks.

3.4.2 Determination of haematological parameters

3.4.2.1. Determination of blood cellular components

Packed cell volume (PCV), erythrocyte count (RBC), thrombocyte count, absolute and differential leucocyte counts was determined as described by Dacie and Lewis (1991). Haemoglobin concentration was determined using cyanmethaemoglobin method. Hb, PCV and RBC count was done as described by Schalm *et al*. (2010).

3.4.3. Determination of Serum Biochemical Parameters

3.4.3.1 Hormonal assay

The serum samples obtained from blood sample collected was analysed for progesterone, using an Enzyme-linked Immunosorbent assay (ELISA) diagnostic kit (Calbiotech Inc. El Cajon, CA. USA).

3.4.6.4 Determination of Oxidant and Antioxidant Biomarkers

The serum samples obtained from blood sample collected was analysed for malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) using a commercial kit (Calbiotech Inc. El Cajon, CA. USA).

3.4.7. Examination of the puppies postpartum

After the bitches' whelped, the average litter size per group was noted, their general physical conditions of the puppies was also be noted. The birth weight for each puppy was recorded and blood samples were collected from the puppies via jugular venupuncture. 6 mls of blood was taken from each puppyand divide into two (3 mls into sample bottles with anticoagulant and the rest in sample bottles without anticoagulant).

3.4.8. Hormonal assay

The serum samples obtained were analysed for triiodothyronine (T3) and Thyroxin (T4) hormones using an Enzyme-linked Immunosorbent assay (ELISA) diagnostic kit (Calbiotech Inc. El Cajon, CA. USA).

3.4.9 Data analysis

All data generated are presented as tables, charts or graphs. Values were expressed as mean± Standard error of mean (SEM). The data was subjected to One-way Analysis of Variance (ANOVA) and Bonferroni post-hoc test using GraphPad Prism version 4.0 for Windows 8 from GraphPad Software, San Diego, California, USA (www. Graphpad.com). Values of $p \leq 0.05$ was considered significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Sonographic features of the reproductive tract of bitches

4.1.1. Sonographic features of the reproductive tract of bitches during phase I

The phase 1 results showed that the bitches were all at various lengths of anoestrus from ten (10) weeks to thirty two (32) weeks (Table 4.1) with mean duration of 26.89 ± 2.29 weeks. The ultrasound examination revealed that the major internal genitalia visualized were the uterus and uterine horns as the absence of follicular activities makes it difficult to visualize the ovaries. The uterus appears as an isoechoic tubular structure with no fluid in the lumen Plate 4.1. The uterine diameter measured less than 1 cm in all the bitches.

Table 4.1 Showing the duration of the anoestrus with reproductive structures visualized on ultrasound examination

Animal	Duration of anoestrus (weeks)	Ovary	Oviduct	Uterus	Cervix
A1	24	-	+	+	+
A2	28	-	+	+	+
A3	10	-	+	+	+
B1	28	-	+	+	+
B2	32	-	+	+	+
B3	28	-	+	+	+
C1	28	-	+	+	+
C2	32	-	+	+	+
C3	32	-	+	+	+
MEAN	**26.89 ± 2.29**				

Key: - = it was not visualized
+ = it was visualized

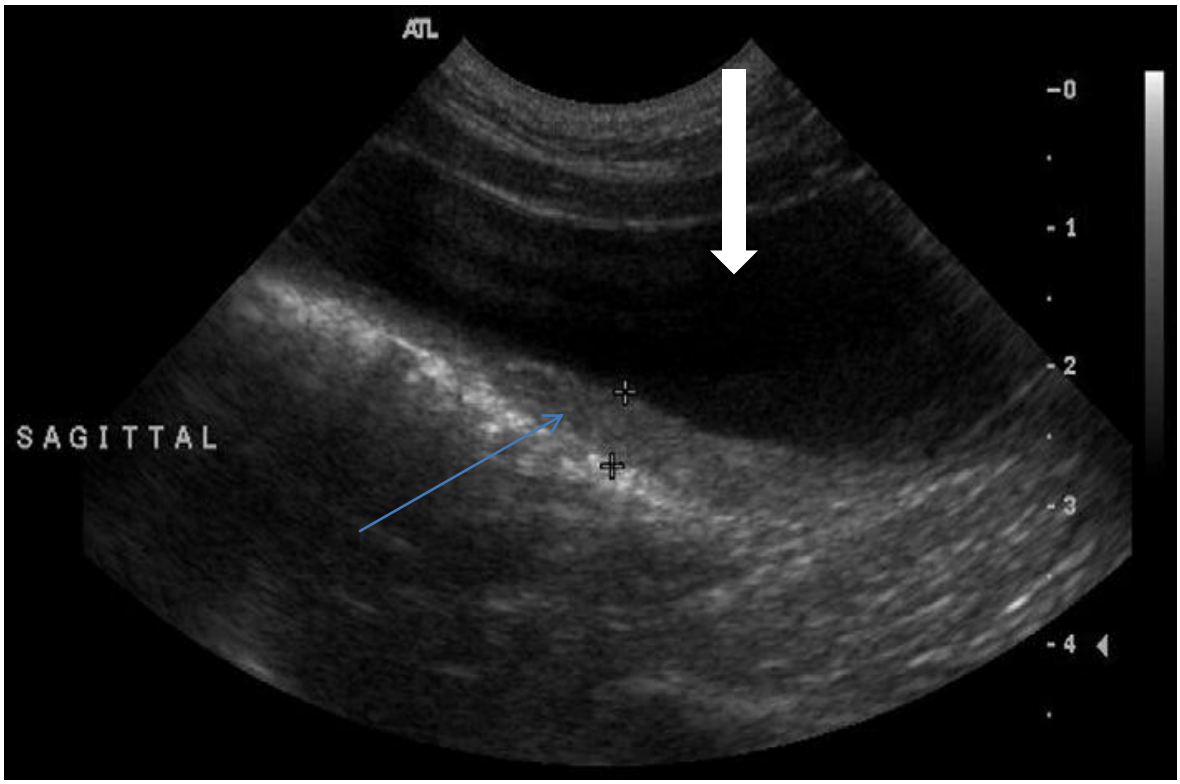


Plate 4.1. Sonographic image of the uterine body which is uniformly echogenic (A-blue arrow) with no fluid within (Arrowed) and the urinary bladder (white broad arrow) containing some volume of urine.

4.1.2. Sonographic features of the reproductive tract of bitches during phase II

We monitored the responses of the various bitches to the reconstituted cabergoline orally administered. The results of this phase showed that the bitches had an uneven response to cabergoline; it took some bitches thirteen (13) days from start of treatment to onset of proestrus. While it took others as late as eighteen (18) days with an average of 15.75 days between start of treatment to onset of proestrus (Table 4.2). The proestrus observed lasted between seven to eleven days in the bitches with an average of 9.38 days between onset of proestrus and the beginning of oestrus. The duration of the induced oestrus was 7-9 days with an average of 8 days. During this period the bitches stood to be mated by the studs we provided. The ultrasound results obtained in this phase of the work, showed that the ovaries which hitherto were not visualized, became visible at the onset of proestrus. The ovaries appeared as roundish structures that had mixed echogenicity. Gradually the follicles became visible and could be differentiated from each other as circular anechoic structures that cluster the ovary. Towards the end of the proestrus, the walls of the follicles became thickened the follicles also became larger and contained more fluid (Plate 4.2). When the bitches manifested oestrus, the follicles were released and the ovaries became relative smaller with more uniform echogenicity. The area where the follicles were became slightly more hyper echoic (from anechoic to isoechoic), they contained the corpora haemorrhagic (Plate 4.3).

Table 4.2. Results of the response of the bitches to Cabergoline treatment.

	Start of treatment to onset of proestrus (Days)	Duration of proestrus (Days)	Duration of oestrus (Days)
Mean ± SEM	15.75 ± 0.68	9.38 ± 0.50	8.0 ± 0.27
Range	13-18	7-11	7-9

Key:
Mean ± SEM = mean with standard error of mean.

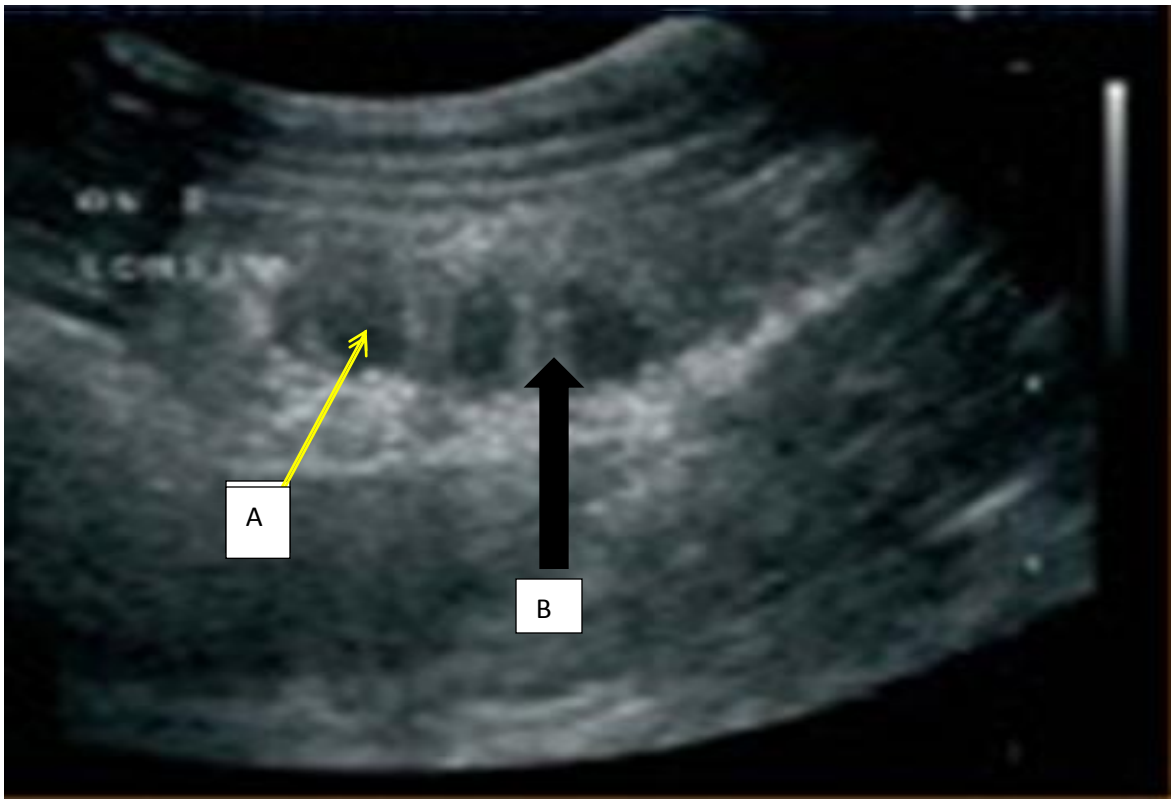


Plate 4.2. Ultrasound appearance of the ovary at late proestrus. There is a large amount of fluid within the follicles (A= yellow arrow). The walls of the follicles are thickened (B=black arrow)

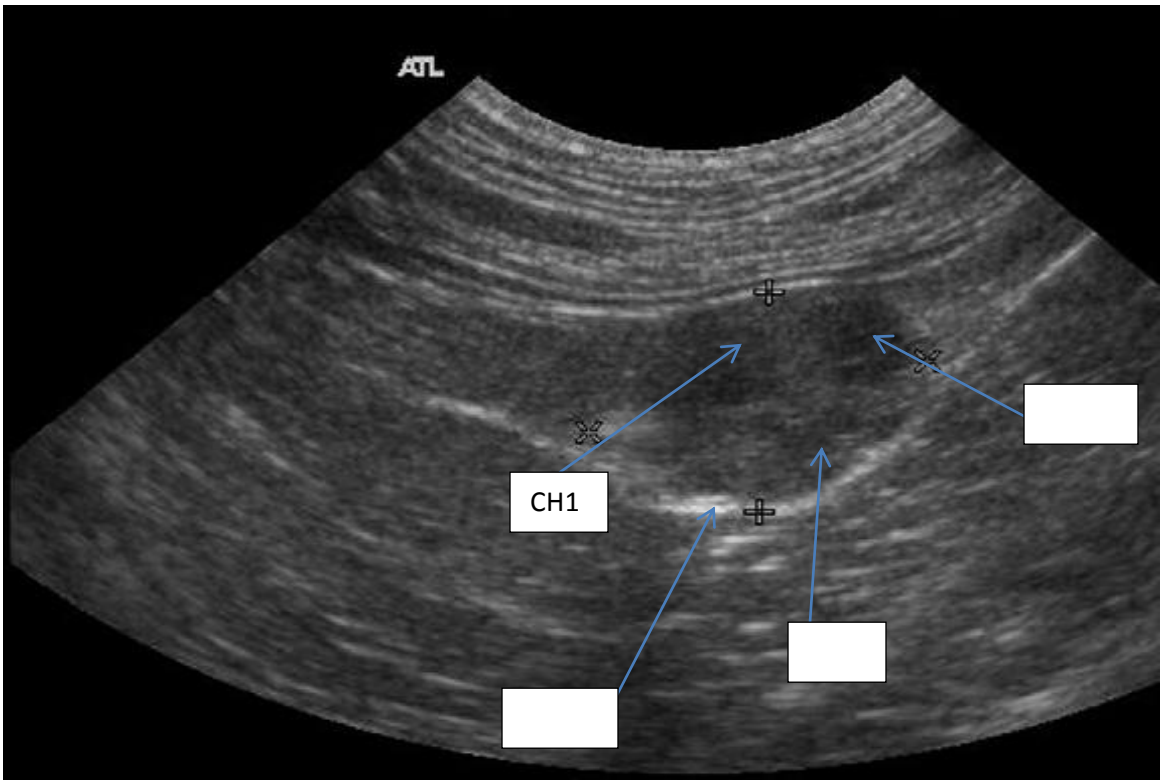


Plate 4.3 Sonograph of ovary post ovulation, the with the copora hemorrhagica are isoechogenic (arrowed, CH1-CH3)

4.1.3. Sonographic features of the reproductive tract of bitches during phase III

The results of ultrasonography in phase 3 showed that the earliest a positive confirmation of pregnancy was made was between days nineteen to twenty-one (19-21 days, Table 4.3) post last service. The sonographic appearance of the gestational sac with anechoic content and a hyper echoic structure at the margins is a definite sign of pregnancy (Plate 4.4). Once pregnancy was confirmed the bitches were then grouped into three A, B and C. Group A was no longer insonated until after whelping while bitches in group B were insonated once a week whiles those of group C were insonated twice a week during this phase. The gestational sac (spherical anechoic structure, with thin and hyper echoic borders) got attached to the wall of the uterus at this point. After which foetal poles then started to develop distinct morphological characteristics (Plate 4.5) by between days 23-25. We started counting the foetal pole at this juncture, the mean maximum foetal poles counted were 8.00 ± 0.58, 9.00 ± 0.58 and 9.00 ± 0.58 for bitches in groups A, B and C respectively (Fig 4.1). It was also at this point that we first visualized foetal cardiac activities. The head became a distinct feature between day 29-31.The head appears as anechoic filled structure just cranial to the forming foetal body.

The ultrasound results of this phase showed the visualization of limb buds which were hyperechoic protrusions from the lateral aspects of both cranial thoracic and caudal abdominal parts. This development was observed between days 34- 37 (with a mean appearance of 35.17 ± 0.48) Plate 4.6. After this was the foetal movement, this was first noticed on day 33 in some bitches while in others it was on day 38 post breeding. The mean period within which this happened was 35.50 ± 0.76. It was observed in most cases

that the urinary bladder becomes visible between days 35- 39 as a spherical anechoic structure at the caudal foetal pole, the mean period was noticed to be 35.50 ± 0.76 days post mating (Plate 4.7, and 4.8). Next structure to become visible was the foetal stomach which appeared as a spherical anechoic organ just caudal to the foetal liver; this happened between day's 35-39 post mating with an average period being 36.83 ± 0.60 days (Table 4.3 and Plate 4.8). The spinal canal became visible as dorsal tubular structure between days 36- 39 with a period mean of 38.00 ± 0.37 days post mating (Plate 4.9). The eyes were visualised as distinct spherical structures with anechoic corpus vitreum, hyper echoic border between sclera and retina and spherical anechoic lens from day 39-40 post mating with a mean of 39.33 ± 0.21 days (Plate 4.10). The foetal heart chambers become visible between day 40- 41 post mating with a mean of 40.33 ± 0.21 day (Plate 4.11). The foetal kidneys appears as a mixed echogenic organ on the lateral aspect of the cranial abdomen of the foetus, This was visible from day 41 -47 post mating with a mean period of 44.83 ± 0.98. The last structure to appear on ultrasound we found was the foetal intestines, they became visible between day 59- 63 post mating and they were the last organ to be visualised before the bitches whelped. The mean period was found to be 60.33 ± 0.61 days. When the bitches whelped we noted each bitches litter size and the birth weight of the puppies. The mean litter size recorded was 7. 33 ± 0.67, 6.67 ± 0.67 and 5.33 ± 0.33 puppies/ litter for bitches in groups A, B and C respectively (Fig 4.2). The mean birth weights for the puppies recorded were 246.4 ± 8.42, 197.5 ± 11.55 and 189.7 ± 10.21 g for puppies whelped by bitches from groups A, B and C respectively (Fig 4.9). The post-partum neonatal deaths were also recorded in the first two weeks post whelping.

4.1.4. Sonographic features of the reproductive tract of bitches during phase IV

The ultrasound results for phase 4 showed that on the long axis ultrasound scanning the post-partum uterus was tubular in shape and initially of mixed echogenicity. In the first week (between days 4-7) postpartum there was a lot of intraluminal fluid which was expelled as involution progressed. There where regions of hyper echogenicity (the uterine walls) which surrounded anechoic substances (Lochia). Measuring the uterine diameter (short axis) was started only after most of the fluid had been expelled the results showed that at the first week postpartum the uterine diameter ranged from 3.510 - 4.510 with a mean of 3.944 ± 0.105 cm this was the highest values obtained during this phase of the study. Involution also involved the reduction in the uterine diameter from the 1st week to means of 3.100 ± 0.116, 2.340 ± 0.114, 1.973 ± 0.058, 1.796 ± 0.042, 1.747 ± 0.025, 1.747 ± 0.027 and 1.727 ± 0.026 cm for weeks 2 to 8 respectively (Fig.4.5). There was also observed a change in the echogenicity from mixed (hyper echoic wall surrounding an echoic fluid) in the first week to almost exclusively isoechoic by the six weeks postpartum.

Table4.3. The summary of the first appearance of various ultrasonographic foetal and extrafoetal features in the bitches post mating

Feature	Days	Average
Gestational sac (spherical anechoic structure, with thin and hyperechoic borders)	19-21	20.11 ± 0.26
Apposition to uterine wall,	23-25	23.5 ± 0.34
Foetal heartbeat	23-25	23.33 ± 0.33
Anechoic area in the foetal head	28-34	29.50 ± 0.96
Limb buds	34-37	35.17 ± 0.48
Foetal movement	33-38	35.50 ± 0.76
Spherical anechoic urinary bladder at the caudal foetal pole	35-39	36.67 ± 0.67
Spherical anechoic stomach caudal to the liver	35-39	36.83 ± 0.60
Spinal canal visible as dorsal tubular structure	36- 39	38.00 ± 0.37
Eyes seen as distinct spherical structures with anechoic corpus vitreum, hyperechoic border.	39-40	39.33 ± 0.21
Lung hyperechoic to liver	39- 42	40.33 ± 0.49
Cardiac chambers	40- 41	40.33 ± 0.21
Kidneys	41- 47	44.83 ± 0.98
Intestines with intestinal layers	59- 63	60.33 ± 0.61

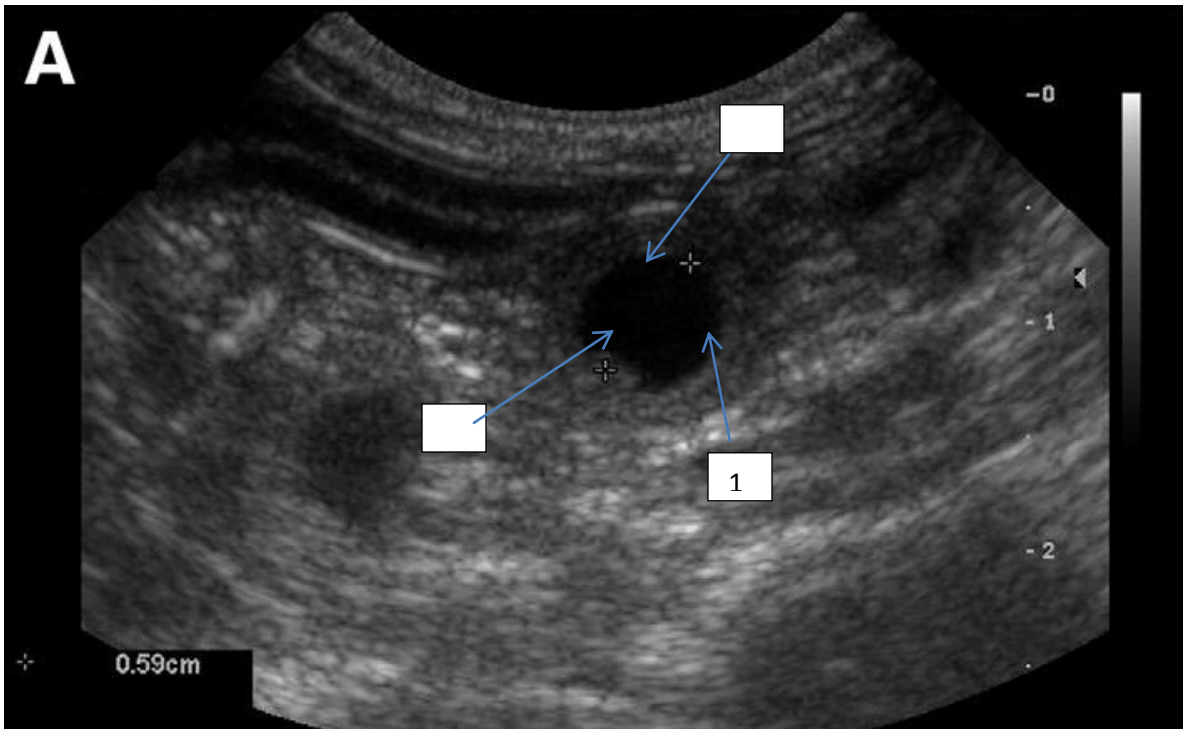


Plate 4.4: Sonograph showing early pregnancy between 19-21 days of gestation. Amniotic sac (1) fluid (2) and foetal pole (3)

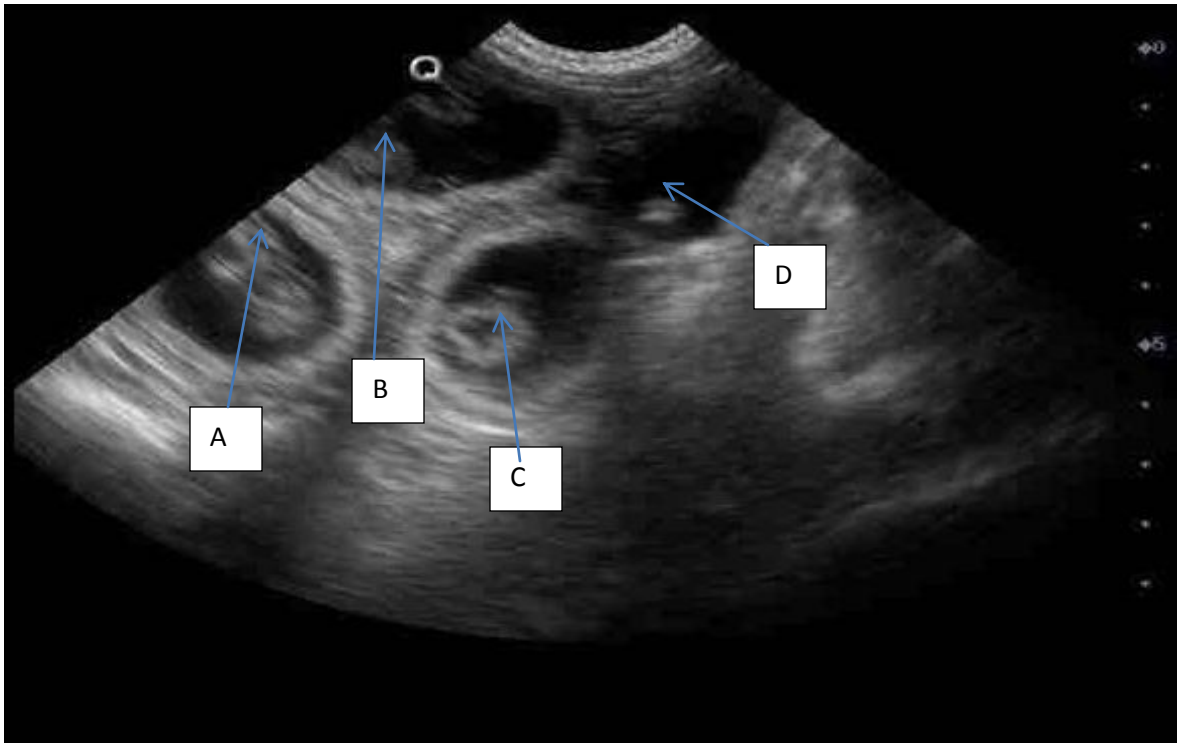


Plate 4.5: Sonograph of foetal poles between days 27-29 . Foetuses have recognizable morphology with their gestational sacs. (Arrows A, B, C and D)

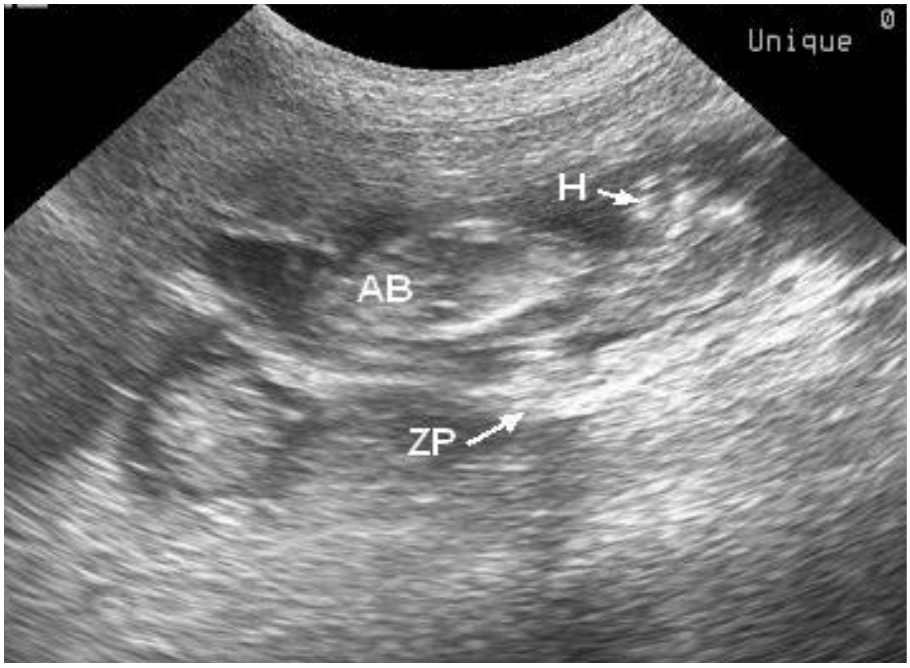


Plate 4.6: Sonogram of the foetal structures in pregnant bitches. Days 28-32 of gestation: Longitudinal image of embryo (H: head, AB, abdomen, ZP: zonary placenta).

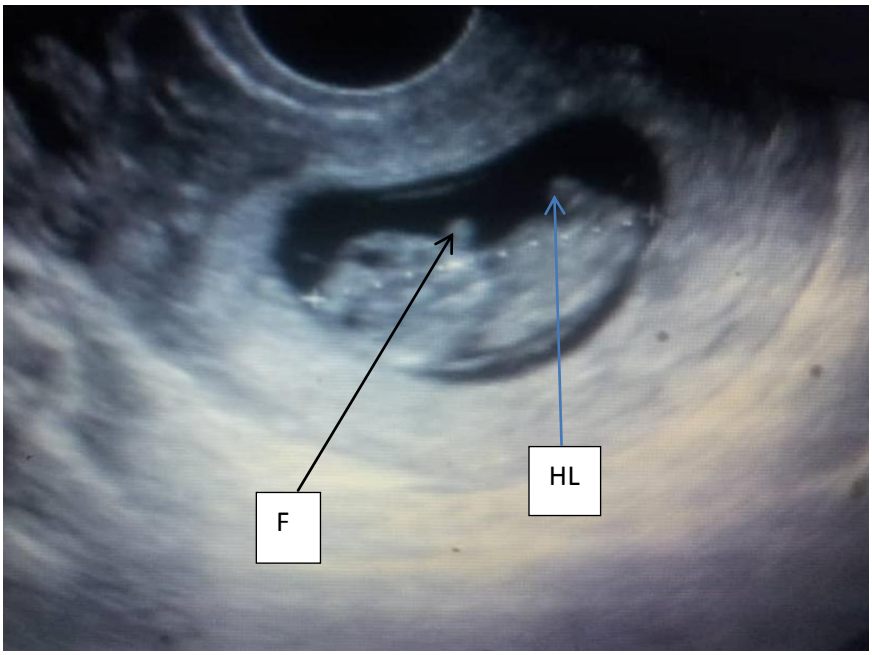


Plate 4.7. Sonogram of the foetal structures in pregnant bitches. between days 34-37 of gestation showing limb bud (FLB= forelimb bud and HLB= Hind limb bud)

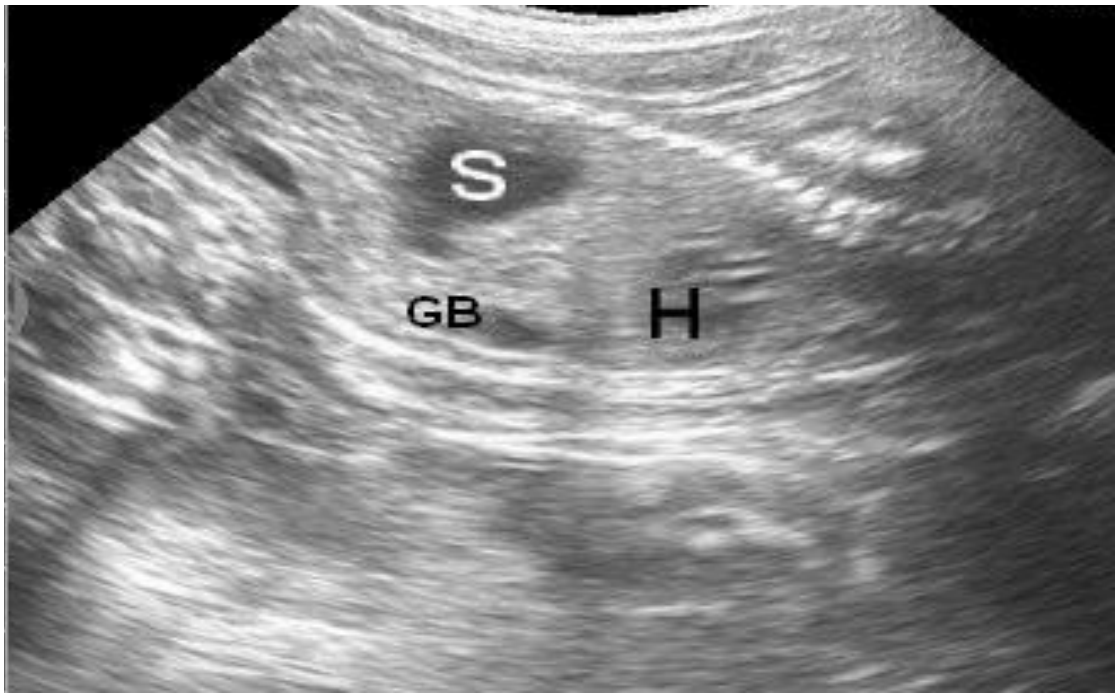


Plate 4.8: Sonogram of the foetal structures in pregnant bitches. Days 35-39 of gestation.Longitudinal image of a foetus with three anechoic area (H: heart, GB: gallbladder, S: stomach).

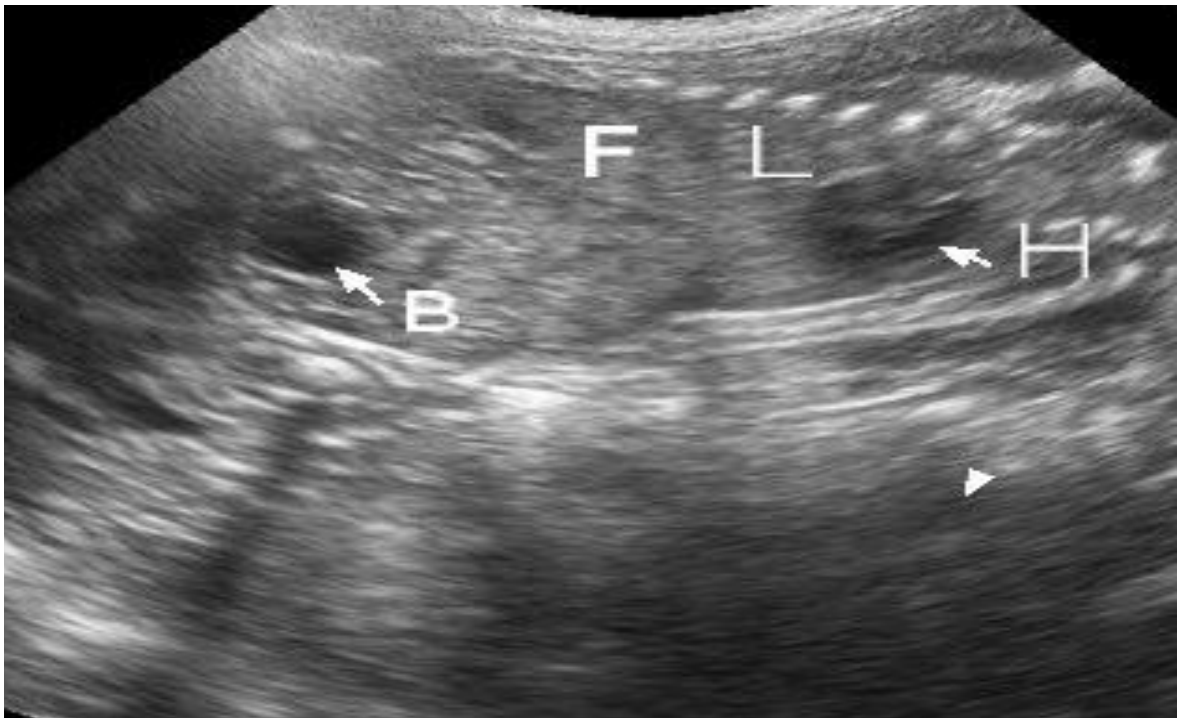


Plate 4.9: Sonograms of the foetal structures in pregnant bitches. Days 39-42 of gestation: Longitudinal image of a foetus (B: bladder, F: liver, L: lung, H: heart).

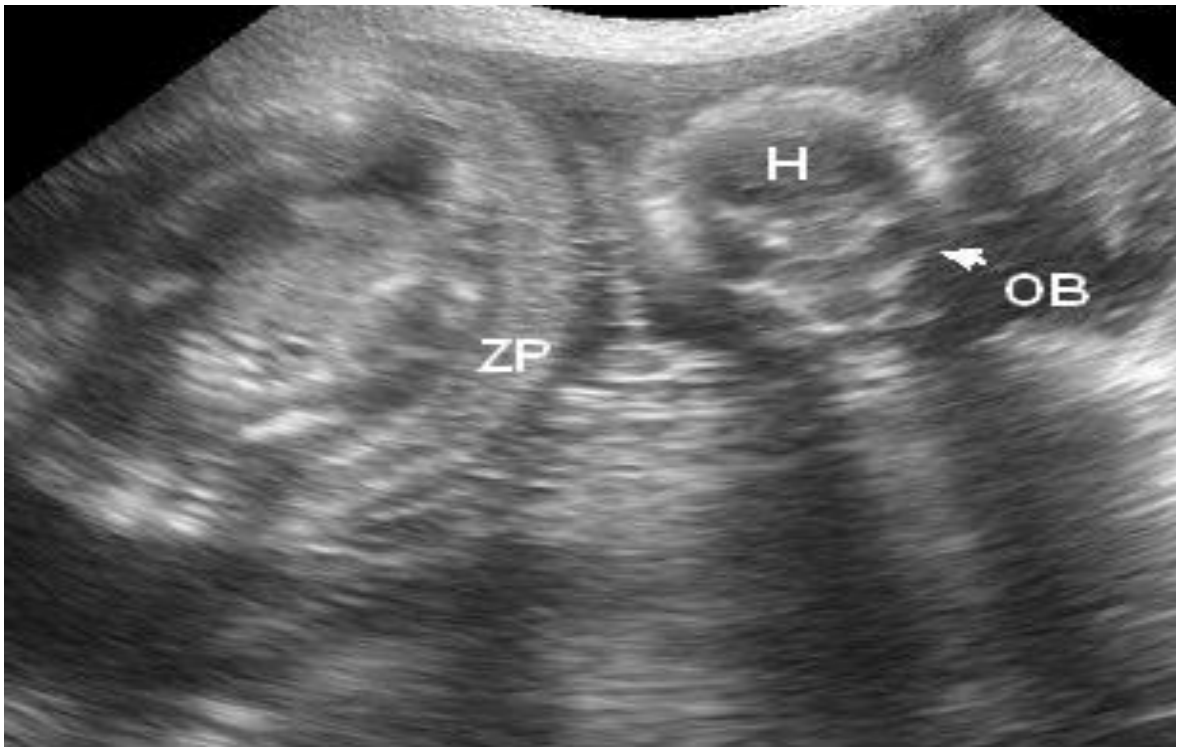


Plate4.10: Sonograms of the foetal structures in pregnant bitches. Days 40-45 of gestation, image of a foetal head (H: head, OB: orbit, ZP: zonary placenta)

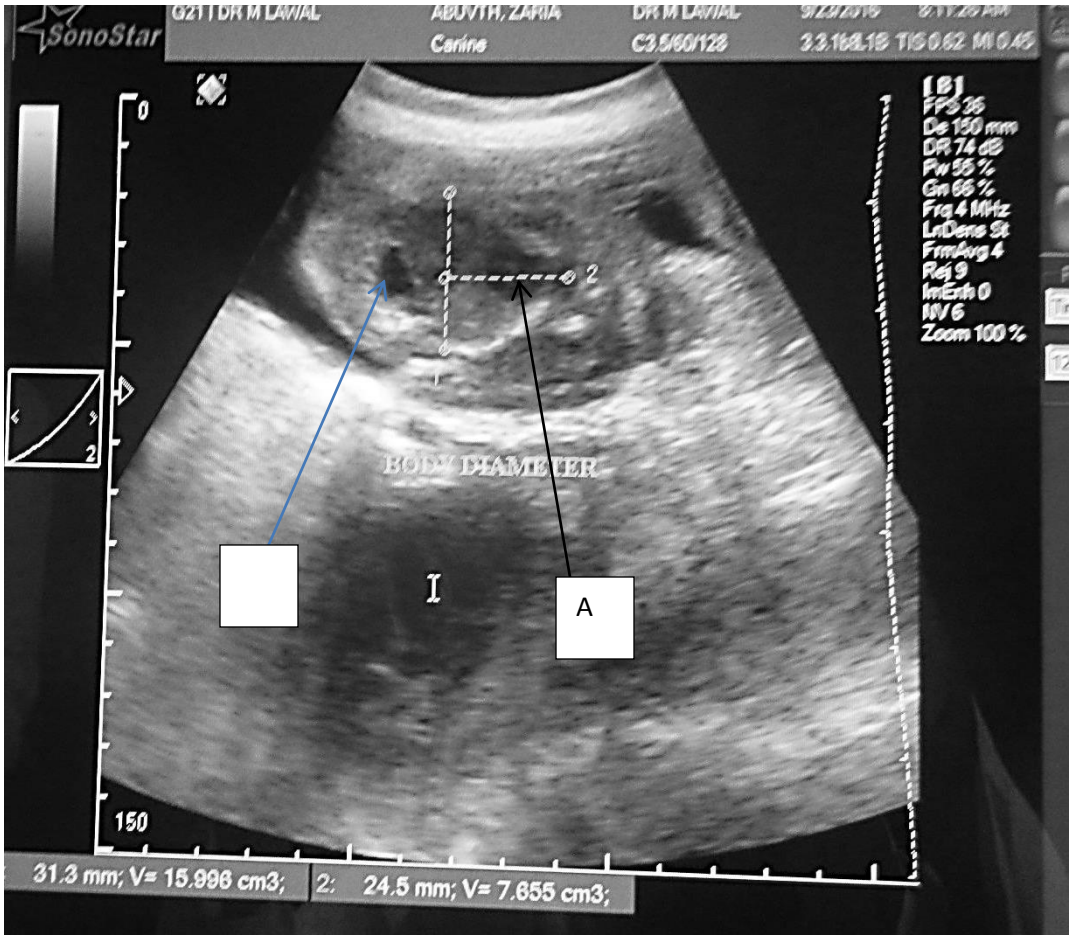


Plate 4.11 Sonogram showing some foetal structures (A= foetal heart with the variouschambers and B = foetal stomach) which was taken between days 41-45 post mating.

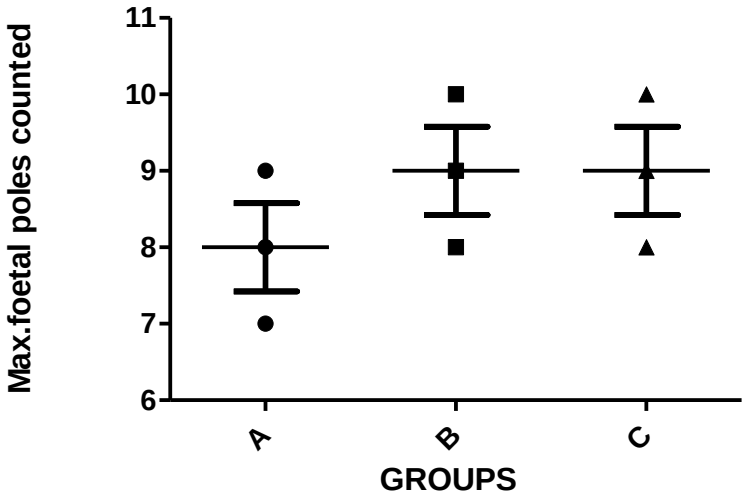


Fig. 4.1. Means of maximum foetal poles visualized at second trimester of gestation

Key: A = not insonated, B= insonated weekly and C = insonated twice a week

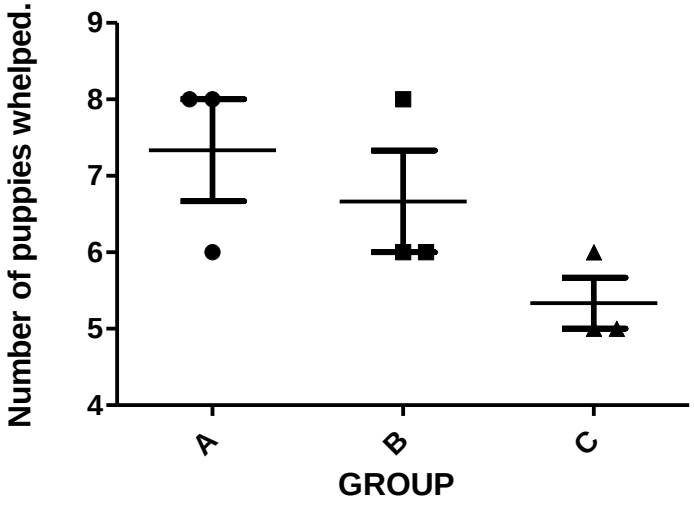


Fig.4. 2. Mean litter size at whelping by the bitches in various groups.

Key: A = not insonated, B= insonated weekly and C = insonated twice a week

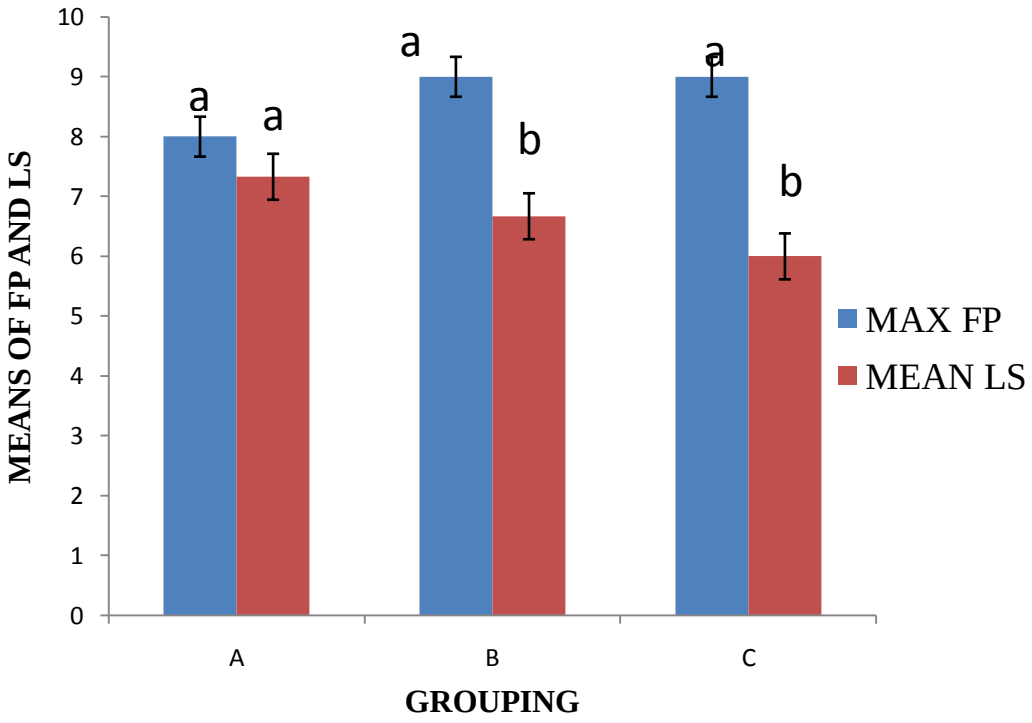


Fig. 4.3. Comparism of the Maximum Fetal poles counted and the mean number of puppies whelped.

Key: A = not insonated, B= insonated weekly and C = insonated twice a week
Max FP= maximum foetal poles counted. LS= litter size

Columns with same alphabets = No statistically significant difference, while those with different alphabets = statistically significant difference.

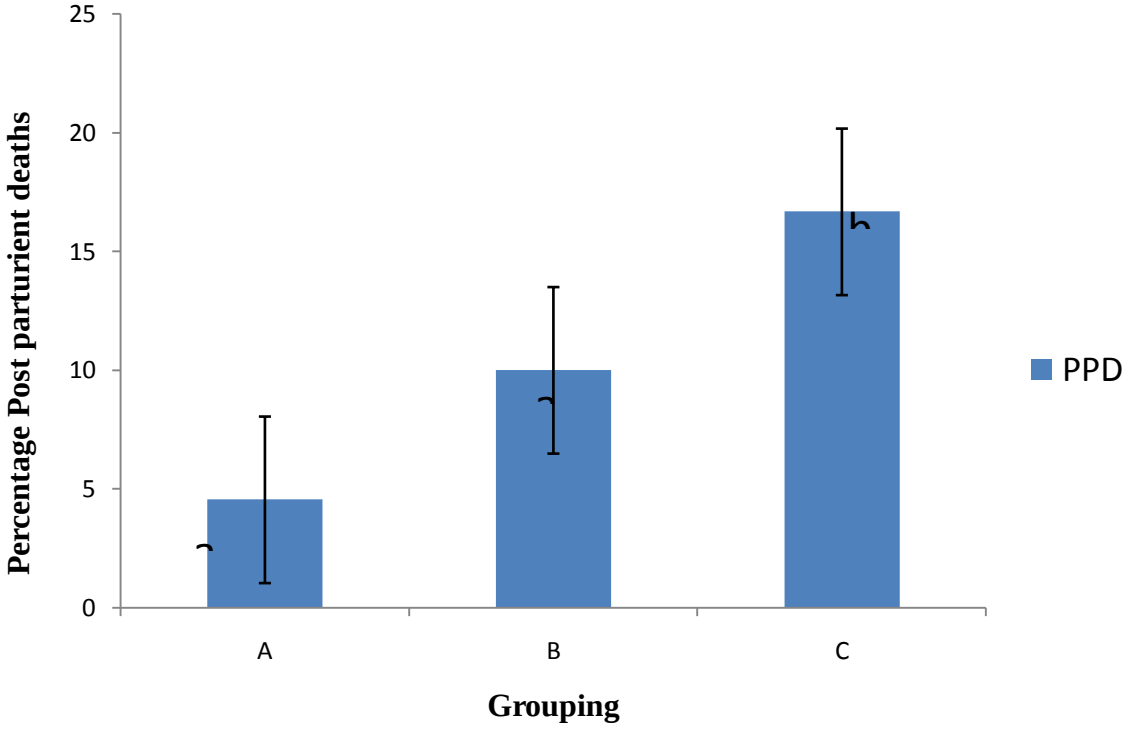


Fig. 4.4. Percentage of post-parturient death recorded two week post whelping

Key: A = not insonated, B= insonated weekly and C = insonated twice a week

PPD= post parturient deaths

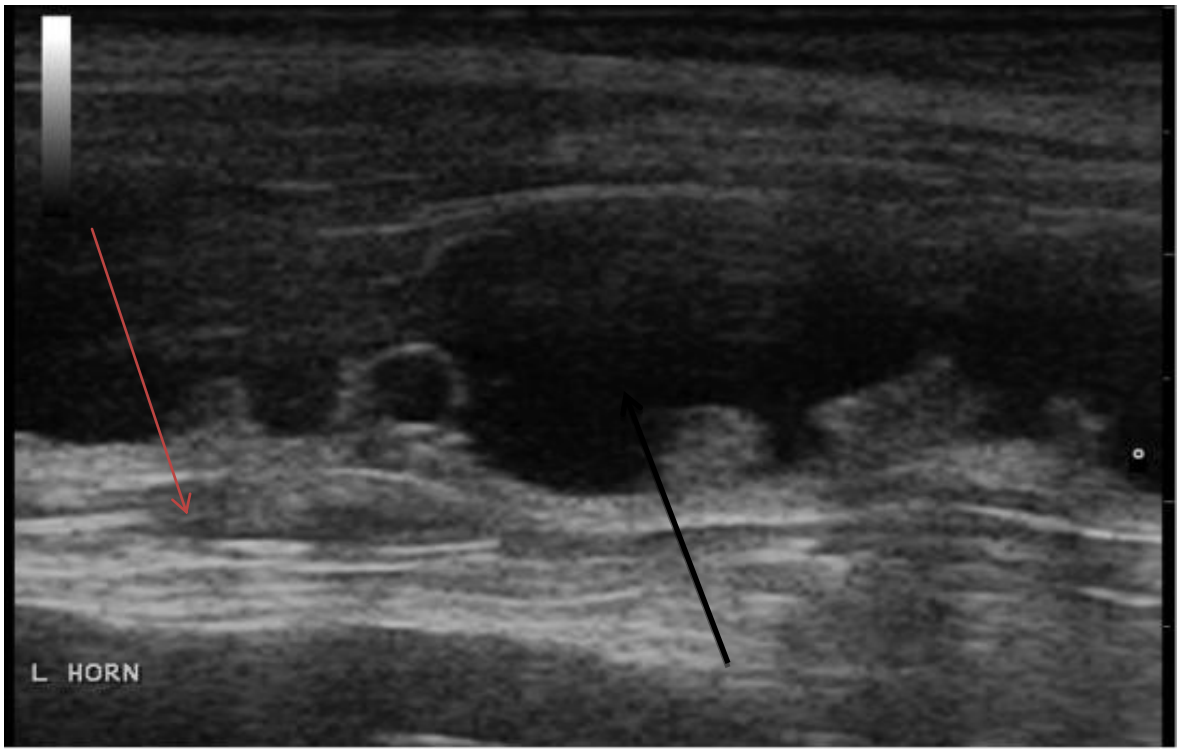


Plate 4.12: Sonographic image of postpartum uterus 3 days after parturition showing thick walls (red arrow) lochia (thick black arrow).

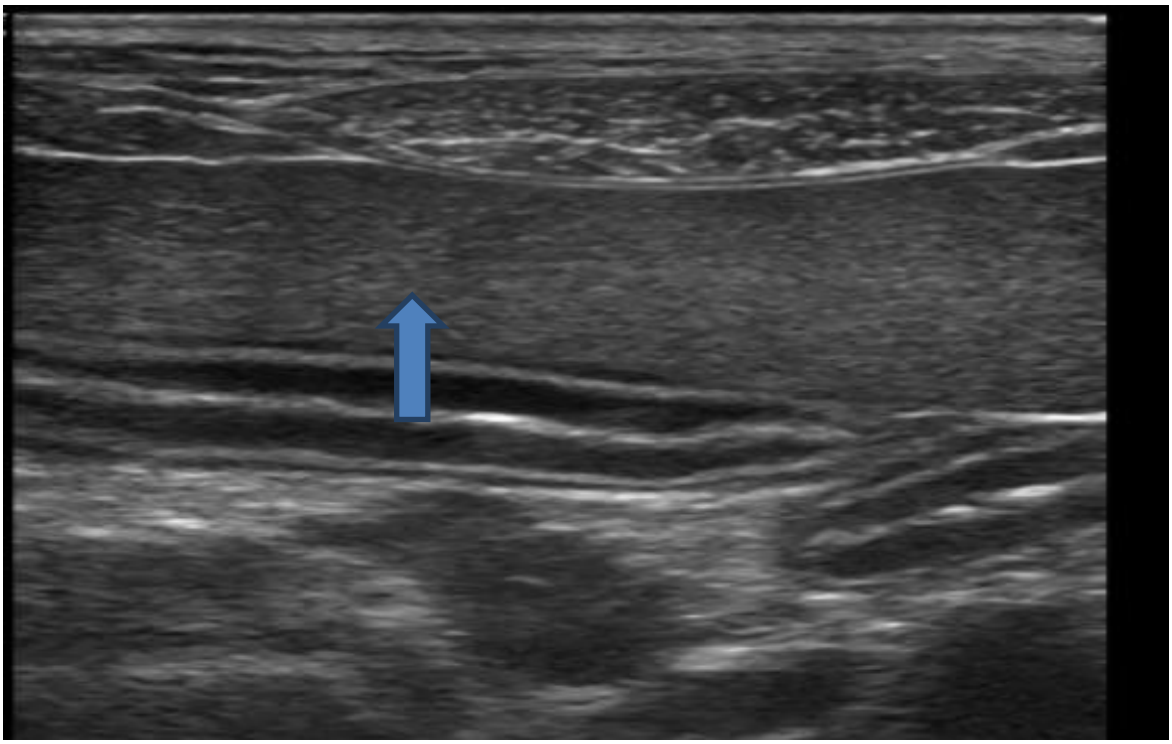


Plate4.13: Sonographic image of postpartum uterus following one month of involution Uterine parenchymal appears isoechogenic (Arrowed)

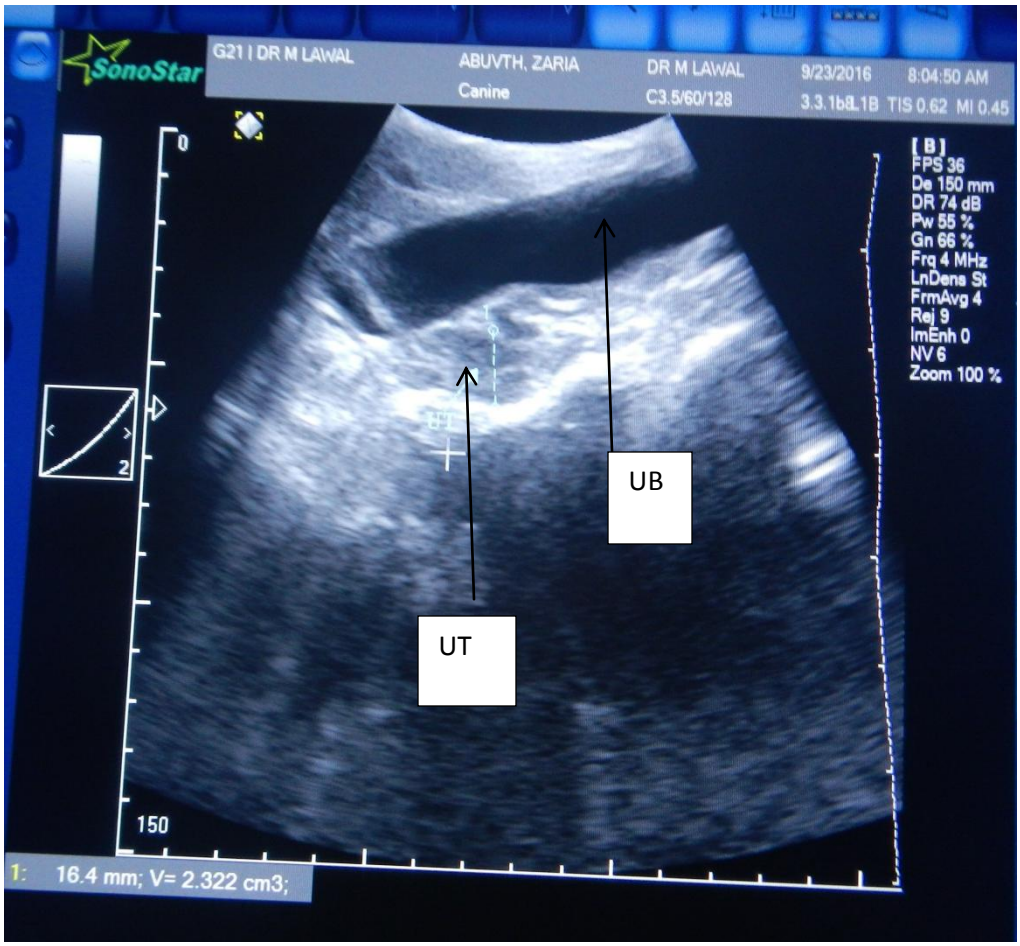


Plate 4.14 Sonograph the urinary bladder (UB) and a mixed echogenic uterus (UT)five weeks postpartum with maximum uterine diameter of 1.64 cm

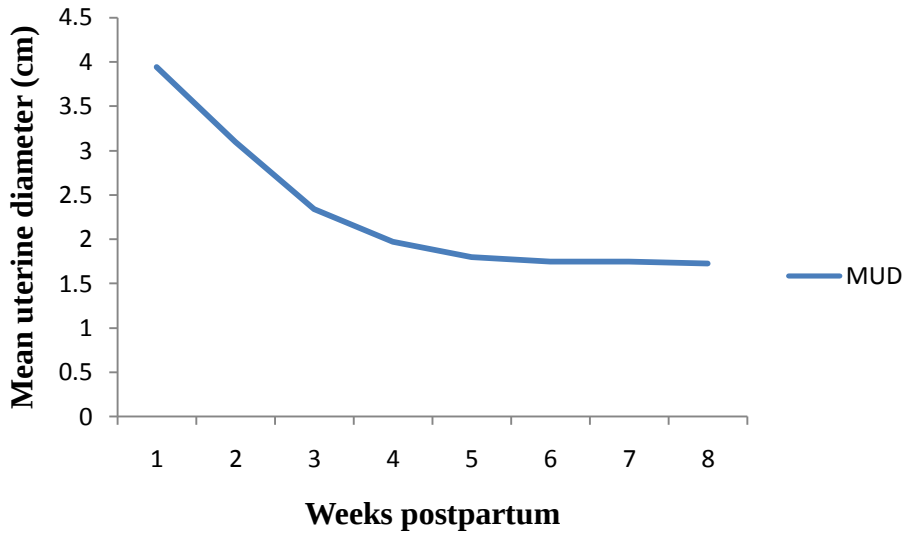


Fig 4.5 The trend in mean diameter of the uterus during part of postpartum (involution) phase of the study plotted against time.

4.2. Haematological parameters of bitches during the reproductive phases

4.2.1. Haematological parameters of bitches during phaseI

The haematology results showed that the mean Packed Cell Volumes for this phase were 38.67 ± 2.81, 39.44 ± 1.72 and 43.00 ± 2.35 for bitches in groups A,B and C respectively (Fig. 4.6). This result was used as baseline for the bitches in this research. Other heamatologic results were: For Haemoglobin (HGB) concentration 12.86 ± .9305, 13.09 ± 0.57 and 14.30 ± 0.78 for groups A, B and C respectively. Leucocyte count was found to be 44.11 ± 4.95, 40.11 ± 4.72 and 44.33 ± 4.61 for groups A, B and C. While the White blood cells counts (WBC) was 9.08 ± 0.83, 12.05 ± 2.20 and 10.98 ± 2.12 for groups A, B and C respectively. The Total protein (TP) result was 9.04 ± 0.58, 7.90 ± 0.21 and 9.27 ± 0.24 A, B and C respectively (Table 4.4).

4.2.2 . Haematological parameters of bitches during phaseII

The haematology results showed that the mean Packed Cell Volumes for thi phase were 36.33 ± 5.64, 49.83 ± 2.37 and 47.33 ± 4.33 % for bitches in groups A, B and C respectively (Fig. 4.6). Other haematological results were: For Haemoglobin (HGB) concentration 12.07 ± 1.88, 16.55 ± 0.80 and 15.72 ± 1.46 for groups A, B and C respectively. Leucocyte count was found to be 23.31 ± 1.45, 39.50 ± 3.02 and 30.17 ± 3.48 for groups A, B and C. While the White blood cells counts (WBC) was 10.68 ± 2.27, 8.90 ± 0.94 and 11.53 ± 1.66 for groups A, B and C respectively. The Total protein (TP) result was 7.37 ± 0.44, 7.15 ± 0.35 and 6.35 ± 0.11 A, B and C respectively (Table 4.4).

4.2.3. Haematological parameters of bitches during phaseIII

The haematology results showed that the mean Packed Cell Volumes for this phase were 47.08 ± 5.64, 45.67 ± 2.37 and 51.42 ± 4.33 % for bitches in groups A, B and C respectively (Fig 4.6). Other heamatologic results were: For Haemoglobin (HGB) concentration 15.69 ± 0.66, 15.18 ± 0.55 and 17.14 ± 0.60 for groups A, B and C respectively. Leucocyte count was found to be 32.83 ± 3.36, 30.25 ± 1.24 and 27.08 ± 2.54 for groups A, B and C. While the White blood cells counts (WBC) was 8.43 ± 0.40, 8.85 ± 0.64 and 10.39 ± 0.78 for groups A, B and C respectively. The Total protein (TP) result was 7.37 ± 0.44, 7.15 ± 0.35 and 6.35 ± 0.11 A, B and C respectively (Table 4.4).

The haematology results in the late gestation phase showed that the mean Packed Cell Volumes for this phase were 42.92 ± 1.70, 40.92 ± 1.46 and 37.25 ± 1.57 % for bitches in groups A, B and C respectively (Fig. 4.6). Other haematological results were: For Haemoglobin (HGB) concentration 14.39 ± 0.55, 13.06 ± 0.80 and 13.68 ± 0.5022 for groups A, B and C respectively. Leucocyte count was found to be 36.50 ± 3.173, 29.75 ± 2.004 and 32.75 ± 2.118 for groups A, B and C. While the White blood cells counts (WBC) was 8.67 ± 0.87, 9.65 ± 1.11 and 9.13 ± 1.40 for groups A, B and C respectively. The Total protein (TP) result was 6.40 ± 0.26, 6.100 ± 0.2339 and 5.900 ± 0.1642 A, B and C respectively (Table 4.4).

4.2.4, Haematological parameters of bitches during phaseIV

The haematology results showed that the mean Packed Cell Volumes for this phase were 34.00 ± 2.51, 43.33 ± 2.34 and 46.89 ± 2.99 % for bitches in groups A, B and C respectively (Fig. 4.6). Other haematological results were: For Haemoglobin (HGB)

concentration 11.33 ± 0.8253, 15.09 ± 0.7804 and 15.59 ± 0.9939 for groups A, B and C respectively. Leucocyte count was found to be 43.11 ± 4.357, 29.22 ± 5.444 and 28.00 ± 4.282 for groups A, B and C. While the White blood cells counts (WBC) was 10.43 ± 1.153, 10.07 ± 1.798 and 7.767 ± 0.6316 for groups A, B and C respectively. The Total protein (TP) result was 8.333 ± 0.51, 8.333 ± 0.5270 and 7.022 ± 0.3968 A, B and C respectively (Table 4.4).

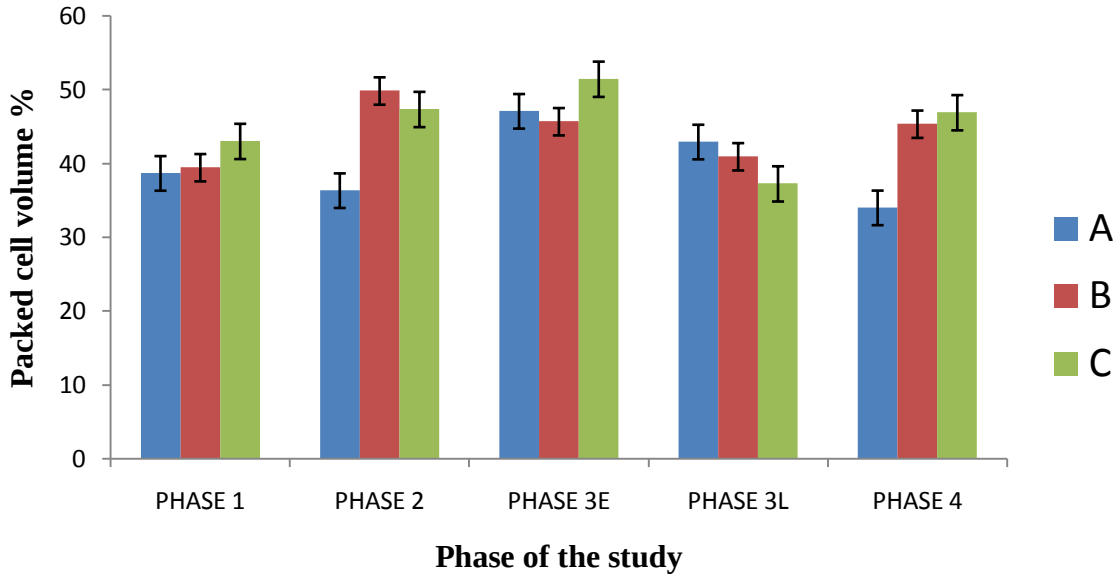


Fig.4 6. Showing the packed cell volumes of the bitches at the various phases of the study.

KEY:

A = group not insonated, B= group insonated once a week in–puppy and C= group insonated twice a week in-pup.

Phase 1 = Acclimatization phase, Phase 2 = Induction phase, Phase 3E = Early gestation phase, Phase 3L = Late gestation phase and Phase 4 = postpartum phase.

Table 4.4. Showing the various means and SEM for the other heamatologic parameters bitches at different phases of the study

	PHASE 1	PHASE 2	PHASE 3E	PHASE 3L	PHASE 4
a	12.86 ± 0.9305[a]	12.07 ± 16.55[a]	15.69 ± 0.6605[a]	14.39 ± 0.5457[a]	11.33 ± 0.8253[a]
b	13.09 ± 0.5738[a]	15.72 ± 0.8007[a]	15.18 ± 0.5546[a]	13.68 ± 0.7965[a]	15.09 ± 0.7804[a]
HGB c	14.30 ± 0.7817[a]	15.72 ± 1.457[a]	17.14 ± 0.5998[a]	13.06 ± 0.5022[a]	15.59 ± 0.9939[a]
a	44.11 ± 4.945[a]	23.33 ± 1.453[a]	32.83 ± 3.355[a]	36.50 ± 3.173[a]	43.11 ± 4.357[a]
b	40.11 ± 4.721[a]	39.50 ± 3.019[a]	30.25 ± 1.238[a]	32.75 ± 2.118[a]	29.22 ± 5.444[a]
LEU c	44.33 ± 4.607[a]	30.17 ± 3.478[a]	27.08 ± 2.536[a]	29.75 ± 2.004[a]	28.00 ± 4.282[a]
a	9.04 ± 0.5819[a]	7.37 ± 0.4364[a]	10.33 ± 3.971[a]	6.40 ± 0.2558[a]	8.33 ± 0.5099[a]
b	7.90 ± 0.2102[a]	7.15 ± 0.3538[a]	6.75 ± 0.1390[a]	6.10 ± 0.2339[a]	8.33 ± 0.5270[a]
TP c	9.27 ± 0.2427[a]	6.35 ± 0.1147[a]	6.35 ± 0.1190[a]	5.90 ± 0.1642[a]	7.02 ± 0.3968[a]
a	9.08 ± 0.8253[a]	10.68 ± 2.265[a]	8.43 ± 0.3966[a]	9.654 ± 1.105[a]	10.43 ± 1.153[a]
b	12.05 ± 2.202[a]	8.90 ± 0.9430[a]	8.85 ± 0.6429[a]	9.13 ± 1.402[a]	10.07 ± 1.798[a]
WBC c	10.98 ± 2.117[a]	11.53 ± 1.661[a]	10.39 ± 0.7762[a]	8.67 ± 0.8737[a]	7.77 ± 0.6316[a]

KEY
HGB = Haemoglobin concentration
LEU = Leucocyte count
TP = Total protein
WBC = White blood cells count

Similar superscripts = no statistically significant difference

4.3. **Serum parameters of theof bitches during the reproductive phases**

4.3.1. Serum parameters of bitches during phaseI

The serum analysis results for this phase showed the progesterone assay was 2.25 ± 0.19, 3.59 ± 0.91 and 2.83 ± 0.79 ng/ml for groups A, B and C respectively. The concentration of Superoxide dismutase was 1.04 ± 0.08, 0.62 ± 0.06 and 0.97 0.07 for Groups A, B and C respectively. Catalase concentration was found to be 6.98 ± 0.12, 6.46 ± 0.03 and 7.78 ± 0.5 µmol/min/ml for groups A, B and C respectively. The concentration of glutathione peroxidase was 28.30 ± 3.04, 21.42 ± 3.15 and 20.09 ± 2.57 µmol/ml for groups A, B and C respectively. While that of malondialdehyde was found to be 3.43 ± 0.50, 4.61 ± 1.58 and 5.57 ± 1.46 µmol/ml for groups A, B and C respectively.

4.3.2. Serum parameters of bitches during phase II

The serology results for this phase showed the progesterone assay was 20.54 ± 4.94, 45.19 ± 24.51 and 70.50 ± 31.50 ng/ml for groups A, B and C respectively. The concentration of Superoxide dismutase was 0.512 ± 0.143, 0.354 ± 0.054 and 0.456 ± 0.14 for Groups A, B and C respectively. Catalase concentration was found to be 5.71 ± 0.717, 5.96 ± 0.324 and 7.123 ± 0.322 µmol/min/ml for groups A, B and C respectively. The concentration of glutathione peroxidase was 12.77 ± 4.493, 9.47 ± 2.17 and 10.5 ± 2.67 µmol/ml for groups A, B and C respectively. While that of malondialdehyde was found to be 0.94 ± 0.27, 1.71 ± 0.51 and 1.876 ± 0.36s µmol/ml for groups A, B and C respectively.

4.3.3. Serum parameters of bitches during phase III

The serum analysis results for early gestation phase showed the progesterone assay was 3.26 ± 0.56, 2.32 ± 0.27 and 3.60 ± 0.33 ng/ml for groups A, B and C respectively (Fig4.7). The concentration of Superoxide dismutase was 1.31 ± 0.04, 1.05 ± 0.05 and

1.47 ± 0.05 for Groups A, B and C respectively (Fig 4.8). Catalase concentration was found to be 10.11 ± 1.41, 11.85 ± 1.20 and 19.12 ± 5.18 µmol/min/ml for groups A, B and C respectively (Fig 4.10). The concentration of glutathione peroxidase was 42.59 ± 2.85, 42.12 ± 3.89 and 41.88 ± 1.72 µmol/ml for groups A, B and C respectively (Fig 4.9). While that of malondialdehyde was found to be 3.43 ± 0.32, 2.31 ± 0.32 and 2.83 ± 0.28 µmol/ml for groups A, B and C respectively (Fig 4.11). The serum analysis results for late gestation phase showed the progesterone assay was 6.54 ± 4.94, 45.19 ± 24.51 and 70.50 ± 31.50 ng/ml for groups A, B and C respectively. The concentration of Superoxide dismutase was 1.50 ± 0.124, 1.86 ± 0.21 and 1.90 ± 0.03 for Groups A, B and C respectively. Catalase concentration was found to be 26.86 ± 4.09, 27.95 ± 4.33 and 33.91 ± 5.83 µmol/min/ml for groups A, B and C respectively. The concentration of glutathione peroxidase was 60.87 ± 3.85, 60.91 ± 5.38 and 76.61 ± 8.98 µmol/ml for groups A, B and C respectively. While that of malondialdehyde was found to be 5.05 ± 0.79, 6.28 ± 2.09 and 7.41 ± 1.62 µmol/ml for groups A, B and C respectively.

4.3.4. Serum parameters of bitches during phase IV

The serology results for this phase showed the progesterone assay was 1.81 ± 0.31, 1.56 ± 0.12 and 1.68 ± 0.13 ng/ml for groups A, B and C respectively (Fig 4.7). The concentration of SOD was 1.53 ± 0.07, 0.898 ± 0.05 and 1.169 ± 0.024 for Groups A, B and C respectively (Fig 4.8). The concentration of GPX was 34.05 ± 3.02, 27.89 ± 2.88 and 32.41 ± 2.04 µmol/ml for groups A, B and C respectively (Fig. 4.9). Catalase concentration was found to be 7.37 ± 4.33, 7.93 ± 0.70 and 12.00 ± 3.24 µmol/min/ml for groups A, B and C respectively (Fig. 4.10). While that of MDAwas found to be 2.049 ± 0.74, 2.83 ± 0.74 and 3.13 ± 0.99 µmol/ml for groups A, B and C respectively (Fig 4.11).

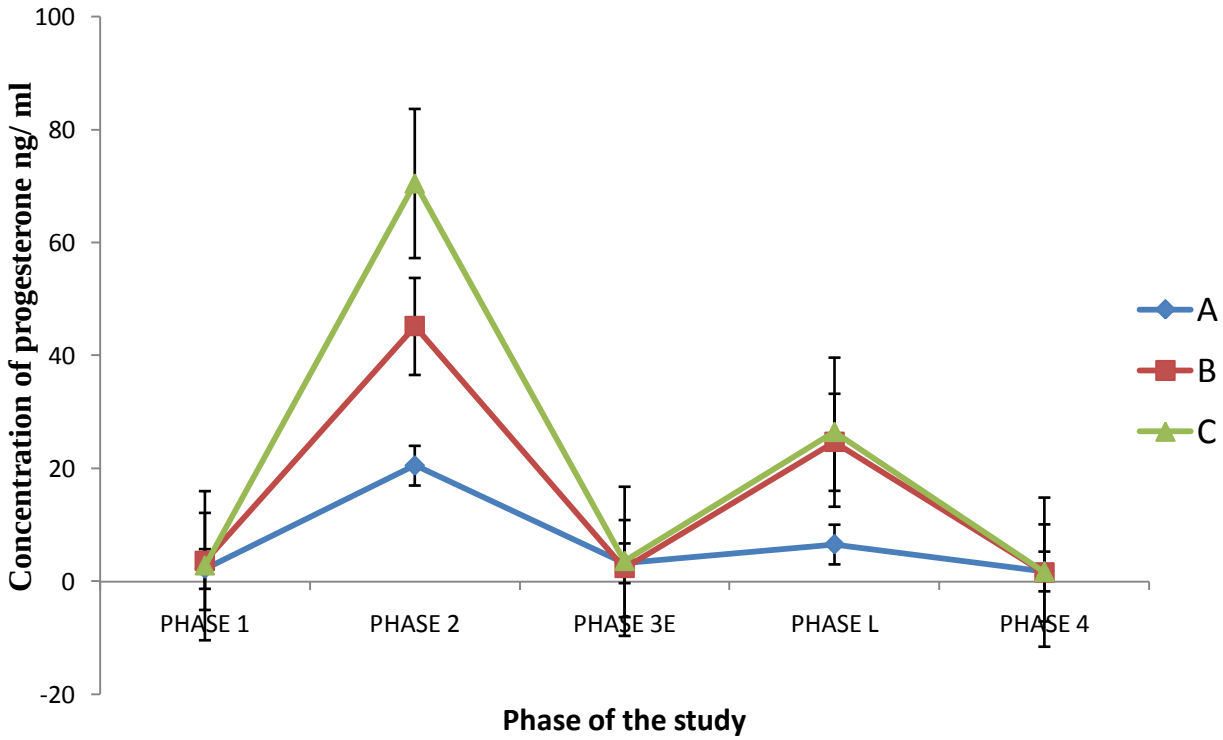


Fig.4.7. Concentration of progesterone of the bitches at the various phases of the study

KEY:

A = group not insonated, B= group insonated once a week in–pup and C= group insonated twice a week in-pup.

Phase 1 = Acclimatization phase, Phase 2 = Induction phase, Phase 3E = Early gestation phase, Phase 3L = Late gestation phase and Phase 4 = postpartum phase.

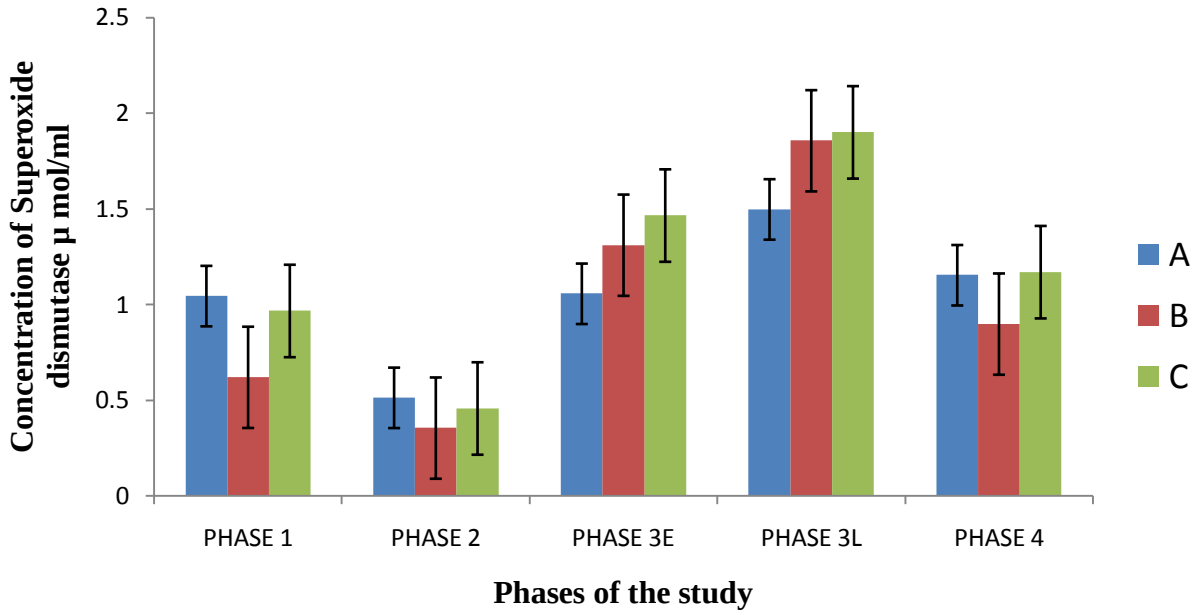


Fig.4.8. Concentration of superoxide dismutase in bitches at various phases of the study

KEY:

A = group not insonated, B= group insonated once a week in–pup and C= group insonated twice a week in-pup.

Phase 1 = Acclimatization phase, Phase 2 = Induction phase, Phase 3E = Early gestation phase, Phase 3L = Late gestation phase and Phase 4 = postpartum phase.

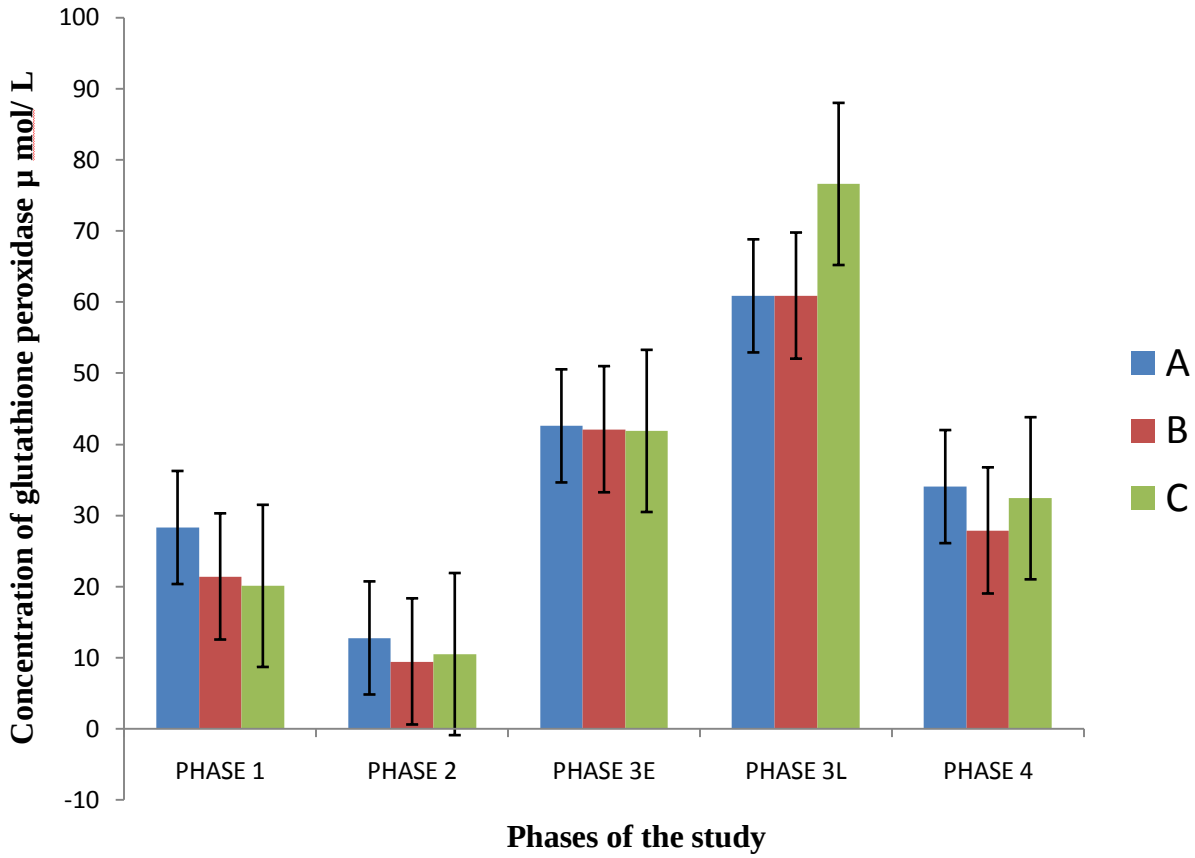


Fig.4.9. Concentration of glutathione peroxidase in the bitches at various phases of the study

KEY:

A = group not insonated, B= group insonated once a week in–pup and C= group insonated twice a week in-pup.

Phase 1 = Acclimatization phase, Phase 2 = Induction phase, Phase 3E = Early gestation phase, Phase 3L = Late gestation phase and Phase 4 = postpartum phase.

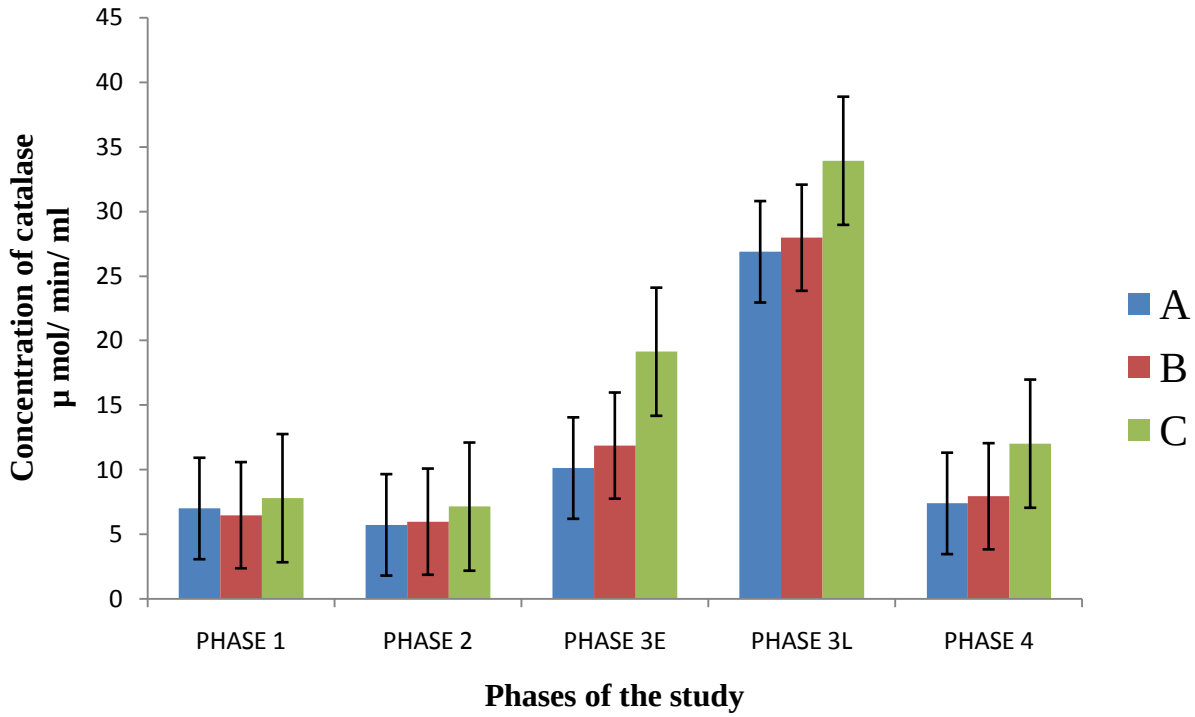


Fig.4.10 Column graph showing concentration of catalase of the bitches at various phases of the study

KEY :

A = group not insonated, B= group insonated once a week in–puppy and C= group insonated twice a week in-puppy.

Phase 1 = Acclimatization phase, Phase 2 = Induction phase, Phase 3E = Early gestation phase, Phase 3L = Late gestation phase and Phase 4 = postpartum phase.

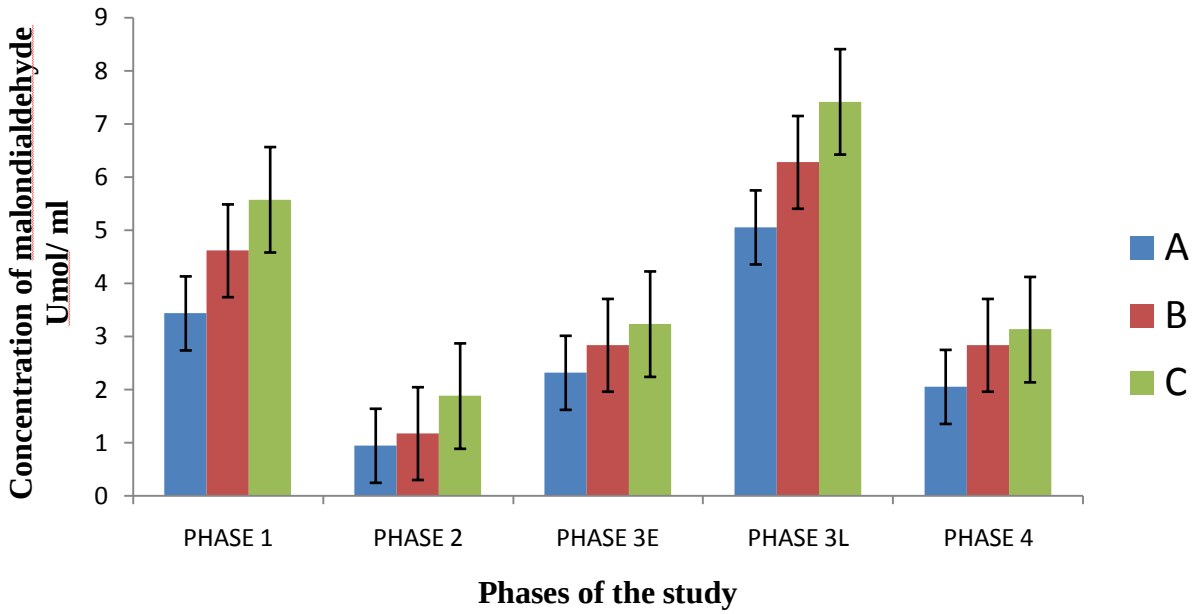


Fig.4.11.Column graph showing concentration of malondialdehyde of the bitches at various phases of the study.

KEY :

A = group not insonated, B= group insonated once a week in–puppy and C= group insonated twice a week in-puppy.

Phase 1 = Acclimatization phase, Phase 2 = Induction phase, Phase 3E = Early gestation phase, Phase 3L = Late gestation phase and Phase 4 = postpartum phase.

4.4. Puppies physical and Serum parameters

4.4.1. Physical parameters postpartum

At whelping the litter size was recorded according to the grouping of the bitches. The total litter size for bitches in group A was 22 (7.33 ± 0.67), group B bitches had a total of 20 puppies (6.67 ± 0.67) while bitches in group C had a litter size of 16 puppies (5.33 ± 0.33). The birth weights of the puppies was also recorded according to the dam's groupings, the mean birth weights for puppies from bitches in group A was 246.4 ± 8.42 g, group B puppies had a mean birth weight of 197.50 ± 11.55 g and those from group C bitches had a mean birth weight of 189.70 ± 10.21 g (Fig. 4.12).

4.4.2. Serum parameters of puppies

The serum analysis results for the puppies showed the mean concentrations of Triiodothyronine were 4.34 ± 0.48, 2.49 ± 0.20 and 1.16 ± 0.20 ng/ml puppies from bitches in groups A, B and C respectively (Fig 4.13). While that of thyroxin was found to be 22.84 ± 6.37, 16.16 ± 1.16 and 14.24 ± 2.19 µg/ml for puppies from bitches in groups A, B and C respectively (Fig 4.14). The concentration of Superoxide dismutase was 0.149 ± 0.045, 0.248 ± 0.0318 and 0.626 ± 0.090 for Groups A, B and C respectively (Fig 4.15). Catalase concentration was found to be 27.03 ± 0.401, 25.11 ± 0.997 and 17.33 ± 5.617 µmol/min/ml for groups A, B and C respectively (Fig 4.16). The concentration of glutathione peroxidase was 74.68 ± 19.33, 71.12 ± 16.66 and 32.03 ± 2.512 µmol/ml for groups A, B and C respectively (Fig 4.17). While that of malondialdehyde was found to be 10.33 ± 0.96, 7.038 ± 0.687 and 4.781 ± 0.853 µmol/ml for groups A, B and C respectively (Fig 4.18).

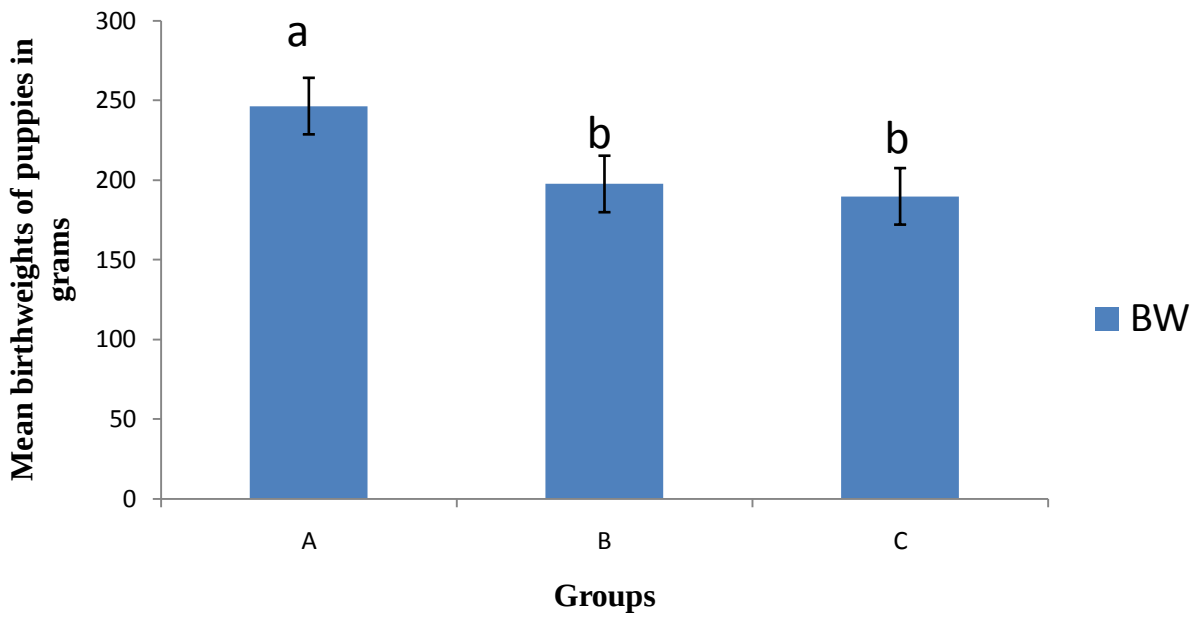


Fig 4.12. Mean birth weights of puppies from the various groups of bitches.

KEY:

A = group not insonated, B= group insonated once a week in –pup and C= group insonated twice a week in-pup

Columns with same alphabets = No statistically significant difference, while those with different alphabets = statistically significant difference.

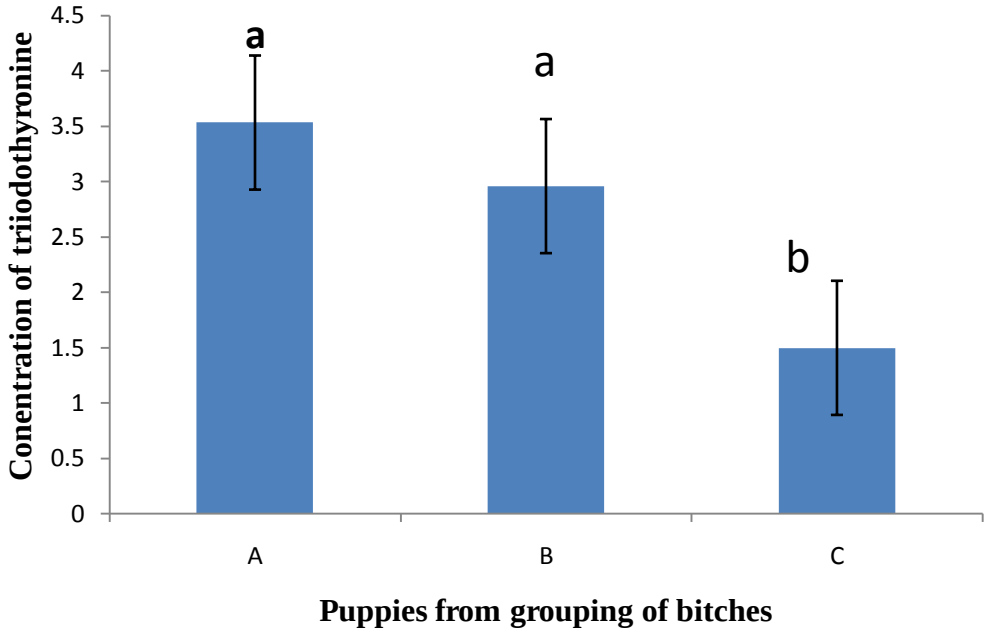


Fig 4.13 Concentration of triiodothyronine of puppies of various bitches

KEY:

A = group not insonated , B= group insonated once a week in–pup
and C= group insonated twice a week in-pup

Columns with same alphabets = No statistically significant difference, while those with different alphabets = statistically significant difference.

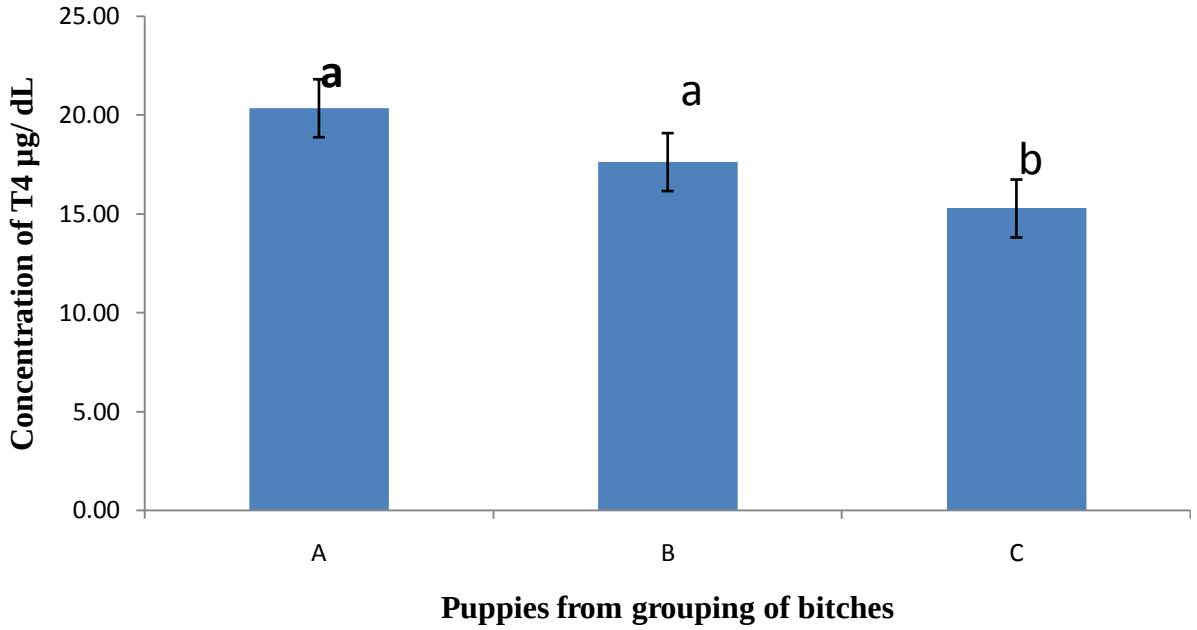


Fig 4.14 Concentration of thyroxin of puppies of various bitches

KEY:

A = group not insonated, B= group insonated once a week in –pup
and C= group insonated twice a week in-pup

Columns with same alphabets = No statistically significant difference, while those with different alphabets = statistically significant difference.

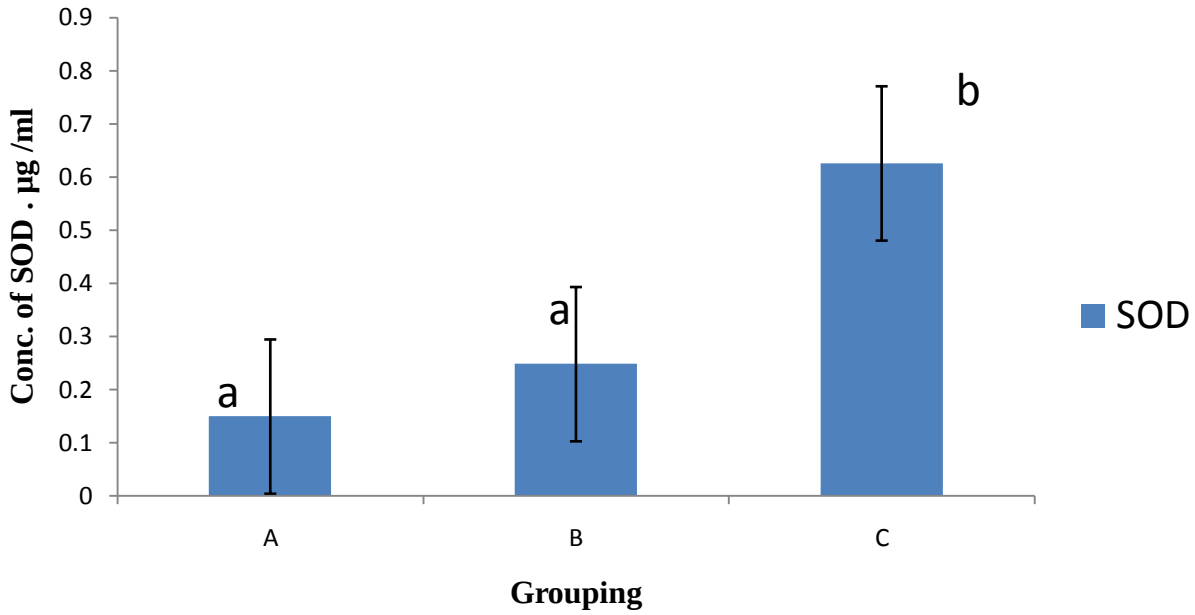


Fig 4.15 Concentration of superoxide dismutase in the puppies from various bitches

KEY:
A = group not insonated, B= group insonated once a week in –pup
and C= group insonated twice a week in-pup

Columns with same alphabets = No statistically significant difference, while those with different alphabets = statistically significant difference.

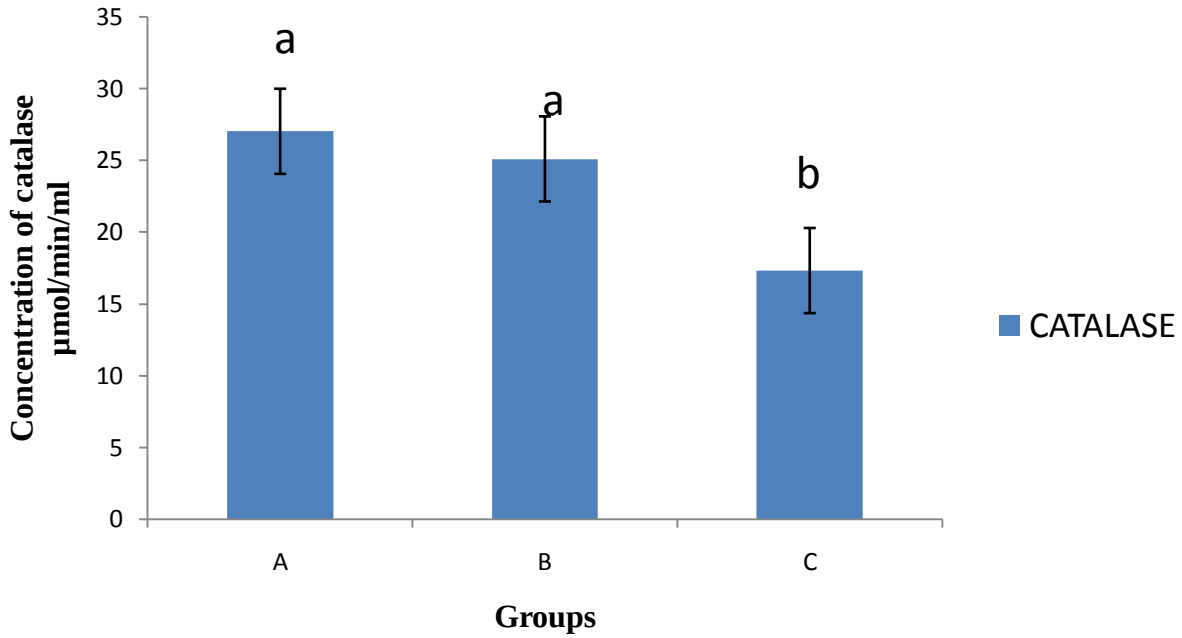


Fig 4.16 Concentration of catalase in the puppies of various bitches

KEY:

A = group not insonated, B= group insonated once a week in –pup
and C= group insonated twice a week in-pup

Columns with same alphabets = No statistically significant difference, while those with different alphabets = statistically significant difference.

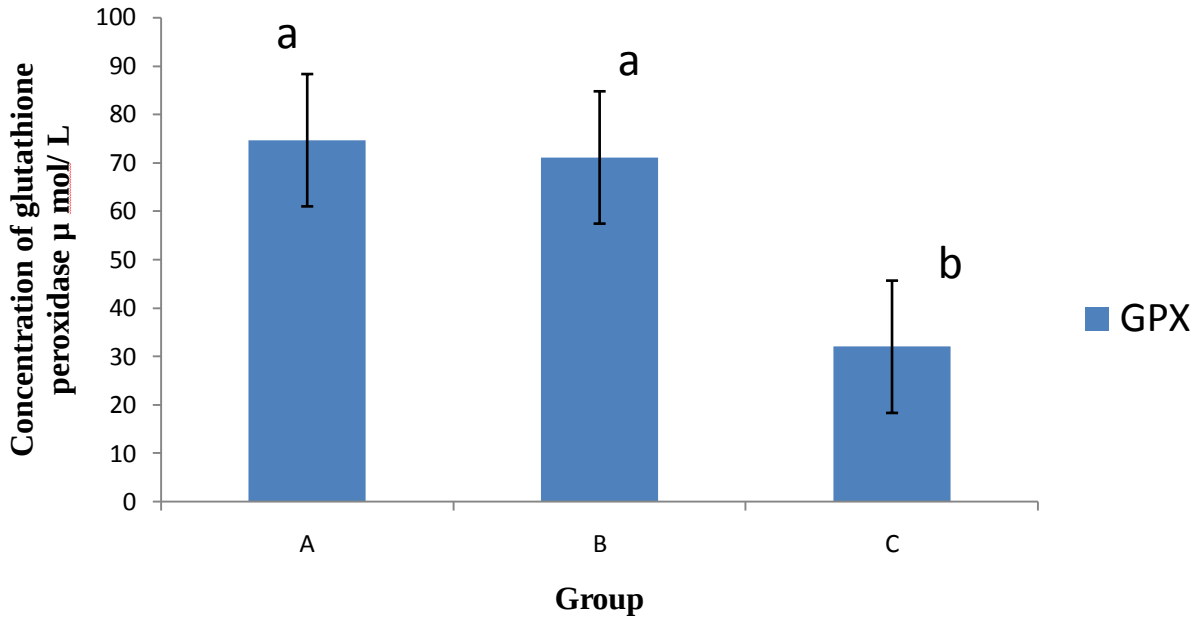


Fig 4.17 Concentration of glutathione peroxidase of puppies of various bitches

KEY:

A = group not insonated, B= group insonated once a week in –pup
and C= group insonated twice a week in-pup

Columns with same alphabets = No statistically significant difference, while those with different alphabets = statistically significant difference.

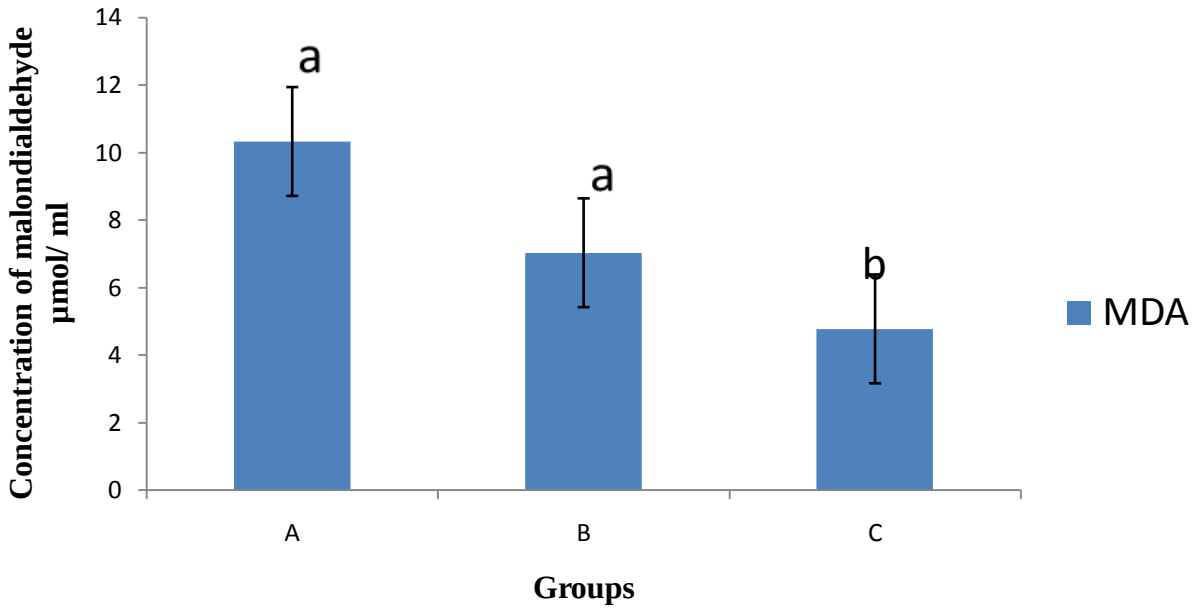


Fig 4.18 Concentration of glutathione peroxidase of puppies of various bitches

KEY:

A = group not insonated, B= group insonated once a week in –pup
and C= group insonated twice a week in-pup

Columns with same alphabets = No statistically significant difference, while those with different alphabets = statistically significant difference

CHAPTER FIVE

5.0 DISCUSSION

Ultrasonography of the internal genitalia in bitches like other mammals revealed that uterine structure are the ones easily visualised when the female is not in season, this is in cornforming with this study. These results agree with that of earlier researcher such as Yeager and Concannon 1990 and Concannon et al 2001, who reported visualising the uterus and uterine horns but not the ovaries or follicles. The failure to visualise the ovaries and follicles was due to their relative small size and absence of follicular activities which would have increased their echogenicity and thereby made them easily visualized.

We monitored the responses of the various bitches to the reconstituted cabergoline orally administered. As early as four (4) days into the treatment, there was evagination of the vulva lips. Followed closely by an increase in the size of the vulva which became larger and turgid initially and this was then followed by serosanguinous vulval discharge. This process took between 13-18 days form start of treatment. These were signs of the onset of proestrus in the bitches. These findings agree with Gobello 2002 and Ajitkumar *et al* 2009. The proestrus response to cabergoline treatment in this study was 100 % which agrees with Verstegen *et al*. (1999) and Ajitkumar *et al*. (2005) who recorded a proestrus response between 75 to 99.33 % in their studies with cabergoline. The mean time taken from initiation of treatment to onset of proestrus was found to be 15.75 ± 0 .675 days though slightly longer than that of, but which agrees with the reports of Ajitkumar *et al*. (2009). The variation seen in the onset of proestrus in our study could be attributed to the variation in the duration of anoestrus which has been known to influence the onset of proestrus in

the bitches when cabergoline was used as an agent of oestrus induction. The proestrus we observed lasted between 7-11 days from onset to the onset of oestrus with a mean period of 9.38 days in the bitches; the duration wass much lower than that reported by England and Yeager 1983 and Concannon 2011, but within the range reported by Ajitkumar *et al* 2009. This difference could be attributed to the fact that the earlier researchers reported natural proestrus duration while our study and that of the latter researchers reported on induced proestrus. Oestrus was considered from the day the bitches stood to be mated by a stud and was considered over when the bitches refused to be mated. The recorded duration for this was found to be between 7-9 days with an average period of 8 days. This was less than that of natural oestrus period but within the induced oestrus as also reported by Ajitkumar *et al* 2009. Initially the ultrasound examination did not differ from the phase 1 part of the study, but by the 10^{th} day the ovaries (right and left) started becoming visible. They appeared as roundish structures with isoechoic tissues. This changed after a few days to have mixed echogenicity due to the development of the follicles which were hyper echoic tissues surrounding an anechoic centre. The anechoic centre increased in size as proestrus advanced and the follicles became visible and could be differentiated from each other as circular anechoic structures that clustered around the ovary. The follicular walls started thickening as the follicles grew larger (and contained more fluid). The follicles were released at oestrus after which the ovaries became smaller and more isoechoic. We observed the area previously occupied by the follicles became first isoechoic and later anechoic due to the release of the follicles which was followed by the development of corpora haemorrhagica. These results agree with the report of previous research finds by Bergeron *et al* (2013), Levy and Fontbonne (2007), Eker and Salmanolu (2006) Fontbonne

and Malandain (2006) and Wallace et al 1992. The ultrasonography in the phaseIII showed that to positively confirm pregnancy diagnosis we needed to closely monitor the serviced bitches for between nineteen to twenty-one days (19-21). The sonographic appearance of the gestational sac was a roundish anechoic structure with a hyper echoic structure at the margins was a definite sign of pregnancy. This structure is usually located around the urinary bladder. The gestational sac (spherical anechoic structure, with thin and hyper echoic borders) got attached to the wall of the uterus at this point; thereafter foetal poles then started to develop distinct morphological characteristics. These morphologic characteristic appeared as hyper echoic non-uniform structures. Then around the period between days 23 to 25, an anechoic part appeared which pulsated (we first visualized foetal cardiac activities). These findings agreed with the reports of Ko, *et. al*; 2004, Concannon *et al* (2001) Kang, *et. al;* 1997, Yeager, *et. al*; 1992 and Yeager et al (1990). We counted the visualised foetal poles and our records show that there was significant difference between the insonated and non-insonated groups. The means of the foetal poles counted were 8.00 ± 0.58, 9.00 ± 0.58 and 9.00 ± 0.58 for bitches in groups A, B and C respectively. The foetuses started to show distinct change in their morphology by separation of the head from the rest of the body a distinct feature between day 29-31.The head appeared as anechoic filled structure just cranial to the forming foetal body. This agreed with the reports of Aissi and Slimani (2008) and Kim and Son (2007). The visualization of limb bud which appeared as short hyper echoic protrusion from the lateral aspects of both cranial thoracic and caudal abdominal parts was first observed between days 34 to 37 (with a mean appearance of 35.17 ± 0.48). The next important occurrence observed on ultrasound examination was foetal movement; this was first noticed on day 33 in some

bitches while in others it was on day 38 post breeding. The mean period within which this happened was 35.50 ± 0.76. These findings agreed with the reports of England and Yeager (1993), Kang et al (1997) and England and Allen (1990). We observed the development of a spherical anechoic structure at the caudal foetal pole between days 35- 39 with the mean period was noticed to be 35.50 ± 0.76 days post mating this was the foetal urinary bladder . Next structure to become visible was the foetal stomach which appeared as a spherical anechoic organ just caudal to the foetal liver; this was visualised between day's 35-39 posts mating with an average period being 36.83 ± 0.60 days. These findings were similar to those of Kim and Son (2007), Ko *et al* (2004) and Yaeger *et al* 1992. The spinal canal became visible as dorsal tubular structure between days 36 – 39g with a period means of 38.00 ± 0.37 days post mating. The eyes were visualised as distinct spherical structures with anechoic corpus vitreum, hyper echoic border between sclera and retina and spherical anechoic lens from day 39-40 post mating with a mean of 39.33 ± 0.21 days. The foetal heart grew larger and the chambers became visible between day 40- 41 post mating with a mean of 40.33 ± 0.21 day. The foetal kidneys appeared as a mixed echogenic structure on the lateral aspect of the cranial abdomen of the foetus. These were visible from day 41 -47 post mating with a mean period of 44.83 ± 0.98. The last structure to appear on ultrasound we found was the foetal intestines; they became visible between day 59- 63 post mating and they were the last organ to be visualised before the bitches whelped. The mean period was found to be 60.33 ± 0.61 days. These findings are in agreement with the reports of Aissi and Slimani (2008) and Kim and Son (2007). Immediately following parturition the uterus appeared tubular on ultrasound with highly thickened walls that were hyper echoic with anechoic intraluminal substances (lochia). The anechoic substances were quickly

expelled four to six postpartum. This agreed with Batista *et al* (2013). In most cases we were able to easily measure the thickness of the walls of the uterus on the short axis. The first week postpartum, the uterine diameter ranged from 3.510 - 4.510 with a mean of 3.944 ± 0.105 cm; this was the highest value obtained during this phase of the study. This was larger than the report of Pharr and Post (1992) who reported uterine diameters sonographically measured in the first week postpartum (2.0-2.8 cm). By second week, we recorded a mean decrease of 21.4 %, to a mean of 3.10 ± 0.116 cm. They uteri were relatively isoechoic. The uterine wall gradually became thinner and less irregular in thickness, and two patterns were noted in the appearance of the uterine contents. In some bitches, the uterine contents became more homogeneous and hypo echoic; while in others, it became more homogeneous and moderately echogenic as fluid components were expelled or resorbed in the uteri of these bitches. By the end of the third week the uteri had reduced by 51 % of the diameter of week 1. This agreed with Ahmed *et al* (2016). Following the third week postpartum uterine involution became more gradual with little or no changes in the echogenicity. The means were 1.973 ± 0.058, 1.796 ± 0.042, 1.747 ± 0.025, 1.747 ± 0.027 and 1.727 ± 0.026 cm for weeks 24 to 8 respectively. These results agree with those of Ahmed *et al* (2016) and Pharr and Post (1992).

The baseline haematological values for this study was obtained at this phase and it showed that the bitches had normal parameters from packed cells volume ranging from 38.67 to 43.00 % , Haemoglobin 12.86 to 14.30, Leucocyte count of between 40.11 to 44.33 with White blood cells counts of 9.08 to 12.05 and Total protein between 7.9 to 9.27. These haematological values served as baseline for the various bitches used in this study. The

haematology results showed an increase from Packed Cell Volume from the baseline data gotten from phase 1, and the values ranged from 36.33 to 49.83 in this phase. There was also a relative increase in other haematological parameters such as Haemoglobin concentration, Leucocyte count, White blood cell count and Total Protein was 12.07 to 16.55, 23.31 to 39.50, 8.90 to 11.53 and 6.35 to 7.37 respectively. These results do not agree with the reports of the findings by Deepthi (2010) who reported a reduction in the haematological parameters during this phase of the study. This could be as a result of the use of cabergoline for the induction of oestrus. The haematology showed that the mean Packed Cell Volumes for this phase increased mildly to between 45.67 to 51.42 % for bitches in groups A, B and C respectively. Other haematological results were: For Haemoglobin (HGB) concentration 15.69 ± 0.66, 15.18 ± 0.55 and 17.14 ± 0.60 for groups A, B and C respectively. Leucocyte count was found to be 32.83 ± 3.36, 30.25 ± 1.24 and 27.08 ± 2.54 for groups A, B and C. While the White blood cells counts (WBC) was 8.43 ± 0.40, 8.85 ± 0.64 and 10.39 ± 0.78 for groups A, B and C respectively. The Total protein (TP) result was 7.37 ± 0.44, 7.15 ± 0.35 and 6.35 ± 0.11 A, B and C respectively. This increment is not in agreement with the reports by Ajala *et al* (2011) and Deepthi (2010) that reported a decrease in the haematological parameters in early pregnancy but agreed with the report of Ahmed *et al* 2015 who reported an increase in the haematological parameters for bitches' pre-treated using cabergoline. The haematology results showed that the mean Packed Cell Volumes for this phase were significantly reduced to 42.92 ± 1.70, 40.92 ± 1.46 and 37.25 ± 1.57 % for bitches in groups A, B and C respectively. This reduction in the PCV is normal in late pregnancy where anaemia is a usual finding. Though the reduction was more in group B than group A bitches which and the most

reduction was observed in group C bitches. This could be attributed to their exposure to ultrasound in-puppy. Ultrasound when introduced to the body was absorbed especially by the bones which led to increase in body temperature which further led to regions of the RBC not containing lipids to melt thereby resulting in haemolysis which was what increased the anaemia we observed in both group B and C bitches. This result agreed with that of Isa and Dom (2016). Other haematological results were: For haemoglobin (HGB) concentration 14.39 ± 0.55, 13.06 ± 0.80 and 13.68 ± 0.5022 for groups A, B and C respectively. Leucocyte count was found to be 36.50 ± 3.173, 29.75 ± 2.004 and 32.75 ± 2.118 for groups A, B and C. While the White blood cells counts (WBC) was 8.67 ± 0.87, 9.65 ± 1.11 and 9.13 ± 1.40 for groups A, B and C respectively. The Total protein (TP) result was 6.40 ± 0.26, 6.100 ± 0.2339 and 5.900 ± 0.1642 A, B and C respectively this result agrees with that of Isa and Dom (2016). There was an increase in the haematological parameters in this phase when compared to the late phase 3. This could be due to the absence of the pregnancy on the haematological values gotten, and the recovery from the pregnancy- induced anaemia. The mean Packed Cell Volumes for this phase were between 34.00 ± 2.51 and 46.89 ± 2.99 %. The Haemoglobin (HGB) concentration ranged from 11.33 ± 0.8253, and 15.59 ± 0.9939, Leucocyte count was found to be between, 28.00 ± 4.282 and 43.11 ± 4.357. While the White blood cells counts (WBC) was 7.767 ± 0.6316 and 10.43 ± 1.153, and finally Total protein (TP) result was between 7.022 ± 0.3968 and 8.333 ± 0.5270.

The serology results which served as the baseline for the bitches showed that progesterone levels at anoestrus ranged from 2.25 to 3.59 ng/ ml, this was higher than those values

reported in other canine by Tsutsui *et al*. (2006) and Concannon 2011 but agrees with the results of Bergeron et al 2013. There are no universally accepted normal ranges for biomarkers of oxidative stress, so the values we got at this phase of served as the baseline for the various bitches. For superoxide dismutase we got a range of 0.62 to 1.04 µ mol/ml that of catalase concentration was found to be 6.46 to 7.78 µmol/min/ml. The concentration of glutathione peroxidase was 20.09 to 28.30 µmol/ml. While that of malondialdehyde was between 3.43 to 5.57 µmol/ml. The serology results showed a statistically significant increase in the serum concentrations of progesterone from that observed at the first phase of the study. The means range from 20.54 to 70.51 ng/ml. This was as a result of the successful induction of oestrus in the bitches using cabergoline. The upwards spike in concentration indicated that the bitches came on heat at this phase of the study. This more than ten (10) folds increment in concentration of progesterone from the initial levels observed in phase 1 of the study agreed with the findings of earlier researchers such as Concannon (2011), England *et al* 2009. The concentrations of all the biomarkers of oxidative stress measured were found to be statistically significantly reduced in this phase of the study. The means of Superoxide dismutase ranged from 0.354 to 0.512 µgm /ml with a p-value of < 0.0001, Catalase concentration was found to be between 5.71 to 7.123 µmol/min/ml with a p-value of < 0.0001. The concentration of glutathione peroxidase was between 9.47 -12.77 µmol/ml had a p-value of 0.013. While concentrations of malondialdehyde was found to be between 0.94 - 1.876 µmol/ml with a p-value of 0.035. These significant reductions in oxidative stress we observed in this phase could be attributed to the antioxidant effects of oral administration of cabergoline which is known to

inhibit lipid peroxidation, activate glutathione peroxidase system and scavenge free radicals. These results complement those of Yoshioka *et al.* (2002).

The serology results for this phase showed the progesterone assay a significant decrease from the previous phase to between 2.32 to 3.60 ng/ml for the bitches. This decrease in progesterone concentration in pregnancy is a unique feature of canine pregnancy. The finding is in agreement with those of Concannon (2011). The concentration of Superoxide dismutase increased significantly from the previous phase to means between 1.05 to 1.47 ± 0.05 bitches at this phase. Mean Catalase concentration was found between 10.11 to 19.12 µmol/ min/ ml for the bitches. The concentration of glutathione peroxidase also significantly increased to between 41.88 to 42.59 µmol/ml. While that of malondialdehyde was found to be from 2.31 to 3.43 µmol/ ml for the various bitches. It is an established fact that pregnancy increases oxygen utilization by the bitches, and this increased demands for oxygen increase production of reactive oxygen species due to increased lipid peroxidation. All of these also led to increase in oxidative stress and an increase in the body's production of antioxidants to try and cope with the stress. These results agree with those of Argarwal *et al* (2008), Vannucci *et al* (2007) Harma *et al* 2004). The serology results for this phase showed the progesterone assay showed an increase in the concentration of progesterone to 6.54 ± 4.94, 45.19 ± 24.51 and 70.50 ± 31.50 ng/ml for groups A, B and C respectively. This phase coincided with the second spike in concentration of progesterone in pregnant bitches. This was a phenomenon only seen in bitches. The increment agrees with the findings of Concannon (2011) and Concannon

(2009). The concentration of Superoxide dismutase was 1.50 ± 0.124, 1.86 ± 0.21 and 1.90 ± 0.03 for Groups A, B and C respectively showed a significant increase as compared to the previous phase. Most especially the bitches in group C, this, we posit could have been due to the greater number of insonation which increased the oxidative stress of the bitches which led to more free radicals being produced and requiring more antioxidants to mop up. This agreed with the finding of Agarwal and Gupta (2014). Catalase concentration was found to be 26.86 ± 4.09, 27.95 ± 4.33 and 33.91 ± 5.83 µmol/min/ml for groups A, B and C respectively. The concentration of glutathione peroxidase was 60.87 ± 3.85, 60.91 ± 5.38 and 76.61 ± 8.98 µmol/ml for groups A, B and C respectively. While that of malondialdehyde was found to be 5.05 ± 0.79, 6.28 ± 2.09 and 7.41 ± 1.62 µmol/ml for groups A, B and C respectively all of these biomarkers showed significant increases in late gestation due to increased oxygen demand and changes in the consumption of the energy substrate by the mother, especially in the foetal–placental unit; this agreed with the finding of Vannucci et al (2007).

The mean progesterone concentration reduced significantly to between 1.56 ± 0.12 to 1.81 ± 0.31 ng/ml. This was the lowest concentration of progesterone we recorded throughout our study. These results agree with the reports of Concannon *et al*, (2006), Concannon (2009) who reported that the concentration of progesterone returns to basal levels postpartum. For biomarkers of oxidative stress we observed a significant reduction in their concentrations as compared to their various levels at the previous phase. With the mean concentration of Superoxide dismutase being between 0.898 ± 0.05 and 1.53 ± 0.07 µmol/ ml. Catalase concentration was found to be between 7.37 ± 4.33, and 12.00 ± 3.24 µmol/min/ml. The concentration of glutathione peroxidase was from 27.89 ± 2.88 and

34.05 ± 3.02, µmol/ml. While that of malondialdehyde was found to be between 2.049 ± 0.74 and 3.13 ± 0.99 µmol/ml.

When the bitches whelped we noted each bitches litter size and the birth weight of the puppies. The mean litter size recorded was 7. 33 ± 0.67, 6.67 ± 0.67 and 5.33 ± 0.33 puppies/ litter for bitches in groups A, B and C respectively. Even though the maximum foetal poles counted showed significant difference between the groups with group A having the lowest means, at parturition the reverse was recorded with group A having the largest litter size. This report agreed with the reports of Idris et al 2017 who reported that insonation had a negative effect on litter size in rabbits. In our case we had three groups with A being non-insonated, B insonated once a week and C insonated twice a week. At parturition the neonatal puppies were weighed and the mean birth weights for the puppies recorded. We recorded a mean birth weight of 246.4 ± 8.42, 197.5 ± 11.55 and 189.7 ± 10.21 g for puppies whelped by bitches from groups A, B and C respectively. This showed that the puppies from dams in group A were the relatively heaviest despite coming from the relatively largest litters. This result did not in agree with the findings of Mila et al (2015), who reported that the larger the litter size the smaller the mean birth weight, but agrees with the finding of Idris et al (2017), who reported a significant difference between the mean birth weights of kittens from dams that were not insonated when in-kitten and dams that were insonated. The post-partum neonatal deaths in the first two weeks showed that the percentage death by group to be 4.55, 10 and 16.67 % for groups A, B and C respectively. We observed that the puppies that died were the mostly the runts of the litter. This agreed with the findings of Mila et al (2015), Devillers et al., (2011) Lawler, (2008);

and Indrebø et al., (2007) who observed that most neonatal deaths occurred in puppies with the least birthweights I n a litter. The increase in the percentage of postpartum deaths in group C could be due to the stress the puppies under went in-utero from multiple insonation when compared to other puppies. This could be due to the fact that in-utero insonation resulted in increased temperature which caused increased oxidative stress which also caused intrauterine growth retardation that led to the puppies being much smaller in birth weight thereby reducing their chances of survival.

The serum collected from the puppies was analysed for Thyroid hormones and biomarkers of oxidative stress, the results showed that both Triiodothyronine (with means of 4.34 ± 0.48, 2.49 ± 0.20 and 1.16 ± 0.20 ng/m for puppies from bitches in groups A,B and C respectively) and thyroxin (with means of 22.84 ± 6.37, 16.16 ± 1.16 and 14.24 ± 2.19 µg/ml for puppies from bitches in groups A, B and C respectively) were significantly different in puppies from the three groups with group A having the highest concentration followed by B and the puppies with the least concentration were those of group C. According to Terranova (2009), formation of thyroid glands begins at the middle of gestation till the end stage of gestation. Hence, external disruption like in-utero heating due to insonation could possibly affect the development of the glands, thus its secretion (Dom *et al*., 2013). These results agree with the findings of Idris *et al* (2017), who reported that there was a difference between the T3 and T4 hormones of the new kittens from dams not insonated and those insonated when pregnant.

The biomarkers of oxidative stress in the puppies showed a significant difference in concentration between the puppies from non-insonated bitches and those of insonated

bitches. The concentration of SOD was 0.149 ± 0.045 µg/ ml for puppies from bitches in group A, 0.248 ± 0.0318 µg/ ml for puppies from group B and 0.626 ± 0.090 µg/ ml for puppies from bitches in group C. Catalase concentration was found to be 27.03 ± 0.401, 25.11 ± 0.997 and 17.33 ± 5.617 µmol/min/ml for groups A, B and C respectively. The concentration of glutathione peroxidase was 74.68 ± 19.33, 71.12 ± 16.66 and 32.03 ± 2.512 µmol/ml for groups A, B and C respectively. While that of malondialdehyde was found to be 10.33 ± 0.96, 7.038 ± 0.687 and 4.781 ± 0.853 µmol/ml for groups A, B and C respectively. These results showed that though the puppies were not directly insonated, they were still exposed to oxidative stress from the in-utero insonation. This result agrees with that of Isa and Dom (2016).

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

1. This study has shown that cabergoline is a very potent agent for inducing oestrus in Nigerian indigenous bitches.
2. There is statistically significant effect of multiple in-utero insonation on maximum fetal poles counted
3. This study has shown that there is statistically significant difference in litter size between the groups
4. There is a significant difference between means of maximum foetal poles visualized and means of litter size in groups with multiple in-utero insonation
5. There is progressive increase of percentage post-parturient deaths from groups A to C.
6. Cabergoline is a very potent antioxidant in our indigenous bitches
7. Multiple in utero insonation significantly affected birth weight of puppies negatively
8. Multiple insonation negatively affected haematological profile of Dams
9. Multiple insonation caused oxidative stress in the dams especially at late gestation
10. In-utero insonation also caused oxidative stress in puppies
11. In-utero insonation negatively affected levels of T3 and T4 hormones in puppies

6.2 Recommendations

1. Further studies should be carried out on a larger population of bitches
2. More reproductive hormones should be studied in the dams
3. More reproductive parameters should be studied
4. More hormone should be studied in the neonatal puppies

6.3 Limitations

1. Funding
2. Lack of adequate electricity supply
3. High cost of laboratory analysis of serum
4. Lack of state of the art laboratory within easy reach and at affordable prices

REFERENCES

Achiron, R., Pinhas-Hamiel, O., Zalel, Y., Rotstein, Z. and Lipitz, S. (1998). Development of foetal male gender: prenatal sonographic measurement of the scrotum and evaluation of testicular descent. *Ultrasound in Obstetrics and Gynecology*, *11*, 242–245.

Adams.G. P. and Ratto, M. H. (2013). Ovulation-inducing factor in seminal plasma: a review.*Animal Reproduction Science, 136*, 148–156.

Agarwal, A., & Gupta, S. (2005). Role of reactive oxygen species in female reproduction. Part I. Oxidative stress: a general overview. Agro Food Industry Hi-Tech, 16(1), 21-25.

Agarwal A, Gupta S, Sekhon L, Shah R.(2008): Redox considerations in female reproductivefunction and assisted reproduction: from molecular mechanisms to health implications. Antioxid Redox Signal;10: 1375–403.

Ahmed. M.N., Ynzeel, J. H and A. F. Majeed. (2016): Ultrasonographic study of uterine involution in of Awassi ewes in Iraq. Al-Anbar J. Vet. Sci., Vol.: 9 No. (1), 16-21

Aissi, A. and Slimani, C. (2008). Time of initial detection of foetal structures and anatomic differentiation by using B-mode ultrasound examination in bitches. *Pakistan Journal of Biological Sciences,11*, 1750–1753.

Aissi, A., & Slimani, C. (2008). Ultrasonographic appearance of the gestational structures throughout pregnancy in bitches. Am J Anim Vet Sciences, 3(1), 32-35.

Ajala O.O., Fayemi, O.O. and Kolawole, T.O.(2011): Erythrocytic parameters as indicators for differentiating between the pregnant and pseudopregnant bitches in Nigeria. *Nig. J. Physiol. Sci*. 26: 019 – 022

Ajala, Oluwatoyin. O., Fayemi, O.E and Oyeyemi, M. O (2012). Some reproductive indices of the Nigerian local bitches in Ibadan, Nigeria. *Niger. J. Physiol. Sci*. 27.049 – 053.

Ajitkumar, G., Sreekumaran, T., Praseeda, R., Mercy, K. A., & Ghosh, K. A. (2010). Comparative efficacy of bromocriptine, cabergoline and thyroxine in inducing oestrus in bitches. *Veterinary research communications*, 34(1), 65-69.

Alatas, C., Aksoy, E., Akarsu, C., Yakin, K. and Bahçeci, M. (1996). Hemodynamic assessment in pelvic inflammatory disease by transvaginal color Doppler ultrasonography. *European Journal of Obstetrics and Gynecology and Reproductive Biology*, *70*, 75–78.

Albrecht, T., Blomley, M. J. K., Heckemann, R. A., Cosgrove, D. O., Jayaram, V., Butler-Barnes, J., Eckersley, R. J., Hoffmann, C. W., and Bauer, A. (2000). Stimulated acoustic emission with the ultrasound contrast agent Levovist: a clinically useful contrast effect with liver-specific properties. *Rofo-Fortschritte Auf dem Gebiet der Rontgenstrahlen und der Bildgebenden Verfahren*, *172*, 61–67.

Allen, A. K. and Stone, L. R. (1990). Equine diagnostic ultrasonography - equipment selection and use. *Compendium of Continueing Education Practicing Veterinarian*. *12*(9), 1307-1311.

Andersen, A. C. and Simpson, M. E. (1973). The ovary and reproductive cycle of the dog (Beagle). Los Altos, California: Geron-X, Inc.

Arbeiter, K., Brass, W., Ballabio, R. and Jöchle, W. (1988). Estrus induction and fertility with cabergoline, a prolactin inhibitor, in the bitch. *Journal of Small Animal Practices,29*, 781-788.

Arnold, S., Arnold, P., Concannon, P. W., Weilenmann, R., Hubler, M., Casal, M., ... & Rüsch, P. (1989). Effect of duration of PMSG treatment on induction of oestrus, pregnancy rates and the complications of hyper-oestrogenism in dogs. Journal of reproduction and fertility. Supplement, 39, 115-122.

Arshadi, R., Yu, A. C. H. and Cobbold, R. S. C. (2007). Coded excitation methods for ultrasoundharmonic imaging. *Canadian Acoustic,35*, 35–46.

Ascoli, M. (2005). Learning new tricks from an old dog: the processing of the intracellular precursor of the luteinizing hormone receptor (LHR) into the mature cell-surface LHR is a regulated process. Endocrinology, 146(8), 3221-3223.

Ascoli, M., Fanelli, F. and Segaloff, D. L. (2002). The lutropin/choriogonadotropin receptor, a perspective. *Endocrine Reviews*, *23*, 141-174.

Assidi, M., Dieleman, S. J. and Sirard, M. A. (2010). Cumulus cell gene expression following the LH surge in bovine preovulatory follicles: potential early markers of oocyte competence. *Reproduction*, *140*, 835-852.

Averkiou, M. A. and Hamilton, M. F. (1997). Nonlinear distortion of short pulses radiated by plane and focused circular pistons. *The Journal of the Acoustical Society of America,102*, 2539–2548.

Baithalu, R. K., Singh, S. K., Gupta, C., Raja, A. K., Saxena, A., Kumar, Y., Singh, R. and Agarwal, S. K. (2013). Cellular and functional characterization of buffalo (*Bubalus bubalis*) corpus luteum during the estrous cycle and pregnancy.*Animal Reproduction Science*, *140*, 138-146.

Barber, M. R., Lee, S. M., Steffens, W. L., Ard, M., & Fayrer-Hosken, R. A. (2001). Immunolocalization of zona pellucida antigens in the ovarian follicle of dogs, cats, horses and elephants. Theriogenology, 55(8), 1705-1717.

Barrett, E. (2011). Bladder and urethra. In: Barr F, Gaschen L, editors. BSAVA manual of canine and feline ultrasonography. Gloucester (United Kingdom). *British Small Animal Veterinary Association,* 155–164

Barthez, P. Y., Le´ veille´, R. and Scrivani, P. V. (1997). Side lobes and grating lobes artefacts in ultrasound imaging. *Veterinary Radiology and Ultrasound*, *38*(5), 387–393.

Batista, P. R., Gobello, C., Corrada, Y., Pons, E., Arias, D. O. and Blanco, P. G. (2013). Doppler ultrasonographic assessment of uterine arteries during normal canine puerperium. *Animal Reproduction Science,141*, 172–176.

Batista, P. R., Gobello, C., Rube, A., Corrada, Y. A., Tórtora, M. and Blanco., P. G. (2016). Uterine blood flow evaluation in bitches suffering from cystic endometrial hyperplasia (CEH) and CEH-pyometra complex. *Theriogenology,85*, 1258–1261

Batista, P.R. Gobello, C. Corradab, Y. Ponsc, E Ariasa, D.O. Blancoa.P.G (2015) Doppler ultrasonographic assessment of uterine arteries during normal canine puerperium. Animal Reproduction Science. 141.172– 176

Baumans, V., Dijkstra, G. and Wensing, C. J. (1981). Testicular descent in the dog. *Anatomia, Histologia, Embryologia*, *10*, 97–110.

Beccaglia, M., and Luvoni, G. C. (2004). Ultrasonographic study during pregnancy of the growth of an encephalic portion in the canine foetus. *Veterinary Research Communications, 28*, 161-164

Beccaglia, M. and Luvoni, G. C. (2006). Comparison of the accuracy of two ultrasonographic measurements in predicting the parturition date in the bitch. *Journal of Small Animal Practice*, *47*, 670–673.

Beccaglia, M., Faustini, M., and Luvoni, G. C. (2008). Ultrasonographic study of deep portion of diencephalo-telencephalic vesicle for the determination of gestational age of the canine foetus. *Reproduction in Domestic Animals, 43*, 367–370.

Beccaglia, M., Alonge, S., Trovo', C. and Luvoni, G. C. (2016). Determination of gestational time and prediction of parturition in dogs and cats: an update. *Reproduction in Domestic Animals, 51*(S1), 12–17.

Bergeron, L. H., Nykamp, S. G., Brisson, B. A., Madan, P. and Gartley, C. J. (2013). An evaluation of B-mode and color Doppler ultrasonography for detecting periovulatory events in the bitch. *Theriogenology, 79*, 274-283.

Bigliardi, E., Parmigiani, E., Cavirani, S., Luppi, A., Bonati, L., & Corradi, A. (2004). Ultrasonography and cystic hyperplasia–pyometra complex in the bitch. *Reproduction in domestic animals*, *39*(3), 136-140.

Blanco, P. G., Rodríguez, R., Batista, P. R., Barrena, J. P., Arias, D. O. and Gobello, C. (2015). Bidimensional and Doppler ultrasonographic evaluation of postpartum uterine involution in the queen. *Theriogenology*, *84*, 82–85

Blanco, P. G., Tórtora, M., Rodríguez, R., Arias, D. and Gobello, C. (2011). Ultrasonographic assessment of maternal cardiac function and peripheral circulation during normal gestation in dogs. *The Veterinary Journal*, *190*, 154–159.

Bouchard, G. F., Gross, S., Ganjam, V. K., Youngquist, R. S., Concannon, P. W., Krause, G. F. and Reddy, C. S. (1993). Oestrus induction in the bitch with the synthetic oestrogen diethylstilboestrol. *Journal of Reproduction and Fertility*,*47*, 515-516.

Bouchard, G. F., Solorzan, N., Concannon, P. W., Youngquist, R. S. and Bierschwal, C. J. (1991). Determination of ovulation time in bitches based on teasing, vaginal cytology and ELISA for progesterone. *Theriogenology*, *35*, 603-611

Boyd, J. S. (1971). The radiographic identification of the various stages of pregnancy in the domestic cat. *Journal of Small Animal Practice*, *12*, 501–506.

Boyd, J. S., Renton, J. P., Harvey, M. J., Nickson, D. A., Eckersall, P. D. and Ferguson, J. M. (1993). Problems associated with ultrasonography of the canine ovary around the time of ovulation.*Journal of Reproduction and Fertility*, *47*, 101-105.

Brown, J. L. (2011). Female reproductive cycles of wild female felids. Anim.Reprod. Sci. 124, 155–162.Concannon, P.W., 2011. Reproductive cycles of the domestic bitch. *Animal Reproduction Science*, *124*, 200–210.

Brugger, N., Otzdorff, C., Walter, B., Hoffmann, B. and Braun, J. (2011). Quantitative determination of progesterone (P4) in canine blood serum using an Enzyme-linked Fluorescence Assay. *Reproduction in Domestic Animals 46*, 870–873.

Bukovsky, A., Indrapichate, K., Fujiwara, H., Cekanova, M., Ayala, M. E., Dominguez, R., Caudle, **……..** Upadhyaya, N. B. (2003). Multiple luteinizing hormone receptor (LHR) protein variants, interspecies reactivity of anti-LHR mAb clone 3B5, subcellular localization of LHR in human placenta, pelvic floor and brain, and possible role for LHR in the development of abnormal pregnancy, pelvic floor disorders and Alzheimer's disease. *Reproductive Biology and Endocrinology*, *1*, 46.

Bürstel, D., Meinecke-Tillman, S. and Meinecke, B. (2002). Ultrasonographic diagnosis of foetal sex in small ruminants bearing multiple fetuses. *Veterinary Record*, *151*, 635–636.

Bysted, B. V., Dieleman, S. J., Hyttel, P. and Greve, T. (2001). Embryonic developmental stages in relation to the LH peak in dogs. *Journal of Reproduction and Fertility*, *57*, 181-186.

Cain, J. L., Cain, G. R., Feldman, E. C., Lasley, B. L. and Stabenfeldt, G. H. (1988). Use of pulsatile intravenous administration of gonadotropin-release hormone to induce fertile estrus in bitches. *American Journal of Veterinary Research,49*, 1993-1996.

Calliada, F., Campani, R., Bottinelli, O., Bozzini, A. and Sommaruga, M. G. (1998). Ultrasound contrast agents: basic principles. *European Journal of Radiology*,*27*(2), S157-S160.

Casanueva, E ; F.E. Viteri (2003). Iron and oxidative stress in pregnancy**.** *Journal of Nutrition*,133. 1700S–1708S.

Chappell, M. A. and Payne, S. J. (2006). A physiological model of gas pockets in crevices and their behavior under compression. *Respiratory Physiology and Neurobiology*, *152*, 100–114.

Chapwanya, A., Clegg, T., Stanley, P. and Vaughan, L. (2008). Comparison of the Immulite and RIA assay methods for measuring peripheral blood progesterone levels in Greyhound bitches. *Theriogenology*,*70*, 795–799.

Chastant-Maillard, S., Chebrout, M., Thoumire, S., Saint-Dizier, M., Chodkiewicz and M., Reynaud, K. (2010). Embryo biotechnology in the dog: a review. *Reproduction, Fertility, and Development,* 22(7), 1049–1056.

Chastant-Maillard, S., de Lesegno, C. V., Chebrout, M., Thoumire, S., Meylheuc, T., Fontbonne, A., Chodkiewicz, M., Saint-Dizier, M. and Reynaud, K. (2011). The canine oocyte: uncommon features of *in vivo* and *in vitro* maturation. *Reproduction, Fertility, and Development*, *23*, 391-402.

Chastant-Maillard, S., Aggouni, C., Albaret, A., Fournier, A., & Mila, H. (2017). Canine and feline colostrum. *Reproduction in Domestic Animals*, 52, 148-152.

Chatdarong, K., Rungsipipat, A., Axnér, E., Linde and Forsberg. C. (2005). Hysterographic appearance and uterine histology at different stages of the reproductive cycle and after progestagen treatment in the domestic cat. *Theriogenology, 64*, 12–29.

Chatdarong, K., Tummaruk, P., Sirivaid-yapong, S. and Raksil, S. (2007). Seasonal and breed effects on reproductive parameters in bitches in the tropics: a retrospective study. *Journal of Small Animal Practice*,*48*:444–448.

Chiao, R. Y. and Hao, X.(2005). Coded excitation for diagnostic ultrasound: a system developer's perspective, *IEEE Transactions on Ultrasonics, Ferroelectrics, and Frequency Control, 52*, 160–170.

Clarke, P. R. and Hill, C. R. (1970). Physical and chemical aspects of ultrasonic disruption of cells. *The Journal of the Acoustical Society of America*, *47*, 649–653.

Coltera, M. (2010): Ultrasound physics in a nutshell. *Otolaryngologic Clinics of North America*, *43*(6), 1149–1159.

Concannon, P. W. and Hansel, W. (1977). Prostaglandin F2alpha induced luteolysis, hypothermia, and abortions in beagle bitches. *Prostaglandins,13*, 533–542.

Concannon, P. W., Powers, M. E., Holder, W. and Hansel, W. (1977). Pregnancy and parturition in the bitch. *Biology of Reproduction, 16*, 517–526.

Concannon, P. W., Butler, W. R., Hansel, W., Knight, P. J., and Hamilton, J. M. (1978). Parturition and lactation in the bitch: Serum progesterone, cortisol and prolactin. *Biology of Reproduction, 19*, 1113–1118

Concannon, P. W. (1989). Induction of fertile oestrus in anoestrus dogs by constant infusion of GnRH agonist. *Journal of Reproduction and Fertility,39*, 149-160.

Concannon, P. W. (1993). Biology of gonadotrophin secretion in adult and prepubertal female dogs. *Journal of Reproduction and Fertility*, *47*(l), 3-27.

Concannon, P. W. (2000) Canine pregnancy: Predicting parturition and timing events of gestation. In P. W. Concannon, G. England, J. Verstegen III and C. Linde-Forsberg (Eds.), Recent advances in small animal reproduction. Ithaca, NY: International Veterinary Information Service. [A1202.0500 (http://www.ivis.org)].

Concannon, P. W. (2009). Endocrinologic control of normal canine ovarian function*Reproduction in Domestic Animals*, *44* (2), 3-15.

Concannon, P. W. (2011). Reproductive cycles of the domestic bitch. *AnimalReproduction Science*, *124*(3/4), 200–210.

Concannon, P. W. (2012). Research challenges in endocrine aspects of canine ovariancycles *Reproduction in Domestic Animals*, *4* (6), 6-12.

Concannon, P. W., McCann, J. P. and Temple, M. (1989). Biology and endocrinology of ovulation, pregnancy and parturition of dog. *Journal of Reproduction and Fertility*, 39, 3-25.

Concannon, P. W., Whaley, S., Lein, D. and Wissler, R. (1983). Canine gestation length: variation related to time of mating and fertile life of sperm. *American Journal of Veterinary Research*, *44*, 1819–1821.

Concannon, P., Lasley, B. and Vanderlip, S. (1997). LH release, induction of oestrus and fertile ovulations in response to pulsate administration of GnRH to anoestrous dogs. *Journal of Reproduction and Fertility*, *51*, 41-54.

Cools, S., Van den Broeck, W., De Vliegher, S., Piepers, S. and Opsomer, G. (2013). The bovine luteal histological composition: a topographic point of view.*Reproduction in Domestic Animals, 48*(2), 29-32.

Cosgrove, D. (2006). Ultrasound contrast agents: An overview. *European Journal of Radiology*, 60, 324–330.

Coussios, C. C. and Roy, R. A. (2008). Applications of acoustics and cavitation to noninvasive therapy and drug delivery. *Annual Review of Fluid Mechanics, 40*, 395–420.

Coussios, C. C., Farny, C. H., ter Haar, G., and Roy, R. A. (2007). Role of acoustic cavitation in the delivery and monitoring of cancer treatment by high-intensity focused ultrasound (HIFU). *International Journal of Hyperthermia, 23*, 105–120.

Coussios, C. C., Holland, C. K., Jakubowska, L., Huang, S. L., MacDonald, R. C., Nagaraj, A., and McPherson, D. D. (2004). *In vitro* characterization of liposomes and OptisonH by acoustic scattering at 3.5 MHz. *Ultrasound in Medicine and Biology, 30*, 181–190.

Culp, W. C., Porter, T. R., Lowery, J., Xie, F., Roberson, P. K., and Marky, L. (2004). Intracranial clot lysis with intravenous micro bubbles and transcranial ultrasound in swine. *Stroke, 35*, 2407–2411.

Curran, S. (1992). Foetal sex determination in cattle and horses by ultrasonography. *Theriogenology, 37*, 17–21.

Curran, S., Kastelic, J. P. and Ginther, O. J. (1989). Determining sex of the bovine fetus by ultrasonic assessment of the relative location of the genital tubercle. *Animal Reproduction Science, 19*, 217–227.

Corso, J. F. (1963). "Bone-conduction thresholds for sonic and ultrasonic frequencies".*Journal of the Acoustical Society of America* **35** (11): 1738–1743.

Dacie, J.V., Lewis, S.M., 1991. In: Dacie, J.V., Lewis, S.M. (Eds.), BasicHaematological Techniques Practical Haematology. , 7th Ed. ChurchillLivingstone, pp. 1–67

Dall'Aglio, C., Pascucci, L., Mercati, F., Polisca, A., Ceccarelli, P. and Boiti, C. (2012a).Immunohistochemical detection of the orexin system in the placenta of cats. *Research in Veterinary Science, 92*, 362–365.

Dall'Aglio, C., Polisca, A., Boiti, C. and Ceccarelli, P. (2012b). Immunolocalization ofleptin and its receptor in the placenta of cats. *Acta Histochemica, 114*, 719–722.

Daniel, G. (2010). Physical principles: In: *BSAVAManual of Canine and Feline Ultrasonography*. Ed. Barr. F.and Gaschen. Pp 15-16. BSAVA Publication Gloucester.

Datta, S., Coussics, C. C., Ammi, A. Y., Mast, T. D., de Court, and Holland, C. K. (2008). Ultrasoundenhanced thrombolysis using DefinityH as a cavitation nucleation agent. *Ultrasound in Medicine and Biology*, *34*, 1421–1433.

Davidson, A. P. and Baker, T. W. (2009). Reproductive ultrasound of the bitch and queen. *Topics in Companion Animal Medicine, 24*(2), 55-63.

de Gier, J., Kooistra, H. S., Djajadiningrat-Laanen, S. C., Dieleman, S. J. and Okkens A. C. (2006). Temporal relations between plasma concetrations of luteinizing hormone,follicle-stimulating hormone, estradiol-17β, progesterone, prolactin, and α-melanocyte-stimulating hormone during the follicular, ovulatory, and early luteal phase in the bitch*Theriogenology*, *65*, 1346-1359.

De los Reyes, M., Palomino, J., Parraguez, V. H. and Ramirez, F. (2017). Analysis of LH receptor in canine ovarian follicles throughout the estrous cycle. *Theriogenology,93*, 71-77.

Deepthi L. (2010). *Diagnostic and Therapeutic approaches for enhancing reproductive efficiency in female dogs.* (Doctoral Thesis). http://krishikosh.egranth.ac.in/handle/1/5810027207

Desser, T. S. and Jeffrey, R. B. (2001). Tissue harmonic imaging techniques: physicalprinciples and clinical applications. *Seminars in Ultrasound, CT, and MRI*, *22*, 1–10.

Devillers, N., J. Le Dividich, and A. Prunier. (2011). Influence of colostrum intake on piglet survival and immunity. Animal 5: pp1605–1612.

Dom SM, Razak HR, Zaiki FW, Saat NH, Manan KA,Isa IN & Hashim UF (2013). Ultrasoundexposure during pregnancy affects rabbitfoetal parathyroid hormone (PTH) level.Quantitative Imaging in Medicine andSurgery, 3(1): 49-53.

Douglass, J. P. (1993). Bladder wall mass effect caused by the intramural portion of the canine ureter. *Veterinary Radiology and Ultrasound*, *34*(2), 107.

Douglass, J. P. and Kremkau, F. W. (1993). Ultrasound corner—the urinary bladder wall hypoechoic pseudolesion. *Veterinary Radiology and Ultrasound*, *34*(1), 45–46.

Ducker, M. J., Thwaites, C. J. and Bowman, J. C. (1970). Photoperiodism in the ewe: 2. The effects of various patterns of decreasing daylength on the onset of oestrus in clun forest ewes. *Animal Production, 12*, 115–123.

EKER. Kemal, SALMANOLU, Mehmet R›fat. (2006). Ultrasonographic Monitoring of Follicular Development, Ovulation and Corpora lutea Formation in a Bitch. *Turk. J. Vet. Anim. Sci*. 30 589-592

Elder, S. A., Kolb, J., and Nyborg, W. L. (1954). Smallscale acoustic streaming effects in liquids. *The Journal of the Acoustical Society of America*, *26*, 933–933.

Elder, S. and Nyborg, W. L. (1956). Acoustic streaming resulting from a resonant bubble. *The Journal of the Acoustical Society of America*, *28*, 155–155.

Elejalde, B. R., de Elejalde, M. M. and Heitman, T. (1985). Visualization of the foetal genitália by ultrasonography: a review of the literature and analysis of its accuracy and ethical implications. *Journal of Ultrasound in Medicine*, *4*, 633–639.

Emoto, M., Tachibana, K., Iwasaki, H., and Kawarabayashi, T. (2007). Antitumor effect of TNP- 470, an angiogenesis inhibitor, combined with ultrasound irradiation for human uterine sarcoma xenografts evaluated using contrast color Doppler ultrasound. *Cancer Science*, *98*, 929–935.

Emoto, M., Tamura, R., Shirota, K., Hachisuga, T. and Kawarabayashi, T. (2002). Clinical usefulness of color Doppler ultrasound in patients with endometrial hiperplasia and carcinoma. *Cancer*, *94*, 700–706.

England, G. C. W., Allen, W. E., and Porter, D. J. (1990). Studies on canine pregnancy using B mode ultrasound: Development of the conceptus and determination of gestational age. *Journal of Small Animal Practice*, *31*, 324–329.

England G.C.W., Yeager AE (1993). Ultrasonographic appearance of the ovary and uterus of the bitch during oestrus, ovulation and early pregnancy. *Journal of Reproduction and Fertility (Suppl)*47: pp107–117. [PubMed]

England, G., Schäfer-Somi, S., Aurich J., Concannon, P. (Eds.), 2009. Canine and feline reproduction VI: reproductive biology and medicine of domestic and exotic Carnivores. ISSN 0936-6768. Reprod. Domest. Anim. 44 (Suppl. 2), 1–380.

England, G. C. W., Moxon, R. and Freeman, S. L. (2012). Delayed uterine fluid clearance and reduced uterine perfusion in bitches with endometrial hyperplasia and clinical management with post mating antibiotic. *Theriogenology*, *78*, 1611–1617.

England, G. C., Russo, M. and Freeman, S. L. (2009). Follicular dynamics, ovulation and conception rates in bitches. *Reproduction in Domestic Animals*, *44*(2), 53-58.

Evans, H. E. and Lahunta, A. (2013). Prenatal development. In: Evans HE, Lahunta A, editors. Miller's anatomy of the dog. Missouri: Elsevier Saunders. 13–60.

Fang, L., Nowicki, B. J., Dong, Y. L. and Yallampalli, C. (1999). Localized increase in nitric oxide production and the expression on nitric oxide syntethase isoforms in rat uterus with experimental intrauterine infection. *American Journal of Obstetrics and Gynecology*, *181*, 601–609.

Feigenbaum, H., Stone, J. M., Lee, D. A., Nasser, W. K., and Chang, S. (1970). Identification of ultrasound echoes from left ventricle by use of intracardiac injections of indocyanine green. *Circulation, 41,* 615–621.

Feldman E. C. and Nelson, R. W. (2004). Canine and feline endocrinology and reproduction. 3rd edn. USA: Saunders.

Feldman, M. K., Katyal, S. and Blackwood, M. S. (2009). Ultrasound artefacts, *Radiographics, 29*, 1179-1187.

Ferguson, J. M., Renton, J. P., Farstad, W. and Douglas, T. A. (1989). Insemination of beagle bitches with frozen semen. *Journal of Reproduction and Fertility*. *39*, 293-298.

Fernandes, P. A., Bowen, R. A., Kostas, A. C., Sawyer, H. R., Nett, T. M. and Olson, P. N. (1987). Luteal function in the bitch: changes during diestrus in pituitary concentration of and the number of luteal receptors for luteinizing hormone and prolactin. *Biology of Reproduction*, *37*, 804-811.

Ferreira, J. C., Gastal, E. L. and Ginther, O. J. (2008). Uterine blood flow and perfusion in mares with uterine cysts: effect of the size of the cystic area and age. *Reproduction, 135*(4), 541-550.

Ferreira, J. C., Ignácio, F. S. and Meira, C. (2010). Uterine vascular perfusion and spectral-Doppler measurements during early gestation in mares: New concepts of evaluation. *Animal Reproduction Science, 121*(S), 281-283.

Ferreira, J. C., Ignácio, F. S. and Meira, C. (2011). Doppler ultrasonography principles and methods of evaluation of the reproductive tract in mares. *Acta Scientiae Veterinariae, 39*(1), s105 - s111. ISSN 1679-9216 (Online)

Ferretti, L. M., Newell, S. M., Graham, J. P. and Roberts, G. D. (2000). Radiographic and ultrasonographic evaluation of the normal feline postpartum uterus. *Veterinary Radiology and Ultrasound*, *41*, 287–291.

Fielder, F. D. and Baker, R. F. (1969). Diagnostic applications of Doppler ultrasound. *Ultrasonics*, *7*, 36–38.

Fitz, T. A., Mayan, M. H., Sawyer, H. R. and Niswender, G. D. (1982). Characterization of two steroidogenic cell types in the ovine corpus luteum. *Biology of Reproduction, 27*(3), 703-711.

Fontbonne A, Malandain E (2006). Ovarian ultrasonography and follow up of estrus in the bitch and queen. Waltham Focus;16:22–29.

Fontbonne, A. (2010). Infertility in bitches and queens: recent advances. *Revista brasileira de reprodução animal,35*, 202-209.

Forcada, F., Abecia, J. A. and Sierra, I. (1992). Seasonal changes in oestrus activity and ovulation rate in Rasa Aragonesa ewes maintained at two different body condition levels. *Small Ruminant Research, 8*, 313–324.

Freeman, S. L., Russo, M. and England, G. C. (2013). Uterine artery blood flow characteristics assessed during oestrus and the early luteal phase of pregnant and non-pregnant bitches. *The Veterinary Journa*l, *197*, 205–210.

Fukuta, N., Kitano, M., Maekawa, K., Chikugo, T. and Kudo, M. (2005). Estimation of the malignant potential of gastrointestinal stromal tumors: The value of contrast-enhanced coded phase inversion harmonic US. *Journal of Gastroenterology, 40*, 247–255.

Fuller, J. L. (1956). Photoperiodic control of estrus in the Basenji. *Journal of Heredity,47*, 179–180.

Garcia, D. A. A., Rompkovski, M. R., Gil, E. M. U., Giannico, A. T. and Froes, T. R.(2015). Accuracy of abdominal radiography to determine fetus numbers in bitches at two gestational ages. Semina: Ciências Agrárias, *Londrina*, *36*(6), 3769-3776.

Geiser, B., Burfeind, O., Heuwieser, W. and Arlt, S. (2014). Prediction of parturition in bitches utilizing continuous vaginal temperature measurement. *Reproduction in Domestic Animals,49*, 109–114.

Giannico, A. T., Gil, E. M., Garcia, D. A., and Froes, T. R. (2015). The use of Doppler evaluation of the canine umbilical artery in prediction of delivery time and foetal distress. *Animal Reproduction Science*, *154*, 105–112.

Gil, E. M. U., Garcia, D. A. A., Giannico, A. T. and Froes, T. R. (2015). Use of B-mode ultrasonography for foetal sex determination in dogs. *Theriogenology,84*, 875–879

Gil, E. M., Garcia, D. A., Giannico, A. T., and Froes, T. R. (2014). Canine foetal heart rate: Do accelerations or decelerations predict the parturition day in bitches? *Theriogenology, 82*, pp 933-941

Ginther, O. J. (2007). Ultrasonic Imaging and Animal Reproduction: Color-Doppler Ultrasonography, Ginther O.J. (ed). Cross Plains: Equiservices Publishing, 258p.

Ginther, O. J. and Utt, M. D. (2004). Doppler Ultrasound in Equine Reproduction: Principles, Techniques, and Potential. *Journal of Equine Veterinary Science, 24*, 516-526.

Ginther, O. J., Gastal, E. L., Gastal, M. O., Utt, M. D. and Beg, M. A. (2007). Luteal blood flow and progesterone production in mares. *Animal Reproduction Science, 99*(1-2), 213-220.

Ginther, O. J., Rodrigues, B. L., Ferreira, J. C., Araujo, R. R. and Beg, M. A. (2008). Characterization of pulses of 13, 14-dihydro- 15-keto-PGF2alpha (PGFM) and relationships between PGFM pulses and luteal blood flow before, during, and after luteolysis in mares. *Reproduction, Fertility and Development, 20*(6), 684-693.

Gniadecka, M. and Quistorff, B. (1996). Assessment of dermal water by high-frequency ultrasound: comparative studies with nuclear magnetic resonance. *British Journal of Dermatology,135*:218-224.

Gobello, C. (2006). Dopamine agonists, anti-progestins, anti-androgens, long-term-release GnRH agonists and anti-estrogens in canine reproduction: A review. Theriogenology,66(6-7), 1560-1567.

Gradial, C. M., Yeager, A. E. and Concannon, P. W. (2000). Pregnancy diagnosis in the bitch. In: Bonagura, J. D. *Kirk's Current Veterinary Therapy XIII Small Animal Practice*. Philadelphia: W. B. Saunders Company. Pp 918-923.

Gramiak, R. and Shah, P. M. (1968). Echocardiography of the aortic root. *Investigative Radiology, 3*, 356–366.

Groppetti, D., Aralla, M., Bronzo, V., Bosi, G., Pecile, A. and Arrighi, S. (2015a). Periovulatory time in the bitch: What's new to know?: Comparison between ovarian histology and clinical features. *Animal Reproduction Science*, *152*, 108-116.

Groppetti, D., Pecile, A., Arrighi, S., Di Giancamillo, A. and Cremonesi, F. (2010). Endometrial cytology and computerized morphometric analysis of epithelial nuclei: a useful tool for reproductive diagnosis in the bitch. *Theriogenology, 73*, 927-941.

Groppetti, D., Vegetti, F., Bronzo, V. and Pecile, A., (2015b). Breed-specific foetal biometry and factors affecting the prediction of whelping date in the German shepherd dog. Animal *Reproduction Science,152*, 117–122.

Grundy, S. A. and Davidson, A. P. (2004). Theriogenology question of the month. Acute metritis secondary to retained foetal membranes and a retained nonviable foetus. *American Veterinary Medical Association,224*, 844-847.

Gudermann, T., Birnbaumer, M. and Birnbaumer, L. (1992). Evidence for dual coupling of the murine luteinizing hormone receptor to adenylyl cyclase and phosphoinositide breakdown and Ca^{2}+ mobilization. Studies with the cloned murine luteinizing hormone receptor expressed in L cells. *Journal of Biological Chemistry, 267*, 4479-4488.

Gunn, R. G. and Doney, J. M. (1979). Fertility in Cheviot ewes. 1. The effect of body condition at mating on ovulation rate and early embryo mortality in North and South Country Cheviot ewes. *Animal Production, 29*, 11–16.

Hamilton, A., Rabbat, M., Jain, P., Belkind, N., Huang, S. L., Nagaraj, A., Klegerman, M., Mac-Donald, R., and McPherson, D. D. (2002). A physiologic flow chamber model to define intravascular ultrasound enhancement of fibrin using echogenic liposomes. *Investigative Radiology, 37,* 215–221.

Hamilton, M. F. and Blackstock, D. T. (1998). Nonlinear Acoustics, Academic press, San Diego, C. A., 25–39.

Haney, D. R., Levy, J. K., Newell, S. M., Graham, J. P., and Gorman, S. P. (2003). Use of foetal skeletal mineralization for prediction of parturition date in cats. *Journal of the American Veterinary Medical Association*, *223*, 1614–1616

Hangiandreou, N. J. (2003). AAPM/RSNA physics tutorial for residents. Topics in US: B-mode US: basic concepts and new technology, *Radiographics, 23,* 1019-1033.

Hariharan, P., Myers, M. R., and Banerjee, R. K. (2007). HIFU procedures at moderate intensities – effect of large blood vessels. *Physics in Medicine and Biology*, *52*, 3493–3513.

Hase, M., Hori, T., Kawakami, E., and Tsutsui, T. (2000). Plasma LH and progesterone levels before and after ovulation and observation of ovarian follicles by ultrasonographic diagnosis system in dogs. *Journal of Veterinary Medical Science*, *62*, 243–248.

Hayer, P., Günzel-Apel, A. R., Lüerssen, D., and Hoppen, H. O. (1993). Ultrasonographic monitoring of follicular development, ovulation and the early luteal phase in the bitch. *Journal of Reproduction and Fertility, 47*, 93–100.

Heap, R. B. and Poyster, N. L. (1975). Prostaglandins in pyometrial fluid from the cow, bitch and ferret. *British Journal of Pharmacology, 55*, 515–518.

Heap, W. (1900). The "sexual season" of mammals and the relation of the "proestrus" to menstruation. *Quarterly Journal of Microbiological Science*, *44*(1), 61-70.

Hecht, S. (2015). Diagnostic Imaging of Lower Urinary Tract Disease. *Veterinary Clinics in Small Animal,45,* 639–663.

Heng, H. G. and Widmer, W. R. (2010). Appearance of common ultrasound artefacts in conventional vs. spatial compound imaging, *Veterinary Radiology of Ultrasound, 51*,615-621.

Heppelmann, M., Weinert, M., Brömmling, A., Piechotta, M., Hoedemaker, M. and Bollwein, H. (2013). The effect of puerperal uterine disease on uterine involution in cows assessed by Doppler sonography of the uterine arteries. *Animal Reproduction Science*, *143,* 1–7.

Hilgenfeldt, S., Lohse, D., and Zomack, M. (2000). Sound scattering and localized heat deposition of pulsedriven micro bubbles. *The Journal of the Acoustical Society of America*, *107*, 3530–3539.

Hill, C. R., Bamber, J., and ter Haar, G. (Eds) (2004). Physical principles of medical ultrasound, ch. 3 (Wiley, Chichester).

Hindi, A., Paterson, C. and Barr, R. G. (2013). Artefacts in diagnostic ultrasound. *Reports in Medical Imaging*,*6*, 29–48.

Hirooka, Y., Naitoh, Y., Goto, H., Ito, A., Taki, T. and Hayakawa, T. (1997). Usefulness of contrast-enhanced endoscopic ultrasonography with intravenous injection of sonicated serum albumin. *Gastrointestinal Endoscopy*,*46*, 166–169.

Hirt, R. A., Kneissl, S. and Teinfalt, M. (2000). Severe hypercalcemia in a dog with a retained fetus and endometritis. *Journal of the American Veterinary Medical Association*, *216*, 1423–1425.

Hohl, C., Schmidt, T., Haage, P., Honnef, D., Blaum, M., Staatz, G. and Guenther, R. W. (2004). Phase-inversion tissue harmonic imaging compared with conventional Bmode ultrasound in the evaluation of pancreatic lesions. *European Radiology*,*14*, 1109–1117.

Holder, R. D. (2003). Foetal sex determination. *Journal of Equine Veterinary Science*, *23*, 414-419.

Holst, P. A. and Phemister, R. D. (1971). The prenatal development of the dog. Preimplantation events. *Biology of Reproduction*, *5*, 771-779.

Hornbuckle, W. E. and Kleine, L. J. (1980). Medical management of prostatic disease. In: Kirk K.W, editor. Current Veterinary Therapy: Small Animal Practice. VII. Philadelphia, PA: W.B. Saunders Co; 1146–1150.

Hou, X., Arvisais, E. W. and Davis, J. S. (2010). Luteinizing hormone stimulates mammalian target of rapamycin signaling in bovine luteal cells via pathways independent of AKT and mitogen-activated protein kinase: modulation of glycogen synthase kinase 3 and AMP-activated protein kinase. *Endocrinology*, *151*, 2846-2857.

Huang, S. L. (2008). Liposomes in ultrasonic drug and gene delivery. *Advanced Drug Delivery Reviews*, *60*, 1167–1176.

Hwang, J. and Simpson, D. H. (1999). Two pulse technique for ultrasonic harmonic imaging, US Patent No. 5, *951*,478.

Idris, S. Y., Audu, H. A., Fadason, S. T., Lawal, M., Ibinaiye, P. O., Muazu, B. N., & Okafor, R. (2017). Effect of multiple in-utero insonation on rabbit foetal thyroid hormonal level. Sokoto Journal of Veterinary Sciences, 15(4), 94-98.

Indrebø, A., C. Trangerud, and L. Moe. (2007). Canine neonatal mortality in four large breeds. Acta Vet. Scand. 49(Suppl. 1):S2. doi:10.1186/1751-0147-49-S1-S2

Inoue, T., Kitano, M., Kudo, M., Sakamoto, H., Kawasaki, T., Yasuda, C. and Maekawa, K. (2007). Diagnosis of gallbladder diseases by contrast-enhanced phase-inversion harmonic ultrasonography. *Ultrasound in Medicine and Biology,33,* 353–361.

Isa, I. N. C., & Dom, S. M. (2016). Evaluation of the thermal effects of prenatal ultrasound on hematological analysis of young Oryctolagus Cuniculus. Journal of Veterinary Medical Science, 78(9), 1399-1403.

Iwanaga, K., Tominaga, K., Yamamoto, K., Habu, M., Maeda, H., Akifusa, S., Tsujisawa, T., Okinaga, T., Fukuda, J., and Nishihara, T. (2007). Local delivery system of cytotoxic agents to tumors by focused sonoporation. *Physics in Medicine and Biology*, *14*, 354–363.

Jauhiainen, J. (2009). The Physical Principles of Medical Imaging: Sprawls Educational Foundation. *Tekniikan yksikkö Hyvinvointiteknologia.* 10-11. Retrieved from www.sprawls.org.

Jeffcoate, I. A. (1993). Endocrinology of anestrous bitches. *Journal of Reproduction and Fertility,47*, 69-76.

Jemec, G. B., Gniadecka, M. and Ulrich, J. (2000). Ultrasound in dermatology. Part I. High frequency ultrasound. *European Journal of Dermatology,10,* 492-497.

Jeppesen, J. V., Kristensen, S. G., Nielsen, M. E., Peter, H., Dal Canto, M., Fadini, R., Schmidt, K. T., Ernst, E. and Yding, A. C. (2012). LH-receptor gene expression in human granulosa and cumulus cells from antral and preovulatory follicles. *The Journal of Clinical Endocrinology and Metabolism,97*, E1524-1531.

Jiang, P., Mao, Z. and Lazenby, J. C. (1998). A new tissue harmonic imaging scheme with better fundamental frequency cancellation and higher signal-to-noise ratio, in: *Proceedings of IEEE Ultrasononic Symposium*, Sendai, Japan, pp. 1589–1594.

Jöchle, W., Arbeiter, K., Post, K., Ballabio, R. and D'Ver, A. S. (1989). Effects on pseudopregnancy, pregnancy and interoestrous intervals of pharmacological suppression of prolactin secretion in female dogs and cats. *Journal of Reproduction and Fertility,39*, 199-207,

Johnson, A. E., Freeman, E. W., Colgin, M., McDonough, C. and Songsasen, N. (2014). Induction of ovarian activity and ovulation in an inducedovulator, the maned wolf (*Chrysocyon brachyurus*), using GnRH agonist and recombinant LH. *Theriogenology, 82*, 71–79.

Johnston, S. D., Root Kustritz, M. V. and Olson, P. N. S. (2001a). The canine estrous cycle Canine and Feline Theriogenology, W.B. Saunders Co, Philadelphia, PA, 16-31

Johnston, S. D., Root Kustritz, M. V. and Olson, P. N. S. (2001b). Canine parturition – eutocia and dystocia. In S. D. Johnston, M. V. RootKustritz, and P. N. S. Olson (Eds.), Canine and feline theriogenology (105–128). Philadelphia: WB Saunders.

Jones, G. E., Boyns, E. T., Bell, D. W., Christie, D. W. and Parkes, M. F. (1973). Immunoreactive luteinizing hormone and progesterone during pregnancy and following gonadotropin administration in beagle bitches. *Acta Endocrinologica,72*, 573-581.

Joone, C. J., De Cramer, K. and Nöthling, J. O. (2016). The first case of genetically confirmed monozygotic twinning in the dog. *Reproduction in domestic animals = Zuchthygiene, 51*, 835-839.

Juffermans, L. J. M., Kamp, O., Dijkmans, P. A., Visser, C. A., and Musters, R. J. P. (2008).Low-intensity ultrasound-exposed micro bubbles provoke local hyperpolarization of the cell membrane via activation of BKCa channels. *Ultrasound in Medicine and Biology, 34*, 502–508.

Kang BK, Choi HS, Son CH, Shin CR, Seo DH, Park IC (1997). Ultrasonographic appearance of the gestational structures throughout pregnancy in pet dogs. I. Time of initial detection of the foetal and extra-foetal structures. *Korean Journal of Veterinary Clinical Medicine* 14: 279–286.

Kato, T., Tsukamoto, Y., Naitoh, Y., Hirooka, Y., Furukawa, T., Hayakawa, T. (1995). Ultrasonographic and endoscopic ultrasonographic angiography in pancreatic mass lesions. *Acta Radiologica, 36*, 381–387.

Kersting, S., Konopke, R., Kersting, F., Volk, A., Distler, M., Bergert, H., Saeger, H.D., Grützmann, R. and Bunk, A. (2009). Quantitative perfusion analysis of transabdominal contrast-enhanced ultrasonography of pancreatic masses and carcinomas. *Gastroenterology,137*(6), 1903–1911.

Kim, B. S., & Son, C. H. (2007). Time of initial detection of foetal and extra-foetal structures by ultrasonographic examination in Miniature Schnauzer bitches. Journal of veterinary science, 8(3), 289-293.

Kim, D. H., Klibanov, A. L., and Needham, D. (2000a). The influence of tiered layersof surface-grafted poly- (ethylene glycol) on receptor-ligand-mediated adhesion between phospholipid monolayer-stabilized micro bubbles and coated class beads. *Langmuir*, *16*, 2808–2817.

Kim, D. Y., Lee, J. C., Kwon, S. J. and Song, T. K. (2000b). Ultrasound secondharmonic imaging with a weighted chirp signal, in: *Proceedings of IEEE Ultrasonic Symposium*, San Juan, Puerto Rico, pp. 1689–1694.

Kim, Y. H., Travis, A. J. and Meyers-Wallen, V. N. (2007). Parturition prediction andtiming of canine pregnancy. *Theriogenology*,*68*, 1177–1182.

Kirberger, R. M. (1995). Imaging artefacts in diagnostic ultrasound—a review. *Veterinary Radiology and Ultrasound*, *36*(4), 297–306.

Kitano, M., Kudo, M., Maekawa, K., Suetomi, Y., Sakamoto, H., Fukuta, N., Nakaoka, R. and Kawasaki, T. (2004). Dynamic imaging of pancreatic diseases by contrast enhanced coded phase inversion harmonic ultrasonography. *Gut*,*53*, 854–859.

Kitano, M., Kudo, M., Sakamoto, H., Nakatani, T., Maekawa, K., Mizuguchi, N., Ito, Y., Miki, M., Matsui, U. and von Schrenck, T. (2008a). Preliminary study of contrast-enhanced harmonic endosonography with second-generation contrast agents. *Journal of Medical Ultrasound*,*35*(1), 11–18.

Kitano, M., Sakamoto, H., Komaki, T. and Kudo, M. (2011). New techniques and future perspective of EUS for theDifferential diagnosis of pancreatic malignancies:Contrast harmonic imaging. *Digestive Endoscopy*,*23*(1), 46–50.

Kitano, M., Sakamoto, H., Matsui, U., Ito, Y., Maekawa, K., von Schrenck, T. and Kudo, M. (2008b). A novel perfusion imaging technique of the pancreas: Contrast-enhanced harmonic EUS (with video). *Gastrointestinal Endoscopy*,*67*, 141–150.

Ko JS, Kim BS, Lee SA, Cho YT, Kim JP, Oh KS, Kim SH…… SonCH (2004). Ultrasonographic apperance of the gestational structures throughout pregnancy in Shih-tzu bitches. I. Time of initial detection of the fetal and extra-fetal structures. *Korean Journal of Veterinary Clinics*. 21: 29–34.

Kooistra, H. S., Okkens, A. C., Bevers, M. M., Popp-Snijders, C., van Haaften, B., Dieleman, S. J. and Schoemaker, J. (1999). Concurrent pulsatile secretion of luteinizing hormone and follicle-stimulating hormone during different phases of the estrous cycle and anestrus in beagle bitches. *Biology of Reproduction*, *60*, 65-71.

Kornbluth, M. (1998). Native tissue harmonic imaging improves endocardial border definition and visualization of cardiac structures. *Journal of the American Society of Echocardiography*,*11*, 693–701.

Kremkau, F. W. (1994). Diagnostic Ultrasound: Principles and Instruments (4th edn*). British Journal of Radiology*, 67(799), 746-746.

Kremkau, F. W. (2011). Artefacts. In Kremkau FW, editor: *Sonography:Principles And Instruments*, 8th Ed., St. Louis, Elsevier Saunders, 171-176.

Kremkau, F. W., Gramiak, R., Carstens, E. L., Shah, P. M., and Kramer, D. H. (1970). Ultrasonic detection of cavitation at catheter tips. *The American Journal of Roentgenology Radium Therapy and Nuclear Medicine,*, *110*, 177–183

Krueger, L., Koerte, J., Tsousis, G., Herzog, K., Flachowsky, G. and Bollwein, H. (2009). Transrectal Doppler sonography of uterine blood flow during the first 12 weeks after parturition in healthy dairy cows. *Animal Reproduction Science*, *114*, 23–31.

Kudo, M. (2003). Various contrast-enhanced imaging modes after administration of levovist. In: Kudo, M. (ed.). *Contrast HarmonicImaging in the Diagnosis and Treatment of HepaticTumors*. Tokyo: Springer, 22–30.

Kumar, P. and Sait, S. F. (2011). Luteinizing hormone and its dilemma in ovulation induction. *Journal of Human Reproductive Sciences*, *4*, 2-7.

Kumari, S., Luthra, R. A., Chandolia, R. K.,Kumar, S., Kumar, P., Devender, Kumar, A., Bishnoi, N. and Bishnoi, S.(2016). Study on development of accessory sex glands in prepubertal kids using two-dimensional ultrasonography. *Vet World*, *9*(4), 346–350.

Kutzler, M. A. (2005). Induction and synchronization of estrus in dogs. *Theriogenology64*(3), 766-775.

Kutzler, M. A. (2007). Estrus induction and synchronization in canids and felids. *Theriogenology,68*(3), 354-374.

Kutzler, M. A., Mohammed, H. O., Lamb, S. V. and Meyers-Wallen, V. N. (2003a). Accuracy of canine parturition date prediction from the initial rise in preovulatory progesterone concentration. *Theriogenology,60*, 1187–1196.

Kutzler, M. A., Wheeler, R. and Volkmann, D. H. (2001). Serum deslorelin concentrations in bitches after Ovuplant administration. *Proceedings of the Annual Meeting of the Society ofTheriogenology,13*.

Kutzler, M. A., Wheeler, R., Lamb, S., & Volkmann, D. H. (2002, May). Deslorelin implant administration beneath the vulvar mucosa for the induction of synchronous estrus in bitches. In Proceedings of Third EVSSAR European Congress (p. 96).

Kutzler, M. A., Yeager, A. E., Mohammed, H. O., and Meyers-Wallen, V. N. (2003b). Accuracy of canine parturition date prediction using foetal measurements obtained by ultrasonography. *Theriogenology*, *60*, 1309–1317

Lamm, C. G. and Makloski, C. L. (2012). Current advances in gestation and parturition in cats and dogs. *Veterinary Clinics of North America: Small Animal Practice,42*, 445–456.

Lawler, D. F. 2008. Neonatal and pediatric care of the puppy and kitten. Theriogenology 70: 384–392. doi:10.1016/j.theriogenology. 2008.04.019

Legan, S. J. and Karsch, F. J. (1980). Photoperiodic control of seasonal breeding in ewes: modulation of the negative feedback action of estradiol. *Biology of Reproduction, 23*, 1061–1068.

Lentacker, I., De Geest, B. G., Vandenbroucke, R. E., Peeters, L., Demeester, J., De Smedt, S. C., and Sanders, N. N. (2006). Ultrasound-responsive polymer- coated micro bubbles that bind and protect DNA. *Langmuir, 22*, 7273–7278.

Leong-Poi, H., Christiansen, J., Klibanov, A. L., Kaul, S., and Lindner, J. R. (2003). Noninvasive assessment of angiogenesis by ultrasound and micro bubbles targeted to alpha(v)-integrins. *Circulation, 107*, 455–460.

Leonhardt, H., Gull, B., Stener-Victorin, E. and Hellstrom, M. (2014). Ovarian volume and antral follicle count assessed by MRI and transvaginal ultrasonography: a methodological study. *Acta Radiologica, 55*, 248-256.

Levine, H. III. (2010): *Medical Imaging*. Santa Barbara, California: ABC-CLIO, LLC. 62.

Levy X, Fontbonne A. Determining the optimal time of mating in bitches: particularities. Rev Bras Reprod Anim 2007;31:128–134.

Lighthill, J. (1978). Acoustic streaming. *Journal of Sound and Vibration, 61*, 391–418.

Linde-Forsberg, C. and Wallén, A. (1992). Effects of whelping and season of the year on the interoestrous intervals in dogs. *Journal of Small Animal Practices, 33*, 67–70.

Linde-Forsberg, C., & Persson, G. (2007). Biology of reproduction of the dog and modern reproductive technology. The Genetics of the Dog. USA: CABI Publishing. 401-432.

Lindner, J. R. (2002). Detection of inflamed plaques with contrast ultrasound. *American Journal of Cardiology, 90*, 32L–35L.

Lindner, J. R., Song, J., Xu, F., Klibanov, A. L., Singbartl, K., Ley, K., and Kaul, S. (2000). Noninvasive ultrasound imaging of inflammation using micro bubbles targeted to activated leukocytes. *Circulation, 102*, 2745–2750.

Liu, Y. B., Kon, T., Li, C. Y., and Zhong, P. (2006). High intensity focused ultrasound-induced gene activation in solid tumours. *The Journal of the Acoustical Society of America*, *120*, 492–501.

Lopate, C. (2008). Estimation of gestational age and assessment of canine foetal maturation using radiology and ultrasonography: a review. *Theriogenology*, *70*(3), 397-402.

Louvet, A. (2006). Twinkling artefact in small animal color-Doppler sonography. *Veterinary Radiology and Ultrasound*, *47*(4), 384–390.

Lu, E., Tom, E. M., Felix, M. M., Gretton, J., Varghese, R. P., Wagner, W. R., & Villanueva, F. S. (2004). 1001-33 In vivo microtubule binding to inflammatory endothelium via selectin targeting by Sialyl Lewis X. Journal of the American College of Cardiology, 5(43), A8.

Luvoni, G. C., and Beccaglia, M. (2006). The prediction of parturition date in canine pregnancy. *Reproduction in Domestic Animals*, *41*, 27–32

Luvoni, G. C., and Grioni, A. (2000). Determination of gestational age in medium and small size bitches using ultrasonographic foetal measurements. *Journal of Small Animal Practice*, *41*, 292-294.

Luvoni, G. C., Chigioni, S., Allievi, E. and Macis, D. (2005). Factors involved *in vivo* and in vitro maturation of canine oocytes. *Theriogenology*, *63*, 41-59.

MacLeod, A. G. and Wisner, E. R. (2011). Computed tomography and magnetic resonance imaging of the urinary tract. In: Bartges J, Polzin DJ, editors. Nephrology and urology of small animals. Ames (IA): Wiley-Blackwell, 146–160.

Maeda, M., Takashima, S., Takasu, M., Mori, T., Goto, N., Matsubara, T., Almunia, J., Imaeda, N., Ando, A. and Kitagawa, H. (2016). Magnetic Resonance Imaging of Ovarian Activity in Microminipigs Showing Normal Estrous Cycles. *In vivo*, *30*, 35-40.

Magata, F., Hartmann, D., Ishii, M., Miura, R., Takahashi, H., Matsui, M., Kida, K., Miyamoto, A. and Bollwein, H. (2013). Effects of exogenous oxytocin on uterine blood flow in puerperal dairy cows: the impact of days after parturition and retained foetal membranes. *The Veterinary Journal*, *196*, 76–80.

Mahi, C. A. and Yanagimachi, R. (1976). Maturation of Sperm penetration of canine ovarian oocyte *in vitro*. *Journal of Experimental Zoology*, 196, 189-196.

Mantis, P. (2008). Ultrasonography of the urinary and genital system of the dog and cat. *Iranian Journal of Veterinary Surgery* Supplement for the 2nd ISVS and 7th ISVSAR, 63-71.

Mar, G., Castagnetti, C. and Belluzzi, S. (2002). Equine foetal sex determination using a single ultrasonic examination under farm conditions. *Theriogenology*, *58*, 1237–43.

Marmottant, P. and Hilgenfeldt, S. (2003). Controlled vesicle deformation and lysis by single oscillating bubbles. *Nature*, *423*, 153–156.

Marmottant, P., Biben, T., and Hilgenfeldt, S. (2008). Deformation and rupture of lipid vesicles in the strong shear flow generated by ultrasound-driven micro bubbles. *Proceedings of the Royal Society A: Mathematical, Physical and Engineering Science*, *464*, 1781–1800.

Mattoon, J. S. and Nyland, T. G. (2014). Small Animal Diagnostic Ultrasound. Saunders Elsevier, Missouri.

McDougall, K., Hay, M. A., Goodrowe, K. L., Gartley, C. J. and King, W. A. (1997). Changes in the number of follicles and of oocytes in ovaries of prepubertal, peripubertal and mature bitches. *Journal of Reproduction and Fertility*, *51*, 25-31.

McLean, S., Brandon, S. and Kirkwood, R. (2007). Stability of cabergoline in fox baits in laboratory and field conditions. *Wildlife Research*, *34*, 239- 246.

Meagher, S. and Davison, G. (1996). Early second-trimester determination of foetal gender by ultrasound. *Ultrasound in Obstetrics and Gynecology*, 8:322–324.

Meairs, S. and Alonso, A. (2007). Ultrasound, micro bubbles and the blood–brain barrier. *Progress in Biophysics and Molecular Biology*, *93*, 354–362.

Meinecke-Tillmann, S.(2016). Basicsof ultrasonographic examination in sheep. Small Ruminant Research, 152, 10-21.

Menon, K. M. J., Clouser, C. L. and Nair, A. K. (2005). Gonadotropin receptors: role of posttranslational modifications and post-transcriptional regulation. *Endocrine*, 26, 249-257.

Menon, K. M., Munshi, U. M., Clouser, C. L. and Nair, A. K. (2004). Regulation of luteinizing hormone/ human chorionic gonadotropin receptor expression: a perspective. *Biology of Reproduction*, *70*, 861-866.

Metoki, R., Moriyasu, F., Kamiyama, N., Sugimoto, K., Iijima, H., Xu, H. X., Aoki, T., Miyata, Y., Yamamoto, K., Kudo, K., Shimizu, M., and Yamada, M. (2006). Quantification of hepatic parenchymal blood flow by contrast ultrasonography with flash-replenishment imaging. *Ultrasound in Medicine and Biology*, *32*, 1459–1466.

Meyer-Lindenberg, A., Ebermaier, C., Wolvekamp, P., Tellhelm, B., Meutstege, F. J., Lang, J., Hartung, K., Fehr, M. and Nolte, I. (2008). Comparative evaluation of six different body regions of the dog using analog and digital radiography. *Berliner Und Munchener Tierarztliche Wochenschrift*, *121*(5-6), 216-227.

Michel, E., Spörri, M., Ohlerth, S. and Reichler, I. M. (2011). Prediction of parturition date in the bitch and queen. *Reproduction in Domestic Animals*, *46*, 926-932.

Michiyo Yoshioka, Ken-ichi Tanaka, Ikuko Miyazaki, Naoko Fujita, Youichirou Higashi, Masato Asanuma, Norio Ogawa. (2002). The dopamine agonist cabergoline provides neuroprotection by activation of the glutathione system and scavenging free radicals.*Neuroscience Research*. 43. 259-267

Mila, H., Grellet, A., Feugier, A., & Chastant-Maillard, S. (2015). Differential impact of birth weight and early growth on neonatal mortality in puppies. *Journal of animal science*,93(9), 4436-4442.

Molina, C. A., Ribo, M., Rubiera, M., Montaner, J., Santamarina, E., Gado-Mederos, R., Arenillas, J. F., Huertas, R., Purroy, F., Delgado, P., and Varez-Sabin, J. (2006). Microbubble administration accelerates clot lysis during continuous 2-MHz ultrasoundmonitoring in stroke patients treated with intravenous tissue plasminogen activator. *Stroke, 37,* 425–429.

Moller, O. M. (1973). Progesterone concentrations in the peripheralplasma of the blue fox (*Alopex lagopus*) during pregnancy and the oestrous cycle. *Journal of Endocrinology,59*, 429–438.

Mondain-Monval, M., Dutourne, B., Bonnin-Laffargue, M., Canivenc, R. and Scholler, R. (1977). Ovarian activity during the anoestrus and thereproductive season of the red fox (*Vulpes vulpes* L.). *Journal of Steroid Biochemistry,8*, 761–769.

Mortensen, C. J., Kelley, D. E. and Warren, L. K. (2011). Supplemental L-arginine shortens gestation length and increases mare uterine blood flow before and after parturition. *Journal of Equine Veterinary Science*, *31*, 514–520.

Moxon, R., Batty, H., Irons, G. and England, G. C. W. (2012). Periovulatory changes in the endoscopic appearance of the reproductive tract and teasing behaviour in the bitch *Theriogenology*, *78*, 1907-1916

Mulic-Lutvica, A., Eurenius, K. and Axelsson, O. (2009). Uterine artery Dopplerultrasound in postpartum women with retained placental tissue. *Acta Obstetricia et Gynecologica Scandinavica*, *88*,724–728.

Mutembei, H. M., Mutiga, E. R. and Tsuma, V. T. (2000). A retrospective study onsome reproductive parameters of German shepherd bitches in Kenya. *Journal of the South African Veterinary Association, 71*, 115–117.

Nagashima, J. B., Sylvester, S. R., Nelson, J. L., Cheong, S. H., Mukai, C., Lambo, C.,Flanders, J. A., Meyers-Wallen, V. N., Songsasen, N. and Travis, A. J. (2015). Live Births from Domestic Dog (*Canis familiaris*) Embryos Produced by *In vitro* Fertilization. *PLoS ONE, 10*(12), e0143930.

Nageotte, M. P. (2015). Foetal heart rate monitoring. *Seminars in Foetal and NeonatalMedicine, 20,* 144–148.

Nakao, T., Yukiyo, A., Fukushima, S., Moriyoshi, M. and Kawata, K. (1985). Inductionof estrus in bitches with exogenous gonadotropins and pregnancy rate and blood progesterone profiles. *The Japanese Journal of Veterinary Science*, *47*, 17-24.

Naughton, J. E., Widmer, W. R., Constable, P. D. and Knapp, D. W. (2012). Accuracyof three-dimensional and two dimensional ultrasonography for measurement of tumor volume in dogs with transitional cell carcinoma of the urinary bladder. *American Journal of Veterinary Research, 73*(12), 1919–1924.

Nett, T. M., Akbar, A. M., Phemister, R. D., Holst, P. A., Reichert, L. E., Niswender, G. D.(1975). Levels of luteinizing hormone, estradiol and progesterone in serum during the estrous cycle and pregnancy in the beagle bitch. *Proceedings of the Society for Experimental Biology and Medicine, 148,* 134–139.

Newnham, R. E., Xu, Q. C., & Yoshikawa, S. (1991). U.S. Patent No. 4,999,819. Washington, DC: U.S. Patent and Trademark Office.Reynaud, 2012

Nothling, J. O., De Cramer, K. G. M., Gerber, D. and Kammer, V. R. (2006). Lutealand follicular count in bitches: assessment by means of magnetic resonance imaging.*Theriogenology,66***,** 1343-1354**.**

Novelline, Robert (1997). Squire's Fundamentals ofRadiology (5th ed.). Harvard University Press. 34–35. ISBN 0-674-83339-2.

Noviana, D., Aliambar, S. H., Ulum, M. F. and Siswandi, R. (2012). Small AnimalDiagnostic Ultrasound [Diagnosis Ultrasonografi pada Hewan Kecil]. Bogor: IPB Press.

Nyland, T. G. and Matton, J. S. (1995). Veterinary Diagnostic Ultrasound. Philadelphia:WB Saunders.143-265.

Nyland, T. G. and Mattoon, J. S. (2002). Ovaries and uterus. In: Nyland, T. G.,Matton, J. S., editors. Small animal diagnostic ultrasound. Second edition. Philadelphia: WB Saunders. 231–249.

O'Donnell, M. (1992). Coded excitation system for improving the penetration of realtime phased array imaging systems, *IEEE Transactions on Ultrasonics, Ferroelectrics, and Frequency Control*,*39,* 341–351.

Okkens, A. C., Teunissen, J. M., Van Osch, W., van den Brom, W. E., Dieleman, S. J. and Kooistra, H. S. (2001). Influence of litter size and breed on the duration of gestation in dogs. *Journal of Reproduction and Fertility, 57*, 193-197.

Oktay, K., Newton, H., Mullan, J., Gosden, R. G. (1998). Development of human primordial follicles to antral stages in SCID/hpg mice stimulated with follicle stimulating hormone. *Human Reproduction, 13*, 1133-1138**.**

Olson, P. N. and Nett, T. M. (1986). Reproductive endocrinology and physiology of the bitch In: Marrow DA, ed. Current therapy in theriogenology: diagnosis, treatment and prevention of reproductive diseases in small and large animals. Philadelphia: Saunders, 453–7.

Olson, P. N., Bowen, R. A., Behrendt, M. D., Olson, J. D. and Nett, T. M. (1982). Concentrations of reproductive hormones in canine serum throughout late anestrus, proestrus and estrus. *Biology of Reproduction,27*, 1196–1206.

Onclin, K., Murphy, B. and Verstegen, J. P. (2002). Comparisons of estradiol, LH and FSH patterns in pregnant and nonpregnant beagle bitches. *Theriogenology, 57*, 1957-1972.

Orfanou, D. C., Ververidis, H. N., Pourlis, A., Fragkou, I. A., Kokoli, A. N., Boscos, C. M., Taitzoglou, A. and Tzora, C. M. N. (2009). Post partum involution of the canine uterus gross anatomical and histological features. *Reproduction in Domestic Animals, 44*, 152–155.

Ortega-Pacheco, A., Rodriguez-Buenfil, *J. C.*, *Segura, C. J. C.*, Bolio-Gonzalez, M. E., *Jiménez-Coello, M. and* Linde- Forsberg, C. (2007), Reproductive patterns and reproductive pathologies of stray bitches in the tropics. *Theriogenology, 67*, 382–390.

Özbay, K. and Deveci, S. (2011). Relationships between transvaginal colour Doppler findings, infectious parameters and visual analogue scale scores in patients with mild acute pelvic inflammatory disease. *European Journal of Obstetrics and Gynecology and Reproductive Biology, 156*, 105–108.

Pakarainen, T., Zhang, F. P., Nurmi, L., Poutanen, M. and Huhtaniemi, I. (2005). Knockout of luteinizing hormone receptor abolishes the effects of follicle-stimulating hormone on preovulatory maturation and ovulation of mouse graafian follicles. *Molecular Endocrinology, 9*, 2591-2602.

Palmowski, M., Huppert, J., Ladewig, G. Hauff, P., Reinhardt, M., Mueller, M. M., Woenne, E. C., ……. and Kiessling, F. (2008). Molecular profiling of angiogenesis with targeted ultrasound imaging: Early assessment of antiangiogenic therapy effects. *Molecular Cancer Therapeutics,7* (1), 101–109.

Park, R. D., Nyland, T. G., Lattimer, J. C., Miller, C. W., and Lebel, J. L. (1981). B-mode grayscale ultrasound: imaging artefacts and interpretation principles, *Veterinary Radiology*. *22*, 204-210.

Patrick, R. K. (2010). Equipment: In: *BSAVAManual of Canine and Feline Ultrasonography*. Ed. Barr. F. and Gaschen. 15-16. BSAVA Publication Gloucester.

Pavlin, C. J. and Foster, F. S. (1998). Ultrasound biomicroscopy: High-frequency ultrasound imaging of the eye at microscopic resolution. *Radiologic Clinics of North America*, 36, 1047 1058.

Payan-Carreira, R. and Pires, M. A. (2008). Multioocyte follicles in domestic dogs: a survey of frequency of occurrence. *Theriogenology*, *69*, 977-982.

Penninck, D. G. (2002). Artefacts. In Nyland TG, Mattoon, J.S, editors: *Small animal diagnostic ultrasound*, ed 2, Philadelphia, Saunders, p 19.

Peter, A. T. (2001). The reproductive system In: Hoskins JD, ed. Veterinary pediatrics: dogs and cats from birth to six months. 3rd edn. Philadelphia, USA: Saunders, 463–475.

Peter, A. T., Pierson, R. A. and Jakovljevic, S. (1992). Use of real-time ultrasonography in bovine and equine reproduction. *Compendium on Continuing Education for the Practicing Veterinarian*, *14*(8), 1116–1124.

Pharr, J. W., and Post, K. (1992). Ultrasonography and radiography of the canine postpartum uterus. Veterinary Radiology & Ultrasound, 33(1), 35-40.

Phillips, P., Holley, G. L., Napolitano, D. J. and Ustuner, K. F. (2001). Medical ultrasound diagnostic imaging method and system with nonlinear phase modulation pulse compression, US Patent No. 6, *241*,674.

Pidoux, G., Gerbaud, P., Tsatsaris, V., Marpeau, O., Ferreira, F., Meduri, G., Guibourdenche, J., Badet, J., Evain-Brion, D. and Frendo, J. L. (2007). Biochemical characterization and modulation of LH/CG receptor during human trophoblast differentiation. *Journal of Cellular Physiology*, *212*(1), 26-35.

Pierson, R. A. and Adams, G. P. (1995). Computer-assisted image analysis, diagnostic ultrasonography and ovulation induction: Strange bed fellows. *Theriogenology*, 43, 105–112.

Piyasena, P., Mohareb, E. and Mckellar, R. C. (2003). Inactivation of microbes using ultrasound: a review. *International Journal of Food Microbiology*, *87*, 207-216.

Porter, M. (1991). Therapeutic ultrasound. *Journal of Equine Veterinary Science*, *11*, 357-362.

Powis, R. L. (1986). Ultrasound science for the veterinarian. *Veterinay Clinic for North American Equine Practice, 2*(1), 3-27.

Powis, R. L. (1993). The saga of the lingering transducer. *Jounal of Equine Veterinary Science*. *13*(6), 323-324.

Prentice, P., Cuschierp, A., Dholakia, K., Prausnitz, M., and Campbell, P. (2005). Membrane disruption by optically controlled microbubble cavitation. *NaturePhysics, 1*, 107–110.

Pretzer, S. D. (2008). Canine embryonic and foetal development: a review. *Theriogenology, 70*, 300–305

Prokop, A. F., Soltani, A., and Roy, R. A. (2007). Cavitational mechanisms in ultrasound accelerated fibrinolysis. *Ultrasound in Medicine and Biology, 33*, 924–933.

Rapoport, N., Gao, Z. G., and Kennedy, A. (2007). Multifunctional nanoparticles for combining ultrasonic tumor imaging and targeted chemotherapy. *Journal of the National Cancer Institute, 99*, 1095–1106.

Reddy, N. K., Ioncicaˇ, A. M., Saˇ ftoiu, A.,Vilmann, P. and Bhutani, M. S. (2011). Contrast-enhanced endoscopic ultrasonography. *World Journal of Gastroenterology,17,* 42–48.

Rehm, S., Stanislaus, D. J. and Williams, A. M. (2007). Estrous cycle-dependent histology and review of sex steroid receptor expression in dog reproductive tissues and mammary gland and associated hormone levels. Birth Defects Res. B Dev. *Reproductive Toxicology, 80*, 233-245.

Rendano, V. T. (1983). Radiographic evaluation of foetal development in the bitch and foetal death in the bitch and queen. In: Kirk, R. W. *Current veterinary therapy*. Philadelphia: WB Saunders Co., v. VIII, 1440 p. 947-952

Rendano, V. T., Lein, D. H. and Concannon, P. W. (1984). Radiographic evaluation of prenatal development in the Beagle: correlation with time of breeding, LH release, and parturition. *Veterinary Radiology, 25*(3), p. 132-141.

Reynaud, K., de Lesegno, C. V., Chebrout, M., Thoumire, S. and Chastant-Maillard, S. (2009). Follicle population, cumulus mucification, and oocyte chromatin configuration during the periovulatory period in the female dog. *Theriogenology, 72*, 1120-1131.

Reynaud, K., Fontbonne, A., Marseloo, N., Thoumire, S., Chebrout, M., de Lesegno, C. V.and Chastant-Maillard S. (2005). *In vivo* meiotic resumption, fertilization and early embryonic development in the bitch. *Reproduction,130*(2), 193–201. doi: 10.1530/rep.1.00500 PMID: 16049157 11.

Reynaud, K., Fontbonne, A., Marseloo, N., Viaris de Lesegno, C., Saint-Dizie, M. and Chastant Maillard, S. (2006). *In vivo* canine oocyte maturation, fertilization and early embryogenesis: a review. *Theriogenology*, *66*, 1685-1693.

Rhind, S. M. and McNeilly, A. S. (1986). Follicle populations, ovulation rates and plasma profiles of LH, FSH and prolactin in Scottish Blackface ewes in high and low levels of body condition. *Animal Reproduction Science, 10*, 105–15.

Richards, R. G., Gadsby, J. E. and Almond, G. W. (1994). Differential effects of LH and PGE2 on progesterone secretion by small and large porcine luteal cells. *Journal of Reproduction and Fertility*, *102*(1), 27-34.

Rooney, J. A. (1970). Hemolysis near an ultrasonically pulsating gas bubble. *Science, 169*, 869–871.

Root, C. R. and Spaulding, K. A. (1994). Diagnostic imaging companion animal theriogenology. *Seminars in Veterinary Medicine and Surgery*, *9*(1), 7-27.

Rosenthal, S. J., Jones, P. H. and Wetzel, L. H. (2001). Phase inversion tissue harmonic sonographic imaging: a clinical utility study. *American Journal of Roentgenology,176*, 1393–1398.

Rosselli, M., Keller, P. J. and Rubey, R. K. (1998). Role of nitric oxide in the biology, physiology and pathophysiology of reproduction. *Human Reproduction Update*, *4*, 3–24.

Rota, A., Charles, C., Cucuzza, A. S. and Pregel, P. (2015) Diagnostic Efficacy of a Single Progesterone Determination to Assess Full-Term Pregnancy in the Bitch. *Reproduction in Domestic Animals,50*, 1028–1031. doi: 10.1111/rda.12631. ISSN 0936–6768.

Rota, A., Milani, C., Romagnoli, S., Zucchini, P. and Mollo, A. (2010). Pregnancy and conception rate after two intravaginal inseminations with dog semen frozen either with 5% glycerol or 5% ethylene glycol. *Animal Reproduction Science*, *118*, 94-97.

Rowlands, I. W. (1950). Some observations on the breeding of dogs. *Proceedings Society for the Study of Fertility, 2*, 40–55.

Rozear, L. and Tidwell, A. S. (2003). Evaluation of the ureter and ureterovesicular junction using helical computed tomographic excretory urography in healthy dogs. *Veterinary Radiology and Ultrasound*, *44*(2), 155–64.

Saint-Dizier, M., Jaffre, N., Reynaud, K., Remy, B., Thoumire, S. and Chastant-Maillard, S. (2008). Expression of follicle-stimulating hormone and luteinising hormone binding sites in the bitch ovary during the follicular phase. *Reproduction, Fertility, and Development*, *20*, 925-934.

Scampini, J. (2017). Overview of Ultrasound Imaging System and Electrical components required formain Subfunction. Available at http://www.maximintegrated.com/en/app-notes/index.mvp/id/4696. Accessed on 30/1/2017@ 12:51 GMT.

Schalm. S.S. Ballif. B.A., Buchanan. S.M., Phillips. G.R., Maniatis. T. (2010)Phosphorylation of protocadherin proteins by the receptor tyrosine kinase RetProc. Natl. Acad. Sci. U. S. A., 107 (31). 13894-13899

Schlief, R. (1991). Ultrasound contrast agents. *Current Opinion in Radiology*, *3*, 198–207.

Schlosser, T., Pohl, C., Veltmann, C., Lohmaier, S., Goenechea, J., Ehlgen, A., Koster, J., Bimmel, D., Kuntz-Hehner, S., Becher, H., and Tiemann, K. (2001). Feasibility of the flash replenishment concept in renal tissue: which parameters affect the assessment of the contrast replenishment?. *Ultrasound in Medicine and Biology*, *27*, 937–944.

Schwarz, T. (2011). Urinary system. In: Schwarz T, Saunders J, editors.Veterinary computed tomography. Chichester (United Kingdom): John Wiley and Sons ltd. Pp 331–338.

Shapiro, R. S., Wagreich, J., Parsons, R. B., Stancato-Pasik, A., Yeh, H. and Lao, R. (1998). Tissue harmonic imaging sonography: evaluation of image quality compared with conventional sonography. *American Journal of Roentgenology*,*171*, 1203–1206**.**

Shille, V. M., and Gontarek, J. (1985). The use of ultrasonography for pregnancy diagnosis in the bitch. *Journal of the American Veterinary Medical Association*, *187*, 1021–1025.

Shille, V. M., Thatcher, M. J. and Simmons, K. J. (1984). Efforts to induce estrus in the bitch, using pituitary gonadotropins. *Journal of the American Veterinary Medical Association, 184,* 1469-1473.

Shille, V., Thatcher, M. J., Lloyd, M., Miller, D., Seyfert, D. and Sherrod, J. (1986). Gondotropin control of follicular development and the use of exogenous gonadotropins for induction of oestrus and ovulation in the bitch. *Journal of Reproduction and Fertility, 39*, 103-113.

Siddharth, S. and Goyal, A. (2007): The origin of echocardiography. *Texas Heart Institute Journal*,*34*(4), 431-438

Siddiqui, M. A., Ferreira, J. C., Gastal, E. L., Beg, M. A., Cooper, D. A. and Ginther, O. J. (2010). Temporal relationships of the LH surge and ovulation to echotexture and powerDoppler signals of blood flow in the wall of the preovulatory follicle in heifers. *Reproduction, Fertility and Development, 22*(7), 1110-1117.

Silva, L. A. and Ginther, O. J. (2006). An early endometrial vascular indicator of completed orientation of the embryo and the role of dorsal endometrial encroachment in mares. *Biology of Reproduction, 74*(2), 337-343.

Silva, L. A., Gastal, E. L., Beg, M. A. and Ginther, O. J. (2005). Changes in vascular perfusion of the endometrium in association with changes in location of the embryonic vesicle in mares. *Biology of Reproduction, 72*(3), 755-761.

Smith, D. A. B., Porter, T. M., Martinez, J., Huang, S. L., MacDonald, R. C., McPherson, D. D., and Holland, C. K. (2007). Destruction thresholds of echogenic liposomes with clinical diagnostic ultrasound. *Ultrasound in Medicine and Biology, 33*, 797– 809.

Smith, F. O. (2007). Challenges in small animal parturition-Timing elective and emergency cesarian sections. *Theriogenology,68*, 348–353.

Socha, P. and Janowski, T. (2014). Predicting the parturition date in bitches of different body weight by ultrasonographic measurements of Inner Chorionic Cavity Diameter and Biparietal Diameter. *Reproduction in Domestic Animals*, *49*, 292–296.

Sokolowski, J. H., Stover, D. G. and VanRavenswaay, F. (1977). Seasonal incidence of estrus and interestrous interval for bitches of seven breeds. *Journal of the American Veterinary Medical Association,171*, 271–273

Song, J., Christiansen, J., Klibanov, A. L., Ley, K., Kaul, S., and Lindner, J. R. (2001). Adhesion of Pselectin- targeted micro bubbles to venules: implications for imaging inflammation. *Journal of the American College of Cardiology*, *37*, 383A–383A.

Song, J., Kim, S., Sohn, H., Song, T. and Yoo, Y. M. (2010). Coded excitation for ultrasound tissue harmonic imaging. *Ultrasonics,50*, 613–619

Songsasen, N., Woodruff, T. K. and Wildt, D. E. (2011). *In vitro* growth and steroidogenesis of dog follicles are influenced by the physical and hormonal microenvironment. *Reproduction*, *142*, 113-122

Steckler, D., De Cramer, K. G. M. and Nöthling, J. O. (2017). Estimated impact of multiple conceptuses per follicle on fecundity in the bitch. *Theriogenology,102,* 108-115

Steckler, D., Nöthling, J. O. and Harper, C. (2013). Prediction of the optimal time for insemination using frozen-thawed semen in a multi-sire insemination trial in bitches. *Animal Reproduction Science*, *142*, 191-197.

Steinetz, B. G., Goldsmith, L. T., Hasan, S. H. and Lust, G. (1990). Diurnal variation of serum progesterone, but not relaxin, prolactin, or estradiol-17 beta in the pregnant bitch. *Endocrinology,127*, 1057–1063.

Steiss, J. E. (2000). Physical therapy modalities: therapeutic ultrasound and phonophoresis. *Compendium on Continuing Education for the Practicing Veterinarian, 22*, 690-693

Stephenson, A. P., Tyler, D. J., Carr, C. A. and Williams, S. A. (2012). Magnetic resonance imaging for the study of ovarian follicles in the mouse. *Theriogenology,78*, 1190-1198.

Stewart, R. A., Pelican, K. M., Crosier, A. E., Pukazhenthi, B. S., Wildt, D. E., Ottinger, M. A. and Howard, J. (2012). Oral progestin priming increases ovarian sensitivity to gonadotropin stimulation and improves luteal function in the cat. *Biology of Reproduction*, *87*, 1–11.

Still, J. G. and Greiss Jr, F. C. (1978). The effect of prostaglandins and other vasoactive substances on uterine blood flow and myometrial activity. *American Journal of Obstetrics and Gynecology,130*, 1–8.

Stride, E. P. and Coussio, C. C. (2009). Cavitation and contrast: the use of bubbles inultrasound imaging and therapy. *Journal of Engineering in Medicine*, *224*, 171-191. Doi: 10.1243/09544119JEIM622

Suslick, K. S. (1990). Sonochemistry. *Science, 247*, 1439–1445.

Szopinski, K. T., Pajk, A. M., Wysocki, M., Amy, D., Szopinska, M. and Jakubowski, W. (2003a). Tissue harmonic imaging: utility in breast sonography. *Ultrasound in Medicine and Biology*, *22*, 479–487.

Szopinski, K. T., Wysocki, M., Pajk, A. M., Slapa, R. Z., Jakubowski, W. and Szopinski, M. (2003b). Tissue harmonic imaging of thyroid nodules: initial experience. *Ultrasound in Medicine and Biology,22,* 5–12.

Tainturier, B. and Tainturier, D. (2004). Bencharif foetal sex determination in cattle by ultrasonography. *Journal of Animal and Veterinary Advances, 3:*136–41.

Takalkar, A. M., Klibanov, A. L., Rychak, J. J., Lindner, J. R., and Ley, K. (2004). Binding and detachment dynamics of micro bubbles targeted to Pselectin under controlled shear flow. *Journal of Controlled Release,96*, 473–482.

Takeuchi, Y. (1996). Coded excitation for harmonic imaging, in: Proceedings. IEEE Ultrasonics Symposium San Antonio, TX, pp. 1433–1436.

Tarantal, A. F., O'Brien, W. D., & Hendrickx, A. G. (1993). Evaluation of the bioeffects of prenatal ultrasound exposure in the cynomolgus macaque (Macaca fascicularis): III. Developmental and hematologic studies. Teratology, 47(2), 159-170.

Tedor, J. B. and Reif, J. S. (1978). Natal patterns among registered dogs in the United States. *Journal of the American Veterinary Medical Association, 172*, 1179–85.

Telfer, E. and Gosden, R. G. (1987). A quantitative cytological study of polyovular follicles in mammalian ovaries with particular reference to the domestic bitch (Canis familiaris). *Journal of Reproduction and Fertility*, *81*, 137-147.

Terranova PF (2009). Fertilization, Pregnancy, andFetal Development: Medical Physiology,Part X, Reproductive Physiology Vol.XXXIX. Saunders, USA. 684-704.

Themmen, A. P. N. and Huhtaniemi, I. T. (2000). Mutations of gonadotropins and gonadotropin receptors: elucidating the physiology and pathophysiology of pituitarygonadal function. *Endocrine Reviews*, *21*, 551-583.

Thomassen, R., Farstad, W., Krogenaes, A., Fougner, J. A. and Berg, K. A. (2001). Artificial insemination with frozen semen in dogs: a retrospective study. *Journal of Reproduction and Fertility*, *57*, 341-346.

Tinkanen, H. and Kujansuu, E. (1993). Doppler ultrasound findings in tuboovarian infectious complex. *Journal of Clinical Ultrasound*, *21*, 175–178.

Tiukinhoy, S. D., Khan, A. A., Huang, S. L., Klegerman, M. E., MacDonald, R. C., and McPherson, D. D. (2004). Novel echogenic drug-immunoliposomes for drug delivery. *Investigative Radiology*, *39*, 104–110.

Toal, R. L., Walker, M. A. and Henry, G. A. (1986). A comparison of real-time ultrasound, palpation and radiography in pregnancy detection and litter size determination in the bitch. *Veterinary Radiology*,*27*(4), 102-108,

Tranquart, F., Grenier, N., Eder, V. and Pourcelot. L. (1999). Clinical use of ultrasound tissue harmonic imaging. *Ultrasound in Medicine and Biology*,*25*, 889–894**.**

Tsang, B. K., Moon, Y. S. and Simpson, C. W. (1979). Androgen biosynthesis in human ovarian follicles: cellular source, gonadotropic control, and adenosine 3959monophosphate mediation. *The Journal of Clinical Endocrinology and Metabolism*, *48*, 153-158.

Tsutsui, T. (1989). Gamete physiology and timing of ovulation and fertilization in dogs. *Journal of Reproduction and Fertility*, *39*, 269–275.

Ulum, M. F., Maharani, A. D. K., Kurniawan, R. E., Sariningrum, A., Frastantie, D., Erwin, Tumbelaka, L. I. and Noviana, D. (2017). Ultrasonography of the external reproductive organs in Tom. *Jurnal Kedokteran Hewan*. *11*(3), 94-100. DOI: https://doi.org/10.21157/j.ked.hewan.v11i3.5700

Unger, E. C., McCreery, T., Sweitzer, R., Vielhauer, G., Wu, G., Shen, D., and Yellowhair, D.(1998). MRX 501: a novel ultrasound contrast agent with therapeutic properties *Academic Radiology*, *5*(1), S247–S249.

Van Haaften, B., Dieleman, S. J., Okkens, A. C., Bevers, M. M. and Willemse, A. H. (1989). Induction of estrus and ovulation in dogs by treatment with PMSG and/or bromocriptine. *Journal of Reproduction and Fertility, 39,* 330-331

van Wamel, A., Kooiman, K., Harteveld, M., Emmer, M., ten Cate, F. J., Versluis, M., and de Jong, N. (2006). Vibrating micro bubbles poking individual cells: drug transfer into cells via sonoporation. *Journal of Controlled Release, 112,* 149–155.

Vanderlip, S. L., Wing, A. E., Felt, P., Linkie, D., Rivier, J., Concannon, P. W. and Lasley, B. L. (1987). Ovulation induction in anestrous bitches by pulsatile administration of GnRH. *Laboratory Animal Science, 27,* 459-468.

Vannucchi, C. I., Jordao, A. A., & Vannucchi, H. (2007). Antioxidant compounds and oxidative stress in female dogs during pregnancy. *Research in veterinary science,* 83(2), 188-193.

Veronesi, M. C., Battocchio, M., Marinelli, L., Faustini, M., Kindahl, H. and, Cairoli, F. (2002).Correlations among body temperature, plasma progesterone, cortisol and prostaglandin F2a of the periparturient bitch. *Journal of veterinary medicine. A, Physiology, pathology, clinical medicine, 49,* 264–268.

Verstegen, M.W.A. and Close, W.H. (1994). The environmental and the growing pigs. In: Principles of Pig Science, Cole, D.J.A., Wiseman, J. and Varley, M.A. (eds). NottinghamUniversity Press, Nottingham, England, pp. 333-353.

Verstegen, J., Onclin, K., Silva, L. and Concannon, P. (1997). Termination of obligate anestrus and induction of fertile ovarian cycles by administration of purified pig. *Journal of Reproduction and Fertility,111,* 35-40.

Verstegen, J.P. K. Onclin, L.D.M. Silva and P.W. Concannon (1999). Effect of stage of anestrus on the induction of estrus by the dopamine agonist cabergoline in dogs. *Theriogenology.* 51: 597-611

Vesely, J. A. (1978). Application of light control to shorten the production cycle in two breeds of sheep. *Animal Production, 26,* 169–176.

Vignoli, M., and Saunders, J. (2011): Image-guided interventional procedures in the dog and cat. *The Veterinary Journal, 187,* 297–303.

Wallace SS, Mahaffey MB, Miller DM, Thompson FN, Chakraborty PK. Ultrasonographic appearance of the ovaries of dogs during the follicular and luteal phases of the estrous cycle. Am J Vet Res 1992; 53: 209–215.

Walter, I. and Schönkyp, S. (2006). Extracellular matrix components and matrix degrading enzymes in the feline placenta during gestation. *Placenta, 27,* 291–306.

Wanke, M., Loza, M., Monachesi, N., and Concannon, P. (1997). Clinical use of dexamethasone for termination of unwanted pregnancy in dogs. *Journal of Reproduction and Fertility*, *51*, 233-238.

Ward, B., Baker, A. C. and Humphrey, V. F. (1997). Nonlinear propagation applied to the improvement of resolution in diagnostic medical ultrasound. *The Journal of the Acoustical Society of America, 101*, 143–154.

Ward, M., Wu, J. R., and Chiu, J. F. (1999). Ultrasound induced cell lysis andsonoporation enhanced by contrast agents. *The Journal of the Acoustical Society of America*, *105*, 2951–2957.

Weilenmann, R., Arnold, S., Dobeli, M., Rüsch, P. and Zerobin, K. (1993). Estrusinduction in bitches by the administration of PMSG and HCG. *Schweizer Archiv Fur Tierheilkunde,135*(8), 236-241.

Whitlow, B. J., Lazanakis, M. S. and Economides, D. L. (1999). The sonographicidentification of foetal gender from 11 to 14 weeks of gestation. *Ultrasound in Obstetrics and Gynecology*, *13*, 301–304.

Whittingham, T. A. (2005). Contrast-specific imaging techniques; technicalperspective. In: Quaia E (ed.). *Contrast Media inUltrasonography. Basic Principles and Clinical Applications*. Berlin: Springer, 43–84.

Wiebe, V. J. and Howard, J. P. (2009). Pharmacologic Advances in Canine and FelineReproduction: Topical Review.*Topics in Companion Animal Medicine*, 24(2), 71-99. doi:10.1053/j.tcam.2008.12.004

Wigham, E. E., Moxon, R. S., England, G. C. W., Wood, J. L. N. and Morters, M. K.(2017). Seasonality in oestrus and litter size in an assistance dog breeding colony in the United Kingdom. *Veterinary Record*, 5-6. Doi: 10.1136/vr.104217

Wildt, D. E., Chakraborty, P. K., Panko, W. B. and Seager, S. W. J. (1978). Relationship of reproductive behavior, serum luteinizing hormone and time of ovulation in the bitch. *Biology of Reproduction*, *18*, 561-570.

Willmann, J. K., Lutz, A. M., Paulmurugan, R., Patel, M. R., Chu, P., Rosenberg, J. and Gambhir, S. S. (2008). Dual-targeted contrast agent for US assessment of tumor angiogenesis *in vivo*. *Radiology,248*, 936–944.

Wright, P. J. (1990). Application of vaginal cytology and plasma progesterone determinations to the management of reproduction in the bitch. *Journal of Small Animal Practices*, *31*, 335-340.

Xu, B. and Feng, H. L. (2016). Ovulation, fertilization and preimplantation embryonic development in raccoon dogs (*Nyctereutes procyonoides*). *Animal Reproduction Science*, xxx. http://dx.doi.org/10.1016/j.anireprosci.2016.12.001

Yeager, A. E. and Concannon, P. W. (1990). Serial ultrasonographic appearance of postpartum uterine involution in beagle dogs. *Theriogenology, 34*, 523–525.

Yeager AE, Mohammed HO, Meyers-Wallen V, Vannerson L, Concannon PW. (1992) Ultrasonographic appearance of the uterus, placenta, fetus, and fetal membranes throughout accurately timed pregnancy in beagels. *Am J Vet Res.;*53: 342–351.

Yoshioka, M., Tanaka, K. I., Miyazaki, I., Fujita, N., Higashi, Y., Asanuma, M.,& Ogawa, N. (2002). The dopamine agonist cabergoline provides neuroprotection by activation of the glutathione system and scavenging free radicals. Neuroscience research, 43(3), 259-267.

Yotov, S. A., Atanasov, A. and Georgiev, P. (2011). Determination of foetal sex in buffaloes through a single sonographic examination. *Bulgarian Journal of Veterinary Medicine*, *14*, 39–44.

Zagzebski, J. A. (2005). Physics and instrumentation in Doppler and B-Mode ultrasonography. Philadelphia: Elsiever Saunders, 752p.

Zambelli, D. and Prati, F. (2006). Ultrasonography for pregnancy diagnosis and evaluation in queens. *Theriogenology, 66*, 135–144.

Zhou, Y. C., Wang, J., Zhang, B. and Su, Y. Q. (2002). Ultrasonic immunization of sea bream, *Pagrus major*, with a mixed vaccine against *Vibrio alginolyticus* and *V. anguillarum*. *Journal of Fish Diseases, 25*, 325-331.

Zoldag, L., Fekete, S., Csaky, I. and Bersényi, A. (2001). Fertile estrus induced in bitches by bromocryptine a dopamine agonist; a clinical trial. *Theriogenology,55* (8), 1657-1666,

Zone, M. A., and Wanke, M. M. (2001). Diagnosis of canine foetal health by ultrasonography. *Journal of Reproduction and Fertility*, *57*, 215–219.

APPENDICES

Appendix 1. Concentration of Superoxide dismutase at various phases for the bitches

PHASE 1			PHASE 2			PHASE 3E		
A	B	C	A	B	C	A	B	C
0.417284	1.1527	0.685289	0.197531	0.150617	0.059259	1.134078	1.294227	1.411546
0.469274	1.197393	0.886406	0.219739	0.620112	0.249383	1.145251	1.3054	1.499069
0.824953	1.197393	1.009311	0.364991	1.022222	0.258845	1.165736	1.307263	1.649907
0.612663	1.147114	0.95717	0.357542	0.091358	0.445065	1.199255	1.318436	1.724395
0.631285	1.266294	1.106145	0.43203	0.901304	0.77037	1.216015	1.364991	1.312849
0.759777	1.288641	1.1527	0.55121	1.111732	0.955307	0.849162	1.366853	1.325885
	0.616387			0.069136		0.865922	1.407821	1.392924
	0.726257			0.088889		0.931099	1.428305	1.402235
	0.804469			0.553073			1.556797	
							1.579143	
							0.998138	
							1.126629	
							1.156425	
							1.171322	
							1.264432	

PHASE 3L			PHASE 4		
A	B	C	A	B	C
1.348231	1.366853	1.979516	0.895717	1.223464	1.055866
1.566108	1.495345	1.979516	1.001862	1.240223	1.206704
1.56797	1.506518	1.988827	1.080074	1.264432	1.214153
1.843575	1.564246	1.996276	0.769088	1.314711	1.165736
1.977654	1.953445	1.769088	0.817505	1.316574	1.165736
1.054321	1.648045	1.785847	0.821229	1.357542	1.208566
1.07635	1.700186	1.839851		0.83054	
1.098696	1.98324	1.86406		0.839851	
1.944134	1.990689			0.992551	
	4.577778				
	1.348231				
	1.379888				
	1.38175				
	1.506518				
	2.437037				

Appendix 2. Concentration of Malondialdehyde at various phases for the bitches

PHASE 1			PHASE 2			PHASE 3E		
A	B	C	A	B	C	A	B	C
7.715	2.203	10.103	1.924	1.205	0.98	2.345	3.722	3.273
2.111	5.991	1.69	0.377	0.038	0.049	4.037	1.228	4.057
10.098	5.159	11.187	1.318	1.945	0.23	2.418	1.637	3.886
4.499	3.461	2.007	2.458	2.609	1.258	5.853	1.639	2.528
3.433	2.405	1.563	2.646	0.053	1.492	2.05	1.652	2.086
	2.891	2.848	2.531		1.637	2.775	2.671	2.214
	3.086	2.878				4.28	3.286	2.294
	6.353					0.117	3.397	2.32
	2.99					4.797	1.594	
	1.648					2.241		
	1.569					4.233		
						3.663		
						2.269		
						6.012		
						1.36		

PHASE 3L			PHASE 4		
A	B	C	A	B	C
0.837	7.406	2.558	0.075	1.034	1.598
2.795	6.959	4.184	0.158	2.703	2.439
8.164	1.492	4.652	3.035	2.89	0.104
2.935	19.939	6.713	4.697	7.655	4.606
11.085	2.165	10.509	2.328	3.407	1.498
3.329	7.602	1.903	3.931	1.073	
4.476	4.463	3.939	6.891		
2.767	6.404	4.146	1.043		
3.061	10.162	4.627	3.327		
33.421	7.545	7.28			
5.272					
2.32					
1.556					
2.358					
9.754					

Appendix 3. Concentration of Catalase at various phases for the bitches

PHASE 1			PHASE 2			PHASE 3E		
A	B	C	A	B	C	A	B	C
7.198205	6.393635	8.34908	6.530334	6.174916	7.1943	8.244928	7.204715	27.69523
7.471603	6.396238	8.533949	6.777693	6.341559	7.840039	8.446722	8.096512	35.61335
7.588773	6.449616	9.581973	6.956053	6.341559	8.250135	9.416633	8.563892	37.00507
6.601938	6.456126	6.685259	0.074128	4.343039	6.260841	18.50906	9.812408	7.646057
6.785505	6.460031	6.725618	5.590366	6.228294	6.510805	23.86234	12.10363	7.721567
6.793316	6.608447	6.805033	6.180124	6.324634	6.682655	7.077129	13.9277	8.944045
6.694372			6.323332			7.398697	14.14772	9.226557
6.792014			6.336351			7.894719	15.5134	
6.877939			6.625372			8.25274	17.28268	
						8.343872		
						7.698133		
						7.985851		
						8.270966		

PHASE 3L			PHASE 4		
A	B	C	A	B	C
27.2733	12.58154	43.36743	7.651264	6.502994	11.30828
36.87749	16.30484	49.17388	7.807492	6.611051	11.97225
40.98887	19.06107	50.71141	7.920756	6.834977	27.35923
45.82931	21.24044	50.76089	6.890958	7.575755	6.900072
51.07464	38.28484	12.6219	6.920902	9.321594	7.006827
8.379024	18.07944	13.41215	6.980789	10.70941	7.45598
8.932328	20.55304	23.8989	7.173469		
26.61205	27.57534	27.35792	7.385678		
27.12098	47.31738		7.61351		
49.85997	47.55302				
8.742251					
8.915403					
12.31986					
38.32259					

Appendix 4. Concentration of Glutathione peroxidase at various phases for the bitches

PHASE 1			PHASE 2			PHASE 3E		
A	B	C	A	B	C	A	B	C
20.36853	11.25333	24.3072	3.0384	5.176533	1.3504	34.4352	41.74987	48.2768
21.38133	12.71627	26.89547	8.1024	5.7392	14.1792	37.24854	42.98773	50.64
21.944	23.85707	32.18453	9.677867	6.864533	15.5296	45.46347	46.47626	52.21547
21.71893	23.85707	11.36587	0.900267	8.327467	5.626667	55.47893	36.01067	56.7168
23.632	24.3072	16.2048	5.176533	15.41707	9.4528	60.65547	38.03627	28.80853
24.6448	24.53227	17.5552	18.2304	21.49387	10.69067	30.3848	46.02613	30.83413
40.1744			1.0128			33.30987		32.7472
40.28693			30.7216			33.98507		36.68587
40.512			38.03627			34.88533		
						46.81387		
						48.72693		
						49.73973		

PHASE 3L			PHASE 4		
A	B	C	A	B	C
64.36906	48.72693	57.16693	28.0208	33.87253	32.63467
70.896	57.50453	62.456	29.0336	34.21013	33.53493
87.4384	82.59946	65.1568	29.93387	39.4992	36.1232
108.8197	46.2512	66.73227	25.6576	25.32	18.9056
136.2779	55.3664	79.11093	27.79573	28.3584	22.84427
38.03627	57.0544	44.11307	27.90827	33.19733	23.2944
41.97493	78.88586	48.952	44.9008		
63.8064		63.24373	46.36374		
89.57653			46.81387		
147.3061					
50.75253					
54.57867					
57.392					
61.33067					

Appendix.5 Showing the uterine diameters of the bitches from week 1 to week 8 postpartum

Dog	WEEK 1	WEEK 2	WEEK 3	WEEK 4	WEEK 5	WEEK 6	WEEK 7	WEEK 8
A1	4.51	3.71	2.85	2.19	2.02	1.85	1.85	1.84
A2	3.9	3.13	2.36	2.03	1.8	1.76	1.76	1.74
A3	3.51	2.54	1.81	1.69	1.62	1.61	1.59	1.58
B1	4.21	3.4	2.68	2.17	1.88	1.82	1.83	1.79
B2	4.04	3.14	2.51	2.04	1.86	1.78	1.78	1.76
B3	3.66	2.8	1.98	1.78	1.64	1.7	1.69	1.68
C1	4.14	3.28	2.56	2.1	1.86	1.79	1.81	1.78
C2	3.86	3.06	2.16	1.89	1.74	1.71	1.71	1.69
C3	3.67	2.84	2.15	1.87	1.74	1.7	1.7	1.68

CHAPTER ONE

2.0 INTRODUCTION

1.1 Preamble

Ultrasound has a frequency greater than 20 kHz, which is above the range of human hearing. It allows communication, food acquisition or orientation in bats and other animals, but can also be used as a technique of diagnostic medical two- and three-dimensional or flow imaging (Meinecke-Tillmann, 2016). Meanwhile, ultrasonography (from Latin words 'ultra': more and 'sono': sound and Greek word 'graphein' ['˛ϕε´ ']: to write) or ultrasound imaging is one of the most widely used powerful tools in animal diagnostics and research, which allows the real-time visualisation of internal organs, tissues, structures or fluid accumulations and the investigation of blood flow without any radiation risk. It depends on the computerised analysis of reflected ultrasound waves. Due to its non-invasive character and apparent high safety, it has become one of the most rapidly advancing technologies in the clinical fields (Meinecke-Tillmann, 2016).

1.1.1 Definition and application of Ultrasound

Ultrasound is an oscillating sound pressure wave with a frequency greater than the upper limit of the human hearing range. It is thus not separated from 'normal' (audible) sound by differences in physical properties, but rather only by the fact that humans cannot hear it. Although this limit varies from person to person, it is approximately 20 kilohertz (20,000 hertz) in healthy, young adults. Ultrasound devices operate with frequencies from 20 kHz up to several gigahertz (Novelline and Robert, 1997). Ultrasound is used in many different fields for example; ultrasonic devices are used to detect objects and measure distances. Furthermore ultrasonic imaging (sonography) is used in both veterinary and human

medicine. In the non-destructive testing of products and structures, ultrasound is used to detect invisible flaws (Novelline and Robert, 1997). Industrially, it is used for cleaning and for mixing, as well as to accelerate chemical processes. Animals such as bats and porpoises use ultrasound for locating prey and obstacles (Novelline and Robert, 1997).

1.1.2. Oestrus cycle and oestrus induction in bitches

Bitches have been classified as non-seasonal monoestrus mammals which have a long period of anoestrus (Verstegen *et al*., 1999). Cyclicity is exhibited only once or twice in a year in normal reproductive physiology of domestic bitches (Verstegen *et al*., 1999). Even with the aforementioned uniqueness, the oestrous cycle of the bitch is a unique physiological period that contains four stages. These stages are proestrus, oestrus, dioestrus, and anoestrus (Sudson, 2011). Proestrus typically lasts for about 9-11 days and then directly leads to oestrus. At this stage, ovarian follicles mature and oestradiol concentration in the blood increases resulting in the swelling of vagina and uterus. The changes in the vaginal epithelium, vulvar oedema and diapedesis of erythrocytes from capillaries of the reproductive tract also occur from the hormonal influence (Sudson, 2011). At this stage the bitch still refuses to stand for mating. Peak concentrations of oestradiol in late proestrus prepare the pituitary and hypothalamus for an ensuing surge-release of luteinizing hormone (LH). Progesterone level begins to increase in late proestrus at the onset of the LH surge. Then, mating is accepted at oestrus that lasts for about 6-11 days. During this period the progesterone gradually rises. This causes the softening of the vulva resulting in mating behaviour. Ovulation takes place spontaneously at the beginning of oestrus. Subsequently, dioestrus starts and the bitch rejects mating. This stage is

predominated by progesterone release thereby inhibiting uterine contraction throughout the period of pregnancy. Dioestrus varies among bitches and can be approximately 9-11 weeks. Anoestrus is a quiescent period for reproductive organs and sexual behaviour. It is a prolonged stage, lasting for 3-5 months (Noakes *et al*., 2001).

Induction of fertile oestrus in the bitch has been achieved using dopamine agonist such as bromocriptine which is traded as a human drug and is not recommended for animals, while Cabergoline is a veterinary drug for treatment of pseudo pregnancy in dogs. Bromocriptine administered orally once or twice per day (50 μg/kg) induces proestrus in 17-28 days (Concannon, 1993) while using cabergoline (5 μg/kg) daily induces fertile proestrus in 15-25 days, 10-20 days, or 4-10 days when administered to bitches in early, middle or late anooestrus, respectively (Verstegen *et al*., 1994). Dopamine agonist may cause unpleasant side effects such as anorexia and vomiting (Concannon, 1993), and may not successfully induce proestrus in cat (Kutzler, 2007).

1.1.3. Ultrasound safety

Present day diagnostic ultrasound machines use such low levels of energy that they appear to be safe, but the safety threshold levels for different tissues, may vary and with the development of more powerful and sophisticated apparatus, the levels may be exceeded for some tissues (Donald, 1979). Despite decades of ultrasonic investigation, the adverse effects of in-utero insonation have not been pinned to a particular point of higher incidence during pregnancy (Newnham *et al*., 1991). The cumulative effect of ultrasound exposure, it's relation to the output of a particular machine, the length of the examination, high frequency of exposures and the mechanisms of its effects on intrauterine growth are yet to

be elucidated through as large-scale study (Newnham *et al*., 1991). As even the relative lower birth weights showed after intra uterine insonation of foetuses, a later study suggested the offspring's soon made up the deficit. Numerous studies on rats, mice and monkeys showed reduced foetal weight in litters that were exposed to in-utero insonation as compared to the controls (Tarantal *et al*., 1993).

1.2 Statement of Research Problems

There are breeding outfits for dogs springing up all over the country, however, there is a shortage of concrete ways of promptly determining pregnancy and possible reproductive problems in the bitches. To the best of our knowledge, no method of accurately determining litter size in-utero has also been established sonographically in our indigenous breed. No records of the effect of insonation on oxidative stress and reproductive parameters in Nigerian Indigenous Dogs (NIDs) have also been established. Likewise thyroid hormone levels in neonates and progesterone profile in the dam,as well as the safety levels following multiple in-utero exposure is yet to be established in NIDs.

1.3 Justification

There is a surge in the use of ultrasonography in clinical veterinary practice due to its affordability and reliability which eases the clinicians' duties. There is therefore an urgent need to establish information on conception, pregnancy and foetal status for clinician on the dominant dog breed in our environment; sonographic features of the non-gravid uterus; data on the sonographic features of the gravid uterus at various stages of foetal development. There is also the need to establish a procedure to determine number of foetuses in-utero so as to help clinicians advise the client who may make such request. Effect of multiple exposures of the foetuses to ultrasound in-utero needs to also be

elucidated. There is also a dearth of information on the association between multiple insonation and clinically observed differences in neonatal puppies.

1.4. Aim of the Study

This study aims to determine the sonographic changes that occur in the reproductive tracts during oestrus cycle, gestation and postpartum and foetuses (during development) in Nigerian Indigenous dogs (NIDs), and the effects of multiple insonation on the bitches in-puppy and the puppies postpartum.

7.5.Objectives of the Study

2. To identify the various sonographic changes that occurs during different phases of the induced oestrus cycle.

8. To sonographically determine the developmental changes that occurs during embryonic and foetal stages in-utero.
9. To provide an accurate method of predicting the number of puppies in-utero sonographically and gestational aging in NIDs
10. To assess the measureable effects of multiple insonation on the biomarkers of oxidative stress in the bitches and puppies.
11. To assess the effects of multiple insonation on birth weight and thyroid hormones profile of the puppies.
12. To assess the quantifiable effects of multiple insonation on the bitches and puppies.
13. To map out various sonographic changes that occurs postpartum in the reproductive tract of NIDs

1.7 Research questions

8. Are there any sonographic changes that occur in the genitalia in induced oestrus?
9. Can insonation map changes during foetal development?
10. Can insonation help in predicting possible litter size?
11. What are the measurable effects of insonation on puppies?
12. What are the measurable effects of insonation on biomarkers of oxidative stress in bitches and puppies?
13. What are the effects of multiple insonation on birth weight and thyroid hormones?
14. Are there any changes in the reproductive tract postpartum?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Anatomy and Physiology

2.1.1 Sexual maturity in bitches

The age of sexual maturity in the bitch may vary with breed.This could however range from approximately 6 months to 2 years, with most reaching maturity at 8–12 months (Kumi- Diaka and Adeyanju,1986; Peter, 2001; Feldman and Nelson, 2004).

2.1.2 The female reproductive organ

The female reproductive organ in bitch comprises of two ovaries, which contain oocytes and the tubular genitalia duct including the oviduct, bicornuate uterus with a short body, the cervix, vagina and vestibule (Linde-Forsberg, 2007). The latter is quite large in this species to be able to accommodate the *bulbusglandis* of the male during the copulatory tie (Linde-Forsberg, 2007).

Unlike in the human and mouse, canine oocytes are dark due to high lipid content making it difficult to identify intracellular structures by light microscopy and to cryopreserve functional eggs and embryos (Chastant-Maillard *et al*., 2010). Canid oocytes are also ovulated at an immature stage compared with other mammalian species (pre-germinal vesicle breakdown), and require 48–72 hrs in the oviduct postovulation to complete nuclear maturation (Reynaud *et al*., 2005).

2.1.3 The oestrous cycle.

The reproductive behaviour of the domestic dog is commonly described as monoestrous with a uniquely prolonged interoestrous interval of approximately seven months and a range of 3.5–13 months (Rowlands, 1950; Olson and Nett, 1986; Feldman and Nelson, 2004). The oestrus cycle of the bitch is classically divided into four stages: the proestrus, oestrus, metoestrus and anoestrus (Heape 1900). It is known that the development of the tertiary follicles, *corpora hemorrhagica* and *corpora lutea* appeared to be related to minimal hormonal variations even within the same stage of the reproductive cycle (Groppetti *et al*., 2015a). The tertiary follicles distribution (small, medium and large) is different among all the stages of the reproductive cycle, except for small follicles (<2 mm), which is always observed from proestrus to anoestrus (Reynaud *et al*. 2009; Groppetti *et al*., 2015a). The number and distribution of large follicles significantly differed between early oestrus (E1), periovulatory oestrus (E2) and oestrus after ovulation (E3) (Groppetti *et al*., 2015a). Moreover, large follicles with a diameter greater than 4 mm characterize the early oestrus up to ovulation (Groppetti *et al*., 2015a). Dioestrus is sometimes used to refer to the metoestrus for the luteal period (Linde-Forsberg, 2007).

2.1.3.1 Proestrus: This is considered to begin on the day when the vaginal haemorrhage appears from the turgid vulva (Linde-Forsberg, 2007). It can last for average of 9 days with a range of 3-27 days (Linde-Forsberg, 2007). The beginning of proestrus is gradual. Firstly the bitch is inviting the male but is not ready to mate. The vulva turgidity and haemorrhage subside towards the end of the proestrus (Linde-Forsberg, 2007).

2.1.3.2 Oestrus: It is usually lasts for an average of 9 days with a range of 2-21 days. The bitch allows mating during this period (Linde-Forsberg, 2007). The end of the fertile period, which coincides with the beginning of dioestrus (D1) is characterized by a mean value of blood progesterone concentration greater than 36.9 ng/mL (Groppetti *et al*., 2015a). The precise identification of the range of the fertile period is essential to plan programs of assisted reproduction technology (ART) in the bitch (Groppetti *et al*., 2015a).

*2.1.3.3 Metoestrus or dioestrus:*The bitch rejects the male during this period (Linde-Forsberg, 2007). The progesterone stimulated uterine epithelium desquamates as the progesterone concentration subsides over 2-3 months (Linde-Forsberg, 2007). The endometrial repair processes is completed after 4.5-5 month (Linde-Forsberg, 2007). Dioestrus is characterized by the development of corpus luteum (CL) the size of which has been reported to be 3–12 mm in diameter (Rehm *et al*., 2007) and 5–6 mm (Groppetti *et al*., 2015a). Small and large luteal cells have been described in the pig (Richards *et al*., 1994), ovine (Fitz *et al*., 1982) and cattle (Cools *et al*., 2013) to originate from theca and granulosa cells, respectively (Baithalu *et al*., 2013). The sizes of small luteal cells (12–23 μm) and large luteal cells (25–55 μm) in cattle increases as the stage of oestrus cycle progressed and during pregnancy (Baithalu *et al*., 2013). This was similarly described in the dog with small luteal cell between 10 and 20 μm of diameter and large luteal cell between 20 and 40 μm of diameter while the size was unchanged between early and late dioestrus (Groppetti *et al*., 2015a).

*2.1.3.4 Anoestrus:*Dogs experience obligatory prolonged ovarian inactivity or anoestrus (Concannon, 2011), and as a result, ovulation only occurs once or twice annually

(Nagashima *et al*., 2015). This lasts for 1-9 months depending on whether the bitch has 1, 2 or 3 cycles per year (Linde-Forsberg, 2007). The interval between two oestrus periods is usually around 2 months longer after a pregnant cycle (Linde-Forsberg and Wallen, 1992). During anoestrus some studies record only follicles smaller than 1 mm in diameter (Reynaud *et al*., 2012), 1.3 ± 0.3 mm of diameter (Groppetti *et al*., 2015a) or ranging from 4 to 6 mm (Reynaud *et al*., 2009).

2.1.4 Oestrus regulation and oestrus synchronisation

It is generally accepted that day length is the main factor regulating breeding cycles in many species including the Basenji breed of dog (Fuller, 1956; Tedor and Reif, 1978; Gunn and Doney, 1979; Rhind and McNeilly, 1986; Forcada *et al*., 1992). For instance, in seasonal breeders such as sheep, the length of anoestrus is synchronised generating seasonal cycles of oestrus in the population. Anoestrus is regulated through exposure of the pineal gland to light, mediating the release of melatonin and, in turn, gonadotrophin-releasing hormone (Wigham *et al*., 2017). This same mechanism is known to drive annual oestrous cycles in the Basenji breed of dogs (Fuller, 1956; Tedor and Reif, 1978; Gunn and Doney, 1979; Rhind and McNeilly, 1986). Controlled studies have demonstrated that manipulating day length using artificial light can modulate the interoestrous period and, therefore, breeding cycles in ewes (Ducker *et al*., 1970; Vesely, 1978; Legan and Karsch, 1980). Although, similar controlled studies have not been carried out in domesticated dogs it may be reasonable to assume that exposure to artificial light, especially during the winter months, may also interfere with the regulation of reproduction in dogs, dampening any normal seasonal variations in oestrus (Wigham *et al*., 2017).

Although it is widely known that photoperiod is the main factor that synchronises oestrus in many species, seasonality in oestrus has also been attributed to other regulatory factors, such as ambient temperature (Chatdarong *et al*., 2007; Ortega-Pacheco *et al*., 2007) and breed (Sokolowski *et al*., 1977). Pregnancy and parity may also influence the length of the interoestrous interval and, thus, subsequent oestrous start dates (Sokolowski *et al*., 1977; Linde-Forsberg and Wallén, 1992; Mutembei *et al*., 2000).

2.1.5 Ovulation

The broad classification of species as either spontaneous or induced ovulators is based on the type of stimulus responsible for eliciting GnRH release from the hypothalamus (Adams and Ratto, 2013). Most canids including domestic dog (Concannon, 2011), blue fox (Moller, 1973) and red fox (Mondain-Monval *et al*., 1977), have been defined as spontaneous ovulators. The known induced ovulating canid is the maned wolf (Johnson *et al*., 2014). In addition, ovulation can be physically induced in most felids (Brown, 2011; Stewart *et al*., 2012) or chemically induced in maned wolf (Johnson *et al*., 2014). There is possibility of male effect such as scent signaling from urine or feces, playing a role in ovulation when female and male were housed together (Xu and Feng, 2016). All ova are not released simultaneously as ovulation may take 24 to 96 hours (Linde-Forsberg, 2007). Boyd *et al*. (1993) using ultrasound notice that the ova were released from one ovary at a time with the whole process taking about 36 hours. Unlike other mammals, dog ovulates primary ooctyes that are at the beginning of the meiotic division at metaphase I (MI) and the germinal vesicle break down shortly thereafter (Linde-Forsberg, 2007). Canine oocytes mature *in vivo* in the oviduct and there is a multilayered and tight cumulus mass around the

oocyte which expands as the oocyte matures (Holst and Phemister, 1971; Mahi and Yanagimachi, 1976; Tsutsui, 1989). The transportation of oocyte in the oviduct takes 5-10 days (Andersen and Simpson 1973; Tsuitsui, 1989). The circulating and local progesterone (P4) levels begin to rise prior to the luteinizing hormone (LH) surge and subsequently leading to ovulation (Olson *et al*., 1982). Canine oocytes are ovulated at an immature stage compared with other mammalian species, and require 48–72 and 96–120 h post ovulation in the oviduct to complete unclear maturation and cytoplasm maturation, respectively (Reynaud *et al*., 2005; Nagashima *et al*., 2015). Previous studies on canine ovarian activity are based on the oocyte evaluation and ultrasonographic measurements of the follicular populations (Payan-Carreira and Pires, 2008; England *et al*., 2009; Reynaud *et al*., 2009; Bergeron *et al*., 2013). However, luteinisation of follicles in the bitch occurs before ovulation and follicles do not collapse immediately after ovulation (Bouchard *et al*., 1991; Concannon, 2009; England *et al*., 2009), making it difficult to distinguish between preovulatory follicles and/or corpora lutea by ultrasound (Bergeron *et al*., 2013). Studying the canine ovary *in situ* is also complicated by some anatomical challenges, especially an encapsulating ovarian bursa that interferes with the ultrasonographic view of the gonads (Wildt *et al*., 1978; England *et al*., 2009; Songsasen *et al*., 2011).

The LH surge commences the first day of the oestrus (E1) (Wright, 1990; Johnston *et al*., 2001a; Kutzler *et al*., 2003a; Michel *et al*., 2011). In the canine species the LH surge is associated with a preluteinisation of the granulosa cells that begin to secrete progesterone (de Gier *et al*., 2006; Concannon, 2009). Early signs of luteinisation became more marked in E1 together with folded and vascularized granulosa layers. This time is characterized by the mucification of the cumulus in the granulosa cells, the intensity of which depends on

the follicular maturity (Reynaud *et al*., 2006). Preliminary histological studies suggested early canine luteal cells may originate primarily from theca in growth, with dispersed mid-cycle luteal cells of a single type as in some rodents (Concannon, 2012).

Ovulation is thought to occur when blood progesterone concentration is between 4 and 10 ng/mL (Wright, 1990; Johnston *et al*., 2001a; Michel *et al*., 2011; Groppetti *et al*., 2015a). In polytocous species not all follicles ripen at the same time and in the bitch ovulations of the various follicles are spread over a 24–36 h period (Boyd *et al*., 1993; Groppetti *et al*., 2015a).

Vaginal cytology alone cannot be used to detect the precise periovulatory events, such as the day of LH surge or ovulation, but together with blood progesterone evaluation, the combination of which allows accurate definition of the stage of the reproductive cycle (Wright, 1990; Johnston *et al*., 2001a; Kutzler *et al*., 2003a; Fontbonne, 2010; Groppetti *et al*., 2010; Michel *et al*., 2011).

2.1.6 Fertilisation

This occurs during the passage of the ovum in the distal part of the oviduct (Linde-Forsberg, 2007). Mature canine ova may remain alive and fertilisable for 2-4.5 days (Concannon *et al*., 1989; Tsutsui, 1989). Canine spermatozoa can fuse immature oocyte (Mahi and Yanagiachi, 1976) whereas, embryonic cleavage between 2 and 16 cells occur more rapidly following fertilization of more mature oocyte (Linde-Forsberg, 2007).

Optimal fertility in the bitch occurs between 2 and 5 days after ovulation when mature oocytes are present (Wildt *et al*., 1978; Concannon, 1993). The ovulation is assumed to

occur approximately 2–3 days after the LH surge (Wildt *et al*., 1978; de Gier *et al*., 2006). Unfortunately, the time of LH surge is highly variable in dogs and takes a relatively long time, ranging from approximately 24 to 60 h (Wildt *et al*., 1978; Concannon, 1993, 2009; Onclin *et al*., 2002). The rapid rise in blood progesterone concentration at the LH surge and ovulation makes its measurement, together with vaginal cytology, the most reliable marker to detect periovulatory events in clinical practice (Okkens *et al*., 2001; Fontbonne, 2010; Moxon *et al*., 2012).

2.1.7 Diagnosis and staging of pregnancy in Canine Spp

The three well-defined stages of pregnancy defined by Wiebe and Howard, (2009) are as discussed below:

2.1.7.1 Stage 1: Canine fertilization to implantation: Positive pregnancy is difficult to confirm at this stage (Wiebe and Howard, 2009). The implantation takes place at approximately 18 days from the LH surge (Wiebe and Howard, 2009). Progesterone is secreted for initiation and maintenance of pregnancy (Wiebe and Howard, 2009). This maintains endometrial integrity and placental attachment, and inhibits myometrial contractility (Wiebe and Howard, 2009).

2.1.7.2 Stage 2: Implantation to Foetal Ossification: Pregnancies are more easily confirmed at this stage (approximately 25-30 days with ultrasound). Embryogenesis and foetal growth and development occur rapidly (Wiebe and Howard, 2009). The foetal ossification similarly occurs at 40 to 42 days.

*2.1.7.3 Stage 3: Foetal Ossification to Parturition:*This is the stage in which the fetus is well developed (Wiebe and Howard, 2009).Prostaglandins are the natural inhibiting factors causing luteal functional arrest before parturition (Wiebe and Howard, 2009).

2.1.8 Reproductive hormone

Luteinizing hormone (LH) plays a major role in reproductive processes (De los Reyes *et al*., 2017). This pituitary gonadotropin regulates follicle/oocyte maturation, ovulation, as well as optimal fertilization and corpus luteum (CL) development (Assidi *et al*., 2010). In the ovary, granulosa, theca, and luteal cells are capable of secreting steroid hormones, such as estrogens, testosterone and progesterone in response to LH (Fernandes *et al*., 1987; De Gier *et al*., 2006). The type and amount of hormone released vary according to the reproductive physiological status of the follicle and CL (Kooistra *et al*., 1999; Concannon, 2011). This gonadotropin exerts its effects through binding to its receptor (LHR), which is one of the seven transmembrane domain Gprotein- coupled receptor (GPCR) superfamily (Ascoli *et al*., 2002). It has been demonstrated in many species that at the ovarian tissue LHR is mainly expressed in theca cells, but it is also present in granulose cells of preovulatory follicles (Themmen and Huhtaniemi, 2000; Pakarainen *et al*., 2005). The interaction LH-LHR activates adenylate cyclase, phospholipase C and ion channels, which in turn control cellular cyclic AMP, inositol phosphates, Ca^{2+} and other secondary messengers (Gudermann *et al*., 1992; Pidoux *et al*., 2007). Through this pathway, the final differentiation of the granulosa cells and the enzymes responsible for androgen production in the theca cells are enhanced (Tsang *et al*., 1979; Oktay *et al*., 1998). However, alternative second messenger pathways, which may not include stimulation of cAMP

levels also exists (Bukovsky *et al*., 2003; Ascoli, 2005); but, none seems to be as effective as cAMP at mediating the induction of steroids synthesis (Hou *et al*., 2010).

In several species the LHR expression levels increase with follicle growth in response to follicle stimulating hormone (FSH), estradiol and paracrine factors, reaching maximum levels prior to ovulation (Menon *et al*., 2005). At this time, granulosa cells also express sufficient receptors for LH. Therefore, the activity of LH increases, supporting the growth and maturation of larger ovarian follicles and the oocyte as a result of specific LH granulosa cell receptors (Menon *et al*., 2004; Jeppesen *et al*., 2012). In dogs, the estrous cycle is longer than that of other mammals, with a non-seasonal anoestrus of about 3-12 months (De Gier *et al*., 2006). The LH pulses appear to occur in all stages of the oestrous cycle, including anoestrus (Kooistra *et al*., 1999). In fact, termination of the anoestrus involves selection of LH sensitive follicles, and this final follicles selection is caused by an increased frequency of high-amplitude LH pulses at the end of anoestrus (Concannon, 2009), since the responsiveness to gonadotropins increase circulating basal LH concentrations (Kumar and Sait, 2011). This period of increased LH pulsatility has been reported as an important determinant in the start of a new follicular phase (Concannon, 1993; De Gier *et al*., 2006).

It has even been suggested that changes in LH secretion may be more important than changes in FSH secretion in the initiation of a follicular phase leading to ovulation (Concannon, 1993). However, the endocrine mechanisms by which a cohort of follicles is recruited in anoestrus for further development in proestrus is unclear, as it is not known when this sensitivity to LH begins in those follicles (De los Reyes *et al*., 2017). Expression

of both LHR gene and LHR protein may be involved in the process of recruitment and selection of anestrous follicles, but unlike the evidences in rodents and other mammals, the canine LHR expression pattern is poorly understood (De los Reyes *et al*., 2017). Luteinization process begins in preovulatory follicles prior to ovulation in the bitch (Chastant-Maillard *et al*., 2011); therefore, canine follicular cells may increase receptors for LH before the end of proestrus (De los Reyes *et al*., 2017). An early acquisition of LH receptors on granulosa cells has been reported in dogs around the period of LH surge, by measuring the binding of radioactive labeled porcine gonadotrophins by autoradiography (Saint-Dizier *et al*., 2008).

2.1.9 Impact of multiple conceptuses per follicle on fecundity in the bitch

Due to the variation in ovulation rate among bitches (Andersen and Simpso, 1973; Nöthling and Volkmann, 1993; Nöthling *et al*., 2005), the relationship between the number of conceptuses and the number of corpora lutea may provide a better measurement of fertility in an experimental setting than the mere number of conceptuses (Nöthling and Volkmann, 1993). Follicles containing more than one oocyte, known as multi-ovular follicles (MOFs) occur in bitches (Telfer and Gosden, 1987; McDougall *et al*., 1997; Barber *et al*., 2001; Luvoni *et al*., 2005 Reynaud *et al*., 2005; Payan-Carreira and Pires, 2008; Reynaud *et al*., 2009). Ovariohysterectomy of the pregnant bitch enables the researcher to precisely and accurately determine the number of corpora lutea and post-implantation conceptuses (Nöthling and Volkmann, 1993; Nöthling 1995; Nöthling *et al*., 2006; Rota A *et al*., 2010; Steckler *et al*., 2013). However, the number of post-implantation conceptuses relative to the number of corpora lutea (implantation ratio)

reflects the net outcome of the number of follicles that ovulated, the number of oocytes that were fertilised and the number of early embryos that died (Ferguson *et al*., 1989). The higher the implantation ratio in a bitch is, the lower the effects of fertilisation failure and embryonic death (Steckler *et al*., 2017).

Multi-ovular follicles may have up to 17 oocytes although 2 or 3 are more common (Telfer and Gosden, 1987). They are more common in Indigenous breeds than in purebred bitches (Payan-Carreira and Pires, 2008), and their prevalence decreases with age of the bitch and with size of the follicle (Telfer and Gosden, 1987; McDougall *et al*., 1997). As many as 7% (Reynaud *et al*., 2009) to 40% (Payan-Carreira and Pires, 2008), of preantral follicles have been found to be MOFs (Steckler *et al*., 2017). Multi-ovular follicles may ovulate (Luvoni *et al*., 2005) because it was observed that the number of oocytes or embryos flushed from bitches was higher than the number of corpora lutea present (Bysted *et al*., 2001; Reynaud *et al*., 2005). However, the number of the oocytes released by ovulation from MOFs that are able to mature and be fertilized is not known (Chastant-Maillard *et al*., 2011) as the oocytes within a MOF can be at different developmental stages, and can be viable or degenerate (Barber *et al*., 2001). However, research suggests that only one oocyte of good quality is contained within a MOF (Payan-Carreira and Pires, 2008; Reynaud *et al*., 2009).

Uterine pathology, mating at the wrong time, sub-fertility of males (Johnston *et al*., 2001a) and various factors pertaining to artificial insemination such as semen quality, site of semen deposition, number of inseminations (Thomassen *et al*., 2001) and semen processing (Nöthling and Volkmann, 1993; Nöthling *et al*., 2005) may result in some

fertile oocytes released at ovulation not yielding conceptuses and thereby reducing the ratio between conceptuses and corpora lutea (Steckler *et al*., 2017). More than one conceptus may derive from the same follicle if a MOF releases more than one fertile oocyte at ovulation or if an embryo splits to yield monozygotic twins (Joone *et al*., 2016).

2.1.10 Gestation period and parturition in the bitch

The length of canine pregnancy apparently varies between 57 and 72 days because bitches show an extended period of receptivity and viability of spermatozoa in the female reproductive tract is prolonged (Concannon *et al*. 1983). Parturition occurs 65 ± 1 day after LH peak and 63 ± 1 day after ovulation (Concannon *et al*., 1983). The preovulatory LH surge can be detected through the measurement of the concomitant increase in serum progesterone concentrations, and this method provided a parturition prediction accuracy of 90% for 65 ± 2 days (Kim *et al*. 2007). Although LH could be measured directly, it is rather impractical because more than one daily samplings are necessary (Hase *et al*. 2000). In clinical settings, veterinarians have frequently to deal with near-term bitches whose history is limited to mating dates (Rota *et al*., 2015). At the end of pregnancy, some parameters can be monitored to predict parturition such as body temperature or progesterone concentration (Rota *et al*., 2015). Rectal and vaginal temperature may show a decline approximately 24 h before the onset of parturition (Concannon and Hansel 1977; Geiser *et al*. 2014), although it does not occur in all bitches (Veronesi *et al*. 2002), a significant decline in progesterone concentration occurs 24–40 h before parturition (Nett *et al*. 1975; Concannon *et al*. 1978; Veronesi *et al*. 2002; Brugger *et al*. 2011; Rota *et al*.,

2015), as a possible response to the rapid changes in circulating hormone concentrations (Concannon and Hansel 1977).

However, both the detection of LH peak and the ultrasonographic identification of ovulation may be unpractical, because of the necessity of daily determinations (Hase *et al*., 2000; Hayer *et al*., 1993). Nevertheless, LH peak and ovulation can be indirectly determined by progesterone rise (Beccaglia *et al*., 2016). It has been demonstrated that serum progesterone ranges between 2 and 3 ng/ml during LH peak and between 4 and 10 ng/ml during ovulation (Kutzler *et al*., 2003a). Progesterone concentration decreases below 2 ng/ml approximately 24 hr before parturition (Concannon *et al*., 1978), and this can be indirectly recognized by the drop in body temperature (Geiser *et al*., 2014; Johnston *et al*., 2001b). Recent studies demonstrated that in dogs, the imminence of parturition could be predicted by evaluating foetal flux and foetal heart rate by ultrasound (Gil *et al*. 2014; Giannico *et al*., 2015). For an accurate prediction of parturition date, the combination of different methods is desirable (Beccaglia *et al*., 2016). However, Concannon, (2000) reported that identification of the onset of dioestrus, through daily vaginal cytology after mating, has been used to predict the parturition. This occurs approximately 57 ± 1 days after the transitional smear from a cytological pattern characterized by a high number of keratinized cells to a pattern with predominance of basal and parabasal cells and leucocytes. It is common opinion that Caesarean sections can be safely performed when progesterone concentration declines below 2 ng/ml, that is, 24–40 h before natural parturition, (Concannon *et al*. 1977; Smith 2007). Progesterone concentration was reported to show diurnal variation in pregnant bitches from weeks 3 to 6, with morning values

higher than afternoon values (Steinetz *et al*. 1990); however, in the eighth and ninth week of pregnancy, morning and afternoon values resulted similar (Steinetz *et al.* 1990).

The gold standard to measure serum progesterone in bitches is radioimmunoassay (RIA), and previous works reporting peri-partum progesterone concentration in bitches have used this methodology (Nett *et al*. 1975; Concannon *et al*. 1978; Veronesi *et al*. 2002). The chemiluminescent (CLIA, Immulite) assay has also been validated for measuring progesterone in canine serum and has the advantage of being safe, fast and accurate (Kutzler *et al*. 2003a; Chapwanya *et al*. 2008). However, comparing RIA and Immulite serum progesterone values, the average Immulite measurement resulted in significantly higher concentrations than the RIA one, with an average difference of 0.69 ng/ml (Chapwanya *et al*. 2008).

2.2 Ultrasound and Ultrasonography

Ultrasonography is a non-invasive, quick and very easy diagnostic imaging modality (Pete *et al*., 1992). It is used to visualize the structure and architectural details of soft tissues and organs (Ulum *et al*., 2017). Its principle is based on the ability to reflect transmitted high-frequency sound waves by tissue (Kumari *et al*., 2016). Ultrasound waves are generated by the piezoelectric effect in a suitable medium such as lead zirconate (Kumari *et al*., 2016). Echoes produced by ultrasound depend on relative density of tissue (Pierson and Adams, 1995). A, B, and M modes are the three basic forms of ultrasound used in soft tissue imaging (Kumari *et al*., 2016). Diagnostic ultrasound frequencies range from 2 to 15 MHz and are inaudible to the human ear (Kumari *et al*., 2016). The image produced in sonogram is created using high-frequency sound wave which is displayed as real-time slices of

images similar to histological images (Pavlin and Foster, 1998). High-frequency sound wave of 20 MHz is commonly used in the medical services for dermatological examination (Gniadecka and Quistorff, 1996). This method is known as dermatosonography (Jemec *et al.*, 2000) while 30-100 MHz was used for eye examination (Pavlin and Foster, 1998). However, such high-frequency transducer is not available in most medical facilities. The most commonly available transducer in medical facilities are 3-15 MHz transducers which are used for obstetrical examination and imaging of other internal organs (Mantis, 2008).

The ultrasonogram is essentially an image of a thin slice of tissue, and therefore, enables serial examinations to monitor the progression of the condition, response to treatment and to practice scanning techniques (Hornbuckle and Kleine, 1980). However, one of the drawbacks of diagnostic ultrasound is that it has a steep learning curve and requires a great deal of skill and experience (Kumari *et al*., 2016).

The sound wave produced and captured by transducers consists of several parts namely Fresnel zone, focal zone and Fraunhoferzone (Noviana *et al.*, 2012; Mattoon and Nyland, 2014). Target organ examination is performed at focal zone to obtain an image with adequate resolution (Ulum *et al*., 2017). However, the limitation of 3-15 MHz transducers is the location of Fresnel zone more than 10 mm, hence the target organ could not be depicted clearly (Ulum *et al*., 2017). Fresnel zone is where complex diffractions patterns occur, located nearest to the transducer as the sound source. Focal zone is where the sound wave is most focused, producing an image with the best resolution. Fraunhofer zone is the part where sound wave diverges and the resolution of the image gradually decreases (Ulum *et al*., 2017). Ideally, the target organ of ultrasonography is directly positioned in the focus

zone by placing the transducer in the imaging area, especially for large organ or dense tissues. Focal zone position can be set to be close to the sound source in the transducer. However, visualizing small organ with direct technique was difficult because of its small size. Organs sized < 10 mm was automatically visualized in Fresnel zone; hence the image produced was not of diagnostic value (Ulum *et al*., 2017).

2.2.1 Historical overview of ultrasonography

World War I saw the first practical use of the piezoelectric effect in the development of sonar using a separate sound generator and detectors (Coltera, 2010). In 1880, the French Physicist; Pierre Currie discovered the Piezoelectric property of certain crystals which upon application of electricity resulted in the deformation and production of sound with a frequency greater than 20KHz known as Ultrasound (Vignoli and Saunders, 2011). Thereafter, in 1940, The American acoustical physicist; Floyd Firestone, devised the first ultrasonic imaging devise, the supersonic reflectoscope, to detect the internal flaws in metal casting. In 1941, the Austrian Neurologist; Karl Theo Dussik and a physicist; Friedriech are the first to use the ultrasound to image the human body (Siddharth, 2007; Levine, 2010). In the 1940s, Dr. George Ludwig was the first person to apply the ultrasonic energy to the human body for medical purposes at the Naval Medical Research Institute, Bethesda, Maryland (Anon, 2006; Anon, 2015). Douglass Howry with the help of Joseph Holmes, an American nephrologist, developed a *B-mode linear compound ultrasound scanner* to include superior transducers, amplifiers, and display imaging. Howry and his team introduced compound scanning in order to eliminate 'false' echoes. Compound scanning allowed Howry to differentiate between structures and tissues which ultimately produced better imaging and were then referred to as '*sonagrams'* (Anon,

2015). Today, the applications of ultrasound are diverse and include therapeutic purposes to stimulate tissue healing (Porter, 1991; Steiss, 2000), food sterilization (Piyasena *et al*., 2003) and vaccination of fish (Zhou *et al*., 2002).

2.2.2 General principles of ultrasonography

2.2.2.1 Resolution: Resolution is the ability of an ultrasound machine to distinguish echoes on the basis of space, time, and strength. (Kremkau, 2011) The better the resolution, the more likely an abnormality can be identified. As the frequency of an ultrasound wave increases, the resolution increases. There are three types of resolution found in ultrasound imaging; axial resolution, lateral resolution and elevation resolution (Kremkau, 2011).

Axial resolution is the resolution of two separate reflectors along the direction of the ultrasound beam (Kremkau, 2011). It is determined by the ability of the transducer to detect two reflecting echoes separately without an overlap in the returning echoes, therefore both echoes have to be separated by half the spatial pulse length (SPL) (Daniel, 2010). Spatial pulse length is the length of space in one pulse of ultrasound. When two reflectors are separated by a distance that is greater than half the SPL, the echoes from these two reflectors do not overlap as they return to the transducer and are interpreted as separate echoes (Daniel, 2010). The shorter the transmitted pulse and likewise its returning echo pulse, the better the axial resolution. The pulse length is determined by the number of cycles emitted multiplied by the wave-length (Daniel, 2010).

Lateral resolution is the ability to separate two adjacent reflectors perpendicular to the beam direction in (Kremkau, 2011). This was determined by the width of the ultrasound

beam which must be narrower than the space separating the reflectors (Daniel, 2010). The width of an ultrasound beam decreases with increasing frequency. In a focused ultrasound beam, where the width of the beam is restricted, the lateral resolution is best at the focal point of the ultrasound beam because this was the narrowest part of the beam (Kremkau, 2011).

*2.2.2.2 Focusing:*Focusing the beam results in reduced beam diameter and improved lateral resolution (Powis, 1986). Focusing of the beam can occur only in the near field of the ultrasound beam (Kremkau, 1993). Beam focusing characteristics are variable for each transducer and frequency and should be obtained from the manufacturer at the time ultrasound equipment is purchased. The focal distance and focal zone can be verified with an ultrassound test object. A number of devices are available commercially for testing imaging performance. Focusing can be performed dynamically or manually (Kremkau, 2011).

Table 2.1:Depth and axial resolutions obtained by different transducer frequencies

Frequency (MHz)	Image depth (cm)	Axial Resolution (mm)
2.0	20	0.77
3.5	17	0.44
5.0	12	0.31
7.5	8	0.20
10.0	6	0.15

Source: (Nyland and Matton, 1995)

2.2.3 Transducers

A transducer is a very important part of an ultrasound imaging system (Scampi, 2017). A typical ultrasound machine uses a wide variety of transducers optimized for specific diagnostic applications (Scampi, 2017). Each transducer is comprised of an array of piezoelectric materials that send focused energy into the body and receive the resulting reflections. Each transducer is connected to the ultrasound imaging system with fine coaxial cables (Scampi, 2017). Typical transducers have up to 32 to as many as 512 piezoelectric elements that operate at frequencies from 1MHz to 15MHz (Scampi, 2017). Most ultrasound systems provide two to as many as four switchable transducer connections to allow the clinician to easily switch among the various transducers for each examination type (Scampi, 2017).

2.2.4 Types of Transducers

2.2.4.1 Array transducers: The array transducers are either linear or curved in shape and their piezoelectric crystals are arranged in a line along the surface of the transducer (Patrick, 2010). They have the advantage of allowing a wide superficial field of view. The linear array is particularly important in viewing superficial tissues. They are used for the examination of the musculoskeletal system, abdominal organs, eyes, body wall and non-cardiac thoracic structures (Patrick, 2010).

2.2.4.2 Phased array transducers: Phased array (sector) transducers are composed of only a small number of crystals in rectangular format that are electronically excited as a group with small time difference (phasing), which results in the sound pulse being sent out in a specific direction that is constantly changing (Patrick, 2010).

They have the advantage of working with small foot prints and creating pie-shape images, which allow deep structures to be view clearly (Patrick, 2010). These transducers have a limited near field visibility compared with the linear array or curvilinear array transducer and are not a good choice for imaging superficial structures (Powis, 1993; Patrick, 2010). Phased array transducers are used for echocardiography and for scanning structures that are deeply located such as the abdominal or thoracic structures in large breeds of dogs (Patrick, 2010).

2.2.5 Transducer frequencies

The ultrasound wave frequency produced by the transducer is a function of the crystals within it (Patrick, 2010). A wide range of transducer frequencies is currently available from low frequency transducers imaging at 2.0 MHz to high frequency transducers imaging at 10.0 MHz (Patrick, 2010). Ultra high frequency transducers exist for specialty imaging. The lower the transducer frequency the deeper the penetration, but less resolution (Patrick, 2010). Routinely, 7.5 MHz penetrates a depth of 4-6 cm, 5 MHz penetrates 10-12 cm, 3.5MHz penetrates 15-20 cm and 2.5 MHZ penetrates 25-30 cm. To reach superficial structures a standoff pad can be used to transfer the focal zone to a higher level (Allen and Stone, 1990). The selection of the appropriate transducer for an examination depends upon the structure being evaluated, and the depth of the area from the transducer surface and the acoustic properties of the intervening tissues (Powis, 1986).

2.2.6 Ultrasound image formation

The formation of image in ultrasonography is based on the principle of pulse echo (Hangiandreou, 2003; Kremkau, 2011). As pulse transmits through the patient, sound waves are reflected back to the transducer from each tissue interface. In between, the

transducer is dampened to stop the piezoelectric crystal from vibrating. The electric signals generated from the returning echoes are amplified to form the final image (Kremkau, 2011). Two modes of echo display are commonly used in ultrasonography: brightness mode (B-mode, B scan, or gray scale) and motion mode (M-mode). B-mode is used commonly in both abdominal and cardiac imaging while M-mode is used only for echocardiography (Kremkau, 2011).

2.2.7 Modes of sonography

2.2.7.1 A-mode: A-mode is the simplest type of ultrasound (Jauhiainen, 2009). A single transducer scans a line through the body with the echoes plotted on screen as a function of depth. Therapeutic ultrasound aimed at a specific tumor or calculus is in A-mode to allow for pinpoint accurate focus of the destructive wave energy (Jauhiainen, 2009).

2.2.7.2 B-Mode: In B-mode ultrasound, a linear array of transducers simultaneously scans a plane through the body that can be viewed as a two-dimensional image on screen (Jauhiainen, 2009). B-mode displays a static image of a section of a tissue (Daniel, 2010). They are made up of collection of dots that corresponds to the amplitude and strength of the returning echo (Kremkau, 2011). These dots are displayed on a black background, and the brightness of the dot is highest (whitest) for the strongest returning echoes (Kremkau, 2011). The greater the amplitude the brighter the dot on the image. B-mode is commonly used in both abdominal and cardiac imaging (Kremkau, 2011).

2.2.7.3 M-mode: M stands for motion. In M-mode a rapid sequence of B-mode scans whose images follow each other in sequence on screen enables doctors to see and measure range of motion, as the organ boundaries that produce reflections move relative to the

probe (Jauhiainen, 2009). M-mode is a time motion displayed on the ultrasound wave along a chosen ultrasound line (Anon, 2017). It displays a static image of a section of a tissue. The echo generated from a single ultrasound wave passes through a moving structure and are acquired and displayed as a function of time on the horizontal axis. The motion pattern allows functional evaluation of moving structures such as the heart (Daniel, 2010). M-mode provides a monodimensional view of the heart and thus used for echocardiography (Kremkau, 2011). It has an advantage of very high sampling rate, which results in a high time resolution so that even very rapid motions can be recorded, displayed, and measured (Anon, 2017).

2.2.7.4 Doppler mode: This mode makes use of the Doppler effect in measuring and visualizing the flow of blood in the body (Jauhiainen, 2009).

2.2.8 Ultrasound artefacts

Ultrasound artefacts are often part of the images, and may lead to misinterpretations (Kirberger, 1995; Feldman *et al*., 2009; Hindi *et al*., 2013). They may be mistaken for lesions or may obscure the area of interest during ultrasound scanning (Hindi *et al*., 2013). For instance, artefacts commonly encountered when imaging the urinary tract include acoustic shadowing, reverberation artefact, distal acoustic enhancement, slice thickness artefact, grating or side-lobe artefact, edge shadowing (refraction) artefact, and twinkle artefact (Douglass, 1993; Douglass and Kremkau, 1993; Kirberger, 1995; Barthez *et al*., 1997; Louvet, 2006; Sutherland-Smith, 2008; Barrett, 2011).

The sound interaction with biological tissues is complex and may be responsible for many explained and unexplained artefacts. Additionally, the understanding of physical properties

of artefacts has been studied *in vitro* by several authors (Barthez *et al*., 1997; Heng and Widmer 2010).

2.2.8.1 Acoustic shadows: Acoustic shadows are areas of decreased echogenicity below a dense object with high reflectivity (Park *et al*., 1981; Penninck, 2002). It occurs if the primary beam is almost completely reflected or absorbed**.** An insufficient quantity of echoes returned from the location distal to the strong reflector makes these regions appear anechoic (black). This type of artefact is useful because it gives information about the composition of the structure causing the shadowing (Heng and Wigner, 2010; Kremkau, 2011). Bone, air, foreign bodies and calcification stops the transmission of sound waves producing a 'sonic shadow' which is a dark region distal to the echogenic obstructing region. Acoustic shadowing artefacts are minimized with spatial compounding (Heng and Wigner, 2010; Kremkau, 2011). This may allow a lesion hidden by a shadowing artefact to be identified. Conversely, the composition of a structure may be misinterpreted because it is not shadowing (Hangiandreou, 2003).

According to Kirberger (1995), acoustic shadowing occurs because of mineral or air interfaces causing loss of echo intensity deep to the interface because of absorption and/or reflection. Mineral interfaces such as uroliths typically cause strong distal hypoechoic to anechoic shadows. Air interfaces typically cause echogenic ("dirty") distal shadows because of concurrent reverberation artefact. This artefact is seen when gas is present within the bladder lumen or wall (Kirberger, 1995).

2.2.8.2 Acoustic enhancement/ distal acoustic enhancement: This is due to passage of sound through an area of decreased attenuation resulting in higher echo intensity from

tissues deep to the area compared with surrounding tissues at the same depth. This artefact is typically seen with fluid-filled structures such as the urinary bladder (Kirberger, 1995). This is an area of increased echogenicity behind structures of low attenuation (Kirberger, 1995; Penninck, 2002; Kremkau, 2011). It mostly occurs at sites distal to cysts, gallbladder and urinary bladder and appears as a hyper intense (hyperechoic) signal (Kirberger, 1995). The attenuation of ultrasound wave in fluid is much lower compared to other tissues; therefore tissues distal to the fluid are enhanced. Artificial enhancement may also be found distal to a homogenous solid tumor surrounded by adipose fat (Kremkau, 2011).

2.2.8.3 Reverberation artefacts: They occur when the sound waves encounter an area of high reflectivity such as bowel gas and the sound is reflected towards the transducer. They are produced from multiple reflections of sound wave, if the acoustic impedances of tissue layers are too much different and the detected echo does not run the shortest sound path because it bounces back and forth between the object and transducer (Park *et al*., 1981; Feldman *et al*., 2009). They appear as multiple hyperechoic foci that occur at regular intervals.

2.2.8.4 Comet tail artefacts: This is another form of reverberation artefact that appears as a series of short, very closely spaced successive echoes that are typically decrease in intensity and width in depth. They are caused by two closely spaced, discrete highly reflective surfaces (Feldman *et al.,* 2009; Kremkau, 2011). When gas bubbles form thin layers separated by liquid just as in the digestive tract, the waves rebound between the layers, resulting in many echoes that return to the probe at regular intervals, making a trail

of echoes in the form of a shadow looking like a comet's tail (Feldman *et al.,* 2009; Kremkau, 2011).

This is also a variation of reverberation artefact that appears as a series of short parallel reflective lines that extends behind a gas collection. It occurs when ultrasound wave reverberates within fluid trapped between collections of air bubbles (Feldman *et al*., 1995; Kirberger, 1995).

2.2.8.6 Mirror-image artefacts: This type of artefact occurs when a highly reflective and obliquely oriented surface reflect sound beam distally instead of returning it to the transducer (Daniel, 2010). It appears as a duplication of a normal structure on the opposite side of a strong reflector (Kremkau, 2011). This goes against the assumption that echoes return to the transducer after a single reflection and that sound wave travel in a straight line This is most commonly encountered when the liver is imaged with the diaphragm/lung interface is acting as a strong reflective surface (Feldman *et al*, 2009).

2.2.8.7 Side-lobe, grating-lobe artefacts and slice thickness: They cause the appearance of echoic material within anechoic areas. These artefacts can give the false impression of sediment within the urinary bladder (Barthez *et al*., 1997). Side lobes and grating lobes are different types of secondary lobes present on the side of the primary sound beam. Side lobes are present in all transducers and originate from additional mode vibrations of the piezoelectric crystal (Barthez *et al*., 1997). They are usually of low intensity and can create spurious echoes in the near field. Grating lobes are associated with the geometric construction of linear probes (Barthez *et al*., 1997). The artefacts created by secondary lobes result in misplacement of reflected echoes. The side or grating lobes are weaker than

the primary sound beam. These lobes must encounter a highly reflective surface and be of sufficient intensity to be noticed. In clinical situations, secondary lobe artefacts are difficult to differentiate from volume-averaging artefacts (Barthez *et al*., 1997).

Slice thickness artefacts occurs when the ultrasound beam is wider than the diameter of the cystic structure being scanned and include normal structures which lie immediately close to the cystic structure, the echoes from the tissues are displayed within the cystic structure mimicking the presence of a pseudo-sludge (Daniel, 2010). This does not occur when the entire beam width includes the cystic structure alone. Thus slice thickness artefact are avoided if the beam width is narrowed, such as in the focal zone of the beam. The surface of a pseudosludge is curved while the surface of a real sludge is flat (Penninck, 2002).

2.2.8.8 Refraction: This occurs when a sound wave changes in direction on passing from one medium to the other (Daniel, 2010). It occurs when sound waves travel from one tissue of different acoustic velocities, its frequency remains the same but its wavelength changes (Daniel, 2010). As the sound wave moves to the new medium, it deviates resulting in display of organs in incorrect locations, usually to the side of their actual location (Feldman *et al*., 2009; Kremkau, 2011). Refraction artefacts may lead to measurement errors because organs may appear wider than normal (Kremkau, 2011).

2.2.8.9: Edge-shadowing artefacts: This is caused by refraction of the ultrasound beam. It occurs at the margin of curved structures and results in a dark line or shadow extending distally. If this artefact involves a curved fluid-filled structure (bladder) surrounded by fluid (abdominal effusion), it will give the impression of a defect in the bladder wall (Douglass and Kremkau, 1993). They are a form of refraction artefacts that are produced

when sound wave deviates as they encounter a curved surface tangentially (Kirberger, 1995). As a result of the sound waves that have been deviated by the curved surfaces, areas of anechoic regions are seen distal to the curved surface. This type of artefacts is most commonly encountered when the kidneys, urinary bladder or gallbladder are imaged and can be eliminated with spatial compounding (Heng and Widmer, 2010).

2.2.8.10 Twinkling artefact: Twinkling artefact is a colour Doppler artefact that occurs behind strongly reflective interfaces such as those produced by urinary tract stones or parenchymal calcifications (Louvet, 2006). It appears as a quickly fluctuating mixture of Doppler signals with an associated spectrum of noisy appearance and can be used to differentiate mineral structures from other hyperechoic material (Louvet, 2006).

2.3 Doppler Ultrasonography

Doppler ultrasonography is a non-invasive real time pulse-wave technique used for the study of the reproductive system hemodynamics (Ferreira *et al*., 2011). This technology allowed re-evaluating previously conceptions considered definitive regarding the physiology of reproduction, and it is effective in the evaluation of animals' reproductive tract for the diagnostic, monitoring, and predictive purposes (Ginther, 2007).

Colour-Doppler ultrasonic imaging is based on the principle that proposes the change in frequency of a wave for an observer moving relative to the source of the respective wave. The frequency is constant when the wave source and observer are stationary (Ferreira *et al*., 2011). However, if there is a motion toward or away from each other, the returning echoes frequency increase and decrease, respectively (Ginther and Utt, 2004). This principle is used for different applications as temperature, velocity and vibration

measurements. In Doppler sonography, the wave source and stationary object are the red blood cells and the transducer respectively (Ferreira *et al*., 2011). During an ultrasonic Doppler examination it is possible to perform a qualitative and/or quantitative evaluation of specific vessels and tissues. The direction and velocity of red-blood cells can be represented by different types and intensities of colours pixels or as a velocity spectral graphic (Ferreira *et al*., 2011).

2.3.1 Coloured ultrasonic imaging

There are two specific approaches for coloured ultrasonic imaging evaluation: Colour-flow and Spectral modes (Ferreira *et al*., 2011). Colour-flow mode uses Colour-Doppler signals superimposed on a B mode image of a structure to estimate its vascularity, while pulsed-Doppler spectral analysis of blood velocities of a specific artery is done on Spectral mode as shown in Plate 2.1. Both methods are based on Doppler-shift frequencies, also called Doppler frequency (Ginther and Utt, 2004). Doppler frequency is the difference between the frequency of received echoes and frequency transmitted by the transducer (Ferreira *et al*., 2011). The transducer transmitted frequency is constant while the frequency of the returning echoes varies according to the Doppler effect principle. Therefore, when the wave source (red blood cells) is stationary or moving parallel to the transducer there is no difference between transmitted and returning frequencies and coloured Doppler signals are not detected (Ferreira *et al*., 2011). If the blood flow moves toward the transducer, the returning frequency is greater than the transmitted frequency resulting in a positive Doppler frequency. A negative Doppler signal is produced when the returning frequency is

lower than the transmitted frequency or, in other words, when the blood red cells move away to the transducer (Zagzebski, 2005).

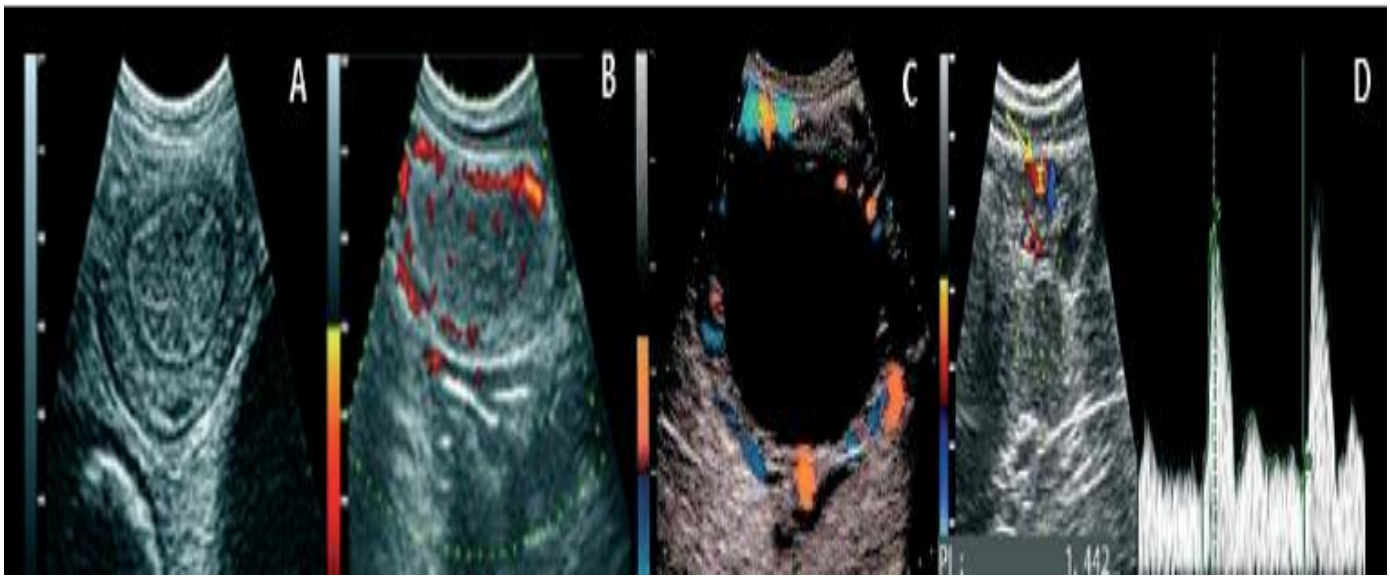


Plate 2.1 Ultrasonic images collection of female reproductive tract in B, Power, Colour and Spectral modes (A, B, C and D, respectively). (A and B) Cross section image, using B-mode and Power-flow Doppler, of a non-pregnant uterine horn in dioestrus. (C) Colour Doppler evaluation of a pre-ovulatory follicle. (B and C) Presence of coloured pixels within uterine tissue (endometrium and myometrium) and follicular wall indicate the red blood cells displacement and have been used to estimate the tissue vascularity. (D) Spectral graph consisting of maximum speeds values of blood flow in mesometrial artery. Source: Ferreira *et al*., (2011).

2.3.1.1 Spectral mode: In the spectral mode, blood flow velocity variations are represented as a graphic wave form called spectrum (Plate 2.2). The spectrum provides maximum velocities values as Peak systolic (PSV), End diastolic (EDV) and Time-average maximum (TAMV) velocities. Peak systolic Velocity is the maximum point along the length of the spectrum, while EDV is the ending point of the cardiac cycle (Ferreira *et al*., 2011). Time-average maximum is the maximum velocity values average. Additionally, the position of the spectrum in relation to the baseline of the monitor indicates the blood flow direction. By convection, wave forms above and under the baseline indicate, respectively, blood-red cells moving toward and away from the transducer (Ginther, 2007; Ferreira *et al*., 2011).

An alternative used in early studies is to calculate the Doppler index (Silva *et al*., 2005; Silva and Ginther, 2006; Ferreira *et al*., 2008; Ferreira *et al*., *et al*., 2010). They are good indicators of vascular perfusion of reproductive organs and are independent of the Doppler angle (Ginther and Utt, 2004). Resistance and Pulsatility indices (RI and PI, respectively) are calculated using the ratio of the three velocities measurements (PSV, EDV and TAMV) and both indices have a negatively correlation with the vascular perfusion of the tissue downstream from the sample gate (Ginther, 2007).

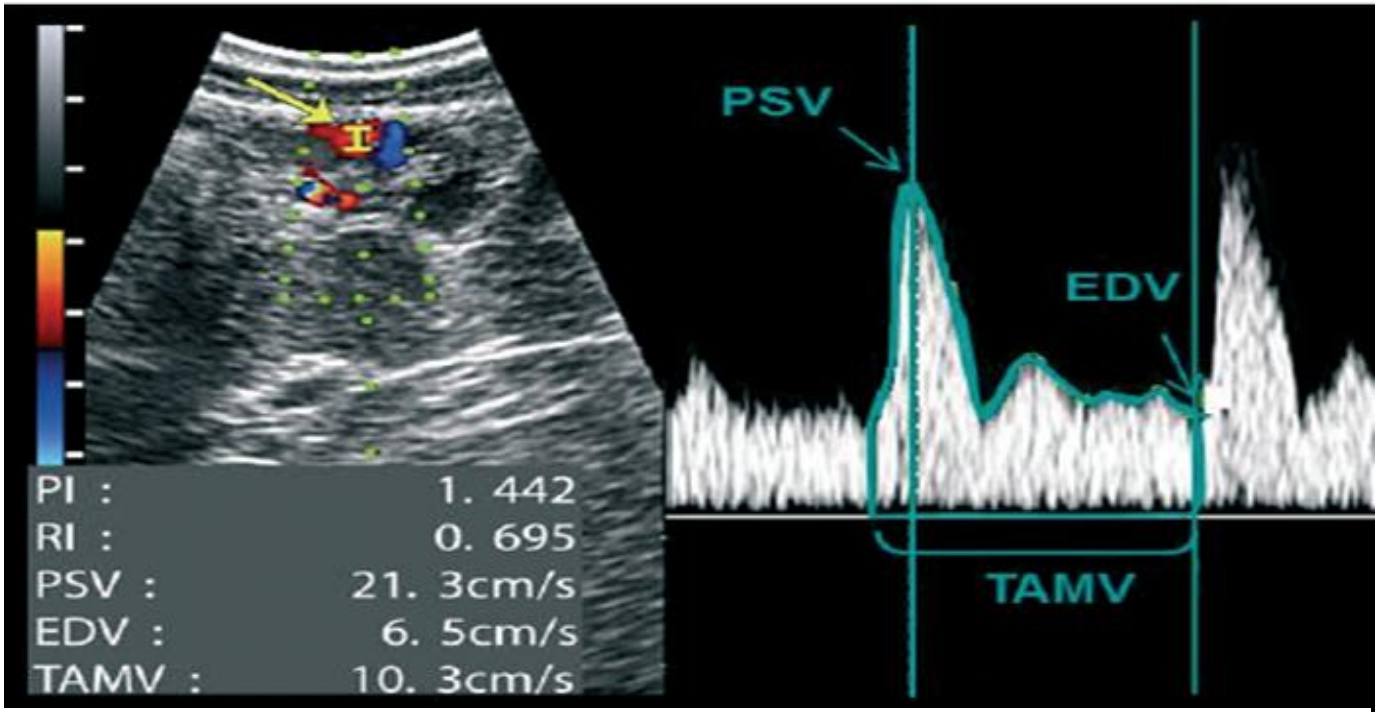


Plate 2.2 Spectral Doppler evaluation of mesometrial attachment artery. The gate (yellow) is placed on an artery of the mesometrial attachment (red) to capture velocities data of uterine blood flow and, posteriorly, produce a spectral graph. Peak systolic (PSV) and end diastolic (EDV) velocities are, respectively, the maximum and ending blood flow velocities of a cardiac cycle. TAMV is the average of an espectral maximum blood flow velocities. Doppler device automatically calculates Pulsatility and Resistance indices (RI and PI, respectively). Spectral graph located above the baseline indicates erythrocytes moving toward to the transducer. Source: Ferreira *et al*., (2011).

2.3.1.2 Colour-flow mode: Colour-flow mode is a simple, rapid and functional method of evaluation, which has been applied for farm practical purposes (Ginther, 2007). Colour-flow Doppler provides an immediately qualitative evaluation of the blood flow of different organs and tissues. The vascularity of a structure is estimated subjectively considering the extension of a tissue with coloured pixels during a continuous real time examination (Ferreira *et al*., 2011).

The current Doppler equipment offers two different modes of colour-flow imaging: Colour-flow and Power-flow modes (Ferreira *et al*., 2011). The classic colour-flow mode uses two distinct colours, usually variations of red and blue colours, to represent the vascular blood perfusion of a structure. Also, coloured pixels indicate the blood red cells direction in relation to the transducer. By convection, red coloured spots indicate blood flow moving toward the transducer, while blue coloured spots represent blood-red cells moving away from the probe. Additionally, the intensity of the coloured pixels suggests the velocity of blood flow ranging from dark to bright tonalities for slower and faster velocities respectively (Ferreira *et al*., 2011).

Power-flow imaging has been used to evaluate uterine and ovarian vascularity of mares and cattle (Silva and Ginther, 2006; Ferreira *et al*., 2008; Ginther *et al*., 2008; Ferreira *et al*., *et al*., 2010). In power-Doppler mode, the blood flow movement is graduated using a single colour and the colour pixels intensity vary according to the power of the Doppler signals (number of blood red cells moving at a specific velocity). Power mode Doppler could improve the evaluation of vessels with small diameters and slow blood flow, as are present in the uterus (mesometrium attachment, endometrium and myometrium) and

ovaries (mesovarium, corpus luteum and follicular wall) of mares (Ferreira *et al*., 2008; 2010; 2011).

2.3.2 Evaluation of the reproductive tract of bitches by Doppler ultrasonography

The use of colour and power Doppler ultrasonography has become one of the best available and reliable techniques for the diagnoses and studies of reproductive haemodynamics (Ginther, 2007; Ferreira *et al*., 2008). Considering the presence of a substantial number of small vessels with slow blood flow that do not appear on a conventional colour-flow image, it suggested the use of power-flow mode for the vascular perfusion evaluation of follicles, corpus luteum and uterus (Ferreira *et al*., 2008; Ginther *et al*., 2008; Siddiqui *et al*., 2010).

The evaluation of uterine vascular perfusion using colour-flow imaging must be done considering all uterine layers (Ferreira *et al*., 2008; Ferreira *et al*., 2010). The vascularity of the uterus is estimated subjectively using the percentage of uterine tissue with colour signals Doppler during a real time cross-sectioning examination of the uterus in a continuous span of 1 minute. Multiple cross sections must be viewed as consequence of animal and uterine movements (Silva *et al*., 2005).

The mesometrium attachment is an important reference point in equine trans-rectal Doppler ultrasound for indicating the area of entry of the blood vessels into the uterus (Ginther, 2007). According to Silva *et al*. (2005), colour Doppler signals within the endometrium are commonly inadequate for the production of spectral waveforms. However, early studies have reported the successful use of mesometrial arteries as an alternative assessment for the spectral evaluation of uterine blood flow velocities of

pregnant and non-pregnant mares (Silva *et al*., 2005; Silva and Ginther, 2006; Ferreira *et al*., *et al*., 2010).

Luteal vascularity is estimated while viewing, in a slow and continuous motion, of the entire CL real-time scan (Ginther *et al*., 2008). Colour spots at the periphery and within the CL must be included in the luteal vascular perfusion estimation. Spectral mode from CL is considered too time consuming hence, it is not used for objective evaluation (Ginther *et al*., 2007). An alternative to validate the subjective examination of CL is determining the number of coloured pixels in a captured selected image representing the largest cross-section of the CL with distinctive coloured areas as described by Ginther and Utt, (2004). Vascular blood perfusion of follicles is estimated considering the coloured Doppler signals from the vessels of the follicular wall (Siddiqui *et al*., 2010). The entire follicle must be scanned several times in a slow and continuous motion (Ferreira *et al*., 2011).

2.4 Contrast-enhanced Ultrasonography

It is well known that the presence of gas bubbles *in vivo* and particularly in the bloodstream can be highly undesirable as this could lead to some pathologic conditions such as air emboli (Chappell and Payne, 2006). However, there are numerous numbers of biomedical applications of gas bubbles particularly in the context of diagnostic and therapeutic ultrasound (Stride and Coussio, 2009). Gas bubbles could be formed *in vivo* by injection into the vein in form of suspension of stabilized micro bubbles or liquid droplets, which subsequently vaporize to form bubbles. These types of micro bubbles produced have been in clinical use for more than two decades as contrast agents for ultrasound imaging (Schlief, 1991; Cosgrove, 2006). Pressure reduction in a given region could also be used to

generate gas bubbles (Stride and Coussio, 2009). Since the solubility of a gas in a liquid falls with pressure, reducing the pressure would drive the gas out of solution (Stride and Coussio, 2009). Similarly, if the pressure falls below the liquid vapour pressure, vapour-filled cavities or bubbles would also be formed. Further reductions in pressure would promote further diffusion of gas and/or vapour into existing bubbles and also growth of the bubbles according to the appropriate gas law (Stride and Coussio, 2009). More so, bubbles can also be formed as a result of an increase in temperature (Stride and Coussio, 2009). It is known that the solubility of most gases falls with increasing liquid temperature while the vapour pressure increases, in both cases promoting the formation and growth of gas- and/or vapour-filled cavities (Stride and Coussio, 2009). The formation and growth of bubbles in tissue or *in vivo* as a result of ultrasound exposure normally involves a combination of changes in both pressure and temperature (Stride and Coussio, 2009).

2.4.1 The use of bubbles in ultrasound imaging and therapy

Ultrasound is the propagation of a pressure disturbance through a medium at a particular frequency, and the pressure at a given location would therefore fluctuate at that frequency (Stride and Coussio, 2009). Similarly, part of the energy carried by the propagating wave was converted into heat by viscous absorption, resulting in a local temperature rise. The formation of and subsequent interaction between bubbles and ultrasound are the key to their exploitation in diagnostic and therapeutic applications (Stride and Coussio, 2009).

Carbon dioxide micro bubbles were used as ultrasound contrast (Kitano *et al*., 2011). It required an angiographic technique that was relatively invasive because the carbon dioxide micro bubbles must be selectively infused into the celiac artery or superior mesenteric

artery (Kitano *et al*., 2011). In a preliminary study using the intravenous ultrasound contrast agent, Albunex® (Shionogi, Osaka, Japan), air-filled micro bubbles stabilized with a thin shell of human albumin and showed that ductal adenocarcinomas were less enhanced than the normal pancreatic parenchyma in contrast enhanced Echo Ultrasound Scan (EUS) after the intravenous infusion of the contrast (Hirooka *et al*., 1997).

There are several reports on the utility of contrast-enhanced EUS with the Doppler mode for diagnosing lesions in esophagus, stomach, gallbladder, pancreas and lymph nodes (Reddy *et al*., 2011). Particularly, power Doppler EUS with or without contrast agents depicted most adenocarcinomas in the pancreas as a hypovascular tumor (Kitano *et al*., 2011). The contrast-enhanced EUS using this carbon dioxide gas depicts ductal carcinomas as hypovasculsr masses whereas it depicts chronic pancreatitis and neuroendocrine tumors as isovascular and hypervascular masses, respectively (Kato *et al*., 1995). Several limitations in contrast enhanced Doppler ultrasonography include blooming artefacts, poor spatial resolution, low sensitivity to slow flow and high sensitivity to motion artefacts (Kudo, 2003; Whittingham, 2005)

2.4.2 The basic principles of micro bubbles as echo contrast agents

The micro bubbles act as echo-enhancers by basically the same mechanism as that determining echo-scattering in all the other cases of diagnostic ultrasound due to the fact that the backscattering echo intensity is proportional to the change in acoustic impedance between the blood and the gas making the bubbles (Calliada *et al*., 1998). The different acoustic impedance at this interface is very high and in fact all of the incident sound is reflected, even though not all of it travels back to the transducer (Calliada *et al*., 1998).

Reflectivity is proportional to the fourth power of a particle diameter and directly proportional to the concentration of the particles themselves (Calliada *et al*., 1998).

2.4.3 Types of micro bubble agent

In the late 1960s, Dr Claude Joyner found that micro bubbles could be used as ultrasound contrast agents while conducting a study on cardiac output by periodically injecting indocyanine green dye into the patient's left ventricle and simultaneously performing an M-mode echocardiogram (Feigenbaum *et al*., 1970). He observed that each injection of dye produced a temporary increase in the ultrasound echo from the ventricle. Initially it was thought that the contrast enhancement was due to the nature of the dye, but further investigation by Gramiak and Shah (1968) and Kremkau *et al*. (1970) showed that formation of gas bubbles at the catheter tip was responsible for the observed contrast enhancement. Since then, series of the contrast agents were developed for use in ultrasonography for diagnostic and therapeutic use.

*2.4.3.1. AlbunexH (Mallinckrodt Inc., Hazelwood, MO, USA**):*** This consists of air micro bubbles coated with a thin stabilizing layer of cross-linked human serum albumin. Although Albunex micro bubbles were stable compared to uncoated bubbles, they were unable to provide prolonged contrast enhancement *in vivo* hence, newer agents containing higher-molecular-weight gases were consequently developed (Stride and Coussio, 2009).

*2.4.3.2. OptisonTM (GE Healthcare Inc., Princeton, NJ, USA)**:***It contains perfluoropropane with a relative molecular mass approximately six times that of air (188 as opposed to 29), resulting in lower diffusion rate of the gas across the bubble wall (Stride and Coussio, 2009).

*2.4.3.3. EchovistH and LevovistH (Schering AG, Berlin, Germany):*They contain suspensions of galactose microcrystals, which dissolved in the blood following injection, thereby releasing air micro bubbles from defects on the crystal surfaces (Stride and Coussio, 2009). Levovist also contained palmitic acid to provide additional stability. Levovist micro bubbles were found to be more echogenic than either Albunex or Optison, but considerably less stable because of higher diffusion rate of their surfactant coatings (Stride and Coussio, 2009). Hence, Levovist was soon superseded by agents like SonoVueH (Bracco International BV, The Netherlands), DefinityH (Bristol- Myers Squibb Medical Imaging Inc., USA) and SonozoidTM (GE Healthcare Inc., Princeton, NJ, USA) containing bubbles stabilised by phospholipid monolayers which had a better compromise between longevity and echogenicity (Stride and Coussio, 2009).

*2.4.3.4. EchoGenH (Sonus Pharmaceuticals Inc., Bothell, USA):*This consists of surfactant-coated droplets of perfluoropentane that have a boiling point coinciding with normal body temperature (37 ºC) (Rapoport *et al*., 2007).

2.4.3.5. Echogenic liposomes: This consists of phospholipid bilayers encapsulating a mixture of liquid and gas (Tiukinhoy *et al*., 2004; Huang, 2008). These are also more stable than monolayer-coated micro bubbles and are particularly attractive for drug delivery applications, as larger quantities of either aqueous or non-aqueous material can be encapsulated. Larger doses of echogenic liposomes are required to obtain equivalent levels of contrast enhancement during imaging, on account of their lower gas content per particle, but such high concentrations are well tolerated physiologically (Hamilton *et al*., 2002). Similarly, specific pulse regimes are required to initiate drug release (Smith *et al*., 2007).

In terms of scattering efficiency, proportion of the incident field is scattered rather than absorbed (Coussios *et al*., 2004). However, it has been reported that the acoustic properties of echogenic liposomes may be superior to those of micro bubbles (Coussios *et al*., 2004).

2.4.4 Use of micro bubbles as quantitative imaging

In general, micro bubbles would remain in the vascular system and potentially therefore can be used as a means of quantifying tissue perfusion and other physiologically relevant parameters such as relative vascular volume and flow velocity (Stride and Coussio, 2009). These measurements are particularly significant for examination of myocardial function, kidney, and tumour vasculature (Stride and Coussio, 2009). Flash replenishment imaging is the most commonly employed technique whereby the micro bubbles in the image plane are initially destroyed by a high-amplitude ultrasound 'release burst', and the rate at which the image plane is replenished with bubbles is then monitored in real time using low amplitude pulses. Fitting the results to an appropriate flow model provides estimates for the blood volume and tissue perfusion (Schlosser *et al*., 2001; Metoki *et al*., 2006).

2.4.5 Using micro bubbles to target tissues and molecular imaging

Some commercial contrast agents such as Levovist have been found to have a tissue (liver and spleen) specific late phase (Albrecht *et al*., 2000). Targeted imaging and therapy of tissues requires some form of functionalization of the microbubble surface. Micro bubbles coated with material carrying a charge have been shown to preferentially locate inflamed tissues (Lindner, 2002), but a more effective method is to attach ligands to the bubble surface that would bind to receptors on particular types of cell (Lindner, 2002). Contrast agents has been used as targetings to activated leucocytes by incorporating

phosphatidylserine in the microbubble coating (Lindner *et al*., 2000), angiogenic markers (Leong-Poi *et al*., 2003; Emoto *et al*., 2007) and antibodies attachments (such as anti-P-selectin monoclonal antibody, anti- ICAM antibody, anti-VCAM antibody) to detect receptors that is expressed during inflammation (Song *et al*., 2001; Lu *et al*., 2004; Takalkar *et al*., 2004).

2.4.6 Therapeutic applications of contrast agents

2.4.6.1 Bubbles as delivery vehicles: There has been increasing interest in the use of micro bubbles for drug and gene delivery. Micro bubbles represent excellent vehicles for this type of application as drugs or DNA can be incorporated into the microbubble coating, traced through the body using low-intensity ultrasound, and then released by destroying the micro bubbles with high-intensity ultrasound at a target site such as a tumour (Stride and Coussio, 2009). The ability of ultrasound to be tightly focused into small tissue volumes ($5mm^3$) provides a physical means of localizing microbubble activity (Stride and Coussio, 2009). With some polymeric micro bubbles it is possible to dissolve or disperse the drug directly in the coating, provided that it is of sufficient thickness for the required dose (Unger *et al*., 1998; Lentacker *et al*., 2006). The drug is released as the coating breaks down when the microbubble oscillates at sufficient amplitude (Stride and Coussio, 2009).

2.4.6.2 Microjetting: In addition to providing a means of encapsulating therapeutic material, there are a number of physical phenomena produced by microbubble oscillations which may contribute to the therapeutic effect by enhancing the rate of uptake of drugs/DNA by cells, and/or increasing the rate of erosion or denaturing of diseased tissue (Stride and Coussio, 2009). The formation of microjets is an example of one of these. It

has been shown *in vitro* that a microjet can easily puncture a cell membrane and this effect has been observed extensively with contrast agent micro bubbles near cell monolayers (Prentice *et al*., 2005). It has been suggested that microjetting could potentially be the cause of the enhanced cell uptake generated by micro bubbles (Prentice *et al*., 2005).

2.4.6.3 Microstreaming: When a sound wave propagates through a liquid, steady currents are set up in the direction of the beam as a result of momentum transfer from the wave to the liquid (Lighthill, 1978; Hill *et al*., 2004). This acoustic streaming also occurs, albeit on a much smaller scale, around micro bubbles undergoing stable oscillations (Elder *et al*., 1954; Elder and Nyborg, 1956). These eddying flows may, in turn, impose shear stresses on nearby surfaces, such as cell membranes, and it is thought that this may promote the uptake of therapeutic components (Marmottant and Hilgenfeldt, 2003; Marmottant *et al*., *et al*., 2008). Microstreaming has also been shown to cause significant damage to cells at higher amplitudes of oscillation (Clarke *et al*., 1970; Rooney, 1970; Ward *et al*., 1999). In addition, microstreaming would contribute to circulating therapeutic agents in the target region, which is also likely to be important in the context of both sonothrombolysis and drug uptake (Prokop *et al*., 2007).

*2.4.6.4 Heating enhancement:*The absorption of energy as ultrasound propagates through a medium also produces a heating effect. In most materials, including tissue, the rate of absorption increases with frequency and thus the presence of bubbles can enhance this heating effect owing to their ability to generate higher harmonics of the excitation frequency (Hill *et al*., 2004). Bubbles also dissipate energy as heat, both as a result of viscous friction in the surrounding liquid and any coating material, and via conduction

during compression (Stride and Coussio, 2009). The relative significance of each of these dissipation mechanisms depends on the size of the bubble, the physical properties of the surrounding liquid, and the frequency and intensity of the ultrasound field (Hilgenfeldt *et al*., 2000; Coussios *et al*., 2008). Similarly, the magnitude of the temperature rise generated also depend on these parameters, as well as on the concentration of bubbles present, the pulse repetition frequency/ duty cycle and proximity to blood vessels (Coussios *et al*., 2007; Hariharan *et al*., 2007).

Large temperature rises are clearly beneficial for tissue ablation, certain types of drug and gene delivery where thermal activation is required (Plathow *et al*., 2005; Liu *et al*., 2006). In diagnostic imaging and where therapeutic agents are temperature sensitive, however, significant heating is normally undesirable, and the relevance of safety indices (e.g. thermal index) for applications employing micro bubbles require further investigation.

2.4.6.5 Chemical effects: The large and rapid reduction in volume experienced by a bubble undergoing inertial collapse can produce a significant rise in pressure and temperature (Kim *et al*., 2000a). These extreme conditions are confined to the centre of the bubble (Unger *et al*., 1998), but highly reactive chemical species may be produced within this space (Suslick, 1990). Of particular interest in the context of medical ultrasound is the potential for the formation of free radicals and toxic chemicals such as hydrogen peroxide (H_2O_2), which may be harmful to cells. For example, Juffermans *et al*. (2008) have shown that rat cardiomyoblast cells experience a calcium ion (Ca^{2+}) influx upon exposure to low-intensity ultrasound with SonoVue, causing localized hyperpolarisation of the cell

membrane, which may promote molecular uptake and this may also be related to the generation of H_2O_2 despite the much lower ultrasound intensities.

Another application undergoing exciting developments is sonothrombolysis, where micro bubbles have been shown to increase markedly the effectiveness of tissue plasminogen activator (t-PA) and the rate of clot lysis *in vitro* and *in vivo* (Culp *et al*., 2004; Molina *et al*., 2006; Datta *et al*., 2008). More so, there is considerable evidence that micro bubbles increase the permeability of not only individual cell membranes but also the endothelium (van Wamel *et al*., 2006; Iwanaga *et al*., 2007) including temporary opening of the blood–brain barrier (Meairs and Alonso, 2007).

2.5 Contrast Harmonic Imaging

This detects signals from micro bubbles and filters signals originating from tissue, by selectively detecting the harmonic components (Kudo, 2003; Whittingham, 2005). This technology can detect signals from micro bubbles in vessels with very slow flow without Doppler-related artefacts and is used to characterize tumor vascularity in liver, pancreas, gallbladder and the gastrointestinal tract during transabdominal ultrasonography (Kitano *et al*., 2004; Fukuta *et al*., 2005; Inoue *et al*., 2007 Kersting 2009). Until recently, a contrast harmonic imaging technique was not available for EUS because the transducer of the echoendoscope was too small to produce enough acoustic power for contrast harmonic imaging using the first generation ultrasound contrast agents like Levovist® (Schering AG, Berlin, Germany) (Kitano *et al*., 2011). Second generation ultrasound contrast agents like SonoVue® (Bracco Imaging, Milan, Italy) and Sonazoid® (Daiichi-Sankyo, Tokyo, Japan; GE Healthcare, Milwaukee, WI, USA) produce harmonic signals at lower acoustic powers

(Kudo, 2003; Whittingham, 2005) and are therefore suitable for EUS imaging at low acoustic powers (Kitano *et al.*, 2011).

2.5.1 Principle of contrast harmonic imaging

Ultrasound contrast agents are micro bubbles of gas covered by a shell of biocompatible material such as a lipid (Kudo, 2003; Whittingham, 2005). When exposed to the ultrasound beams, the micro bubbles are disrupted or resonated, which releases a large amount of harmonic signals (Kudo, 2003; Whittingham, 2005). When the tissue and the micro bubbles receive transmitted ultrasound waves, both produce harmonic components that are integer multiples of the fundamental frequency, but the harmonic content from the micro bubbles is higher than that from the tissues (Kitano *et al.*, 2011). Selective depiction of the second harmonic component visualizes the signals from the micro bubbles more strongly than those from the tissue. In addition, signals from the contrast agents vary greatly in phase with no relation to its motion when ultrasound pulses are transmitted multiple times successively (Kitano *et al*., 2011). However, signals are hardly changed between multiple transmissions in the part without the contrast agent (Kitano *et al*., 2011). The extended pure harmonic detection mode equipped with the EUS system specific for contrast harmonic imaging receives not only the second harmonic components but also signals produced by the relative phase shifts of the received signals by transmitting and receiving ultrasound beams multiple times (Kitano *et al*.,2008a and b). This processing is capable of enhancing the imaging of signals from contrast agents (Kitano *et al*.,2008a and b).

2.5.2 Second harmonic imaging

The micro bubbles reached by an ultrasound signal resonate with a specific frequency depending on microbubble diameter (Calliada *et al*., 1998). However, the main resonance frequency is not the only resonance frequency of the bubble itself and multiple frequencies of the fundamental one are emitted, just like in a musical instrument (Calliada *et al*., 1998). These harmonic frequencies have decreasing intensity, but the second frequency, known as the second harmonic, is still strong enough to be used for diagnostic purposes (Calliada *et al*., 1998). The theoretical advantage of the harmonic over the fundamental frequency is that only contrast agent micro bubbles resonate with harmonic frequencies, while adjacent tissues do not resonate, or else their harmonic resonation is very little (Calliada *et al*., 1998). Thus, using a unit especially set to produce ultrasounds at a given frequency (3.5 MHz) and receive an ultrasound signal twice as powerful (7 MHz). Moreover, second harmonic imaging permits to show extremely small vessels (down to 40µ) with very slow flow, which was missed with a conventional method (Calliada *et al*., 1998).

2.5.3 Ultrasound tissue harmonic imaging (THI)

In THI nonlinear distortion of the transmitted frequencies within the body is useful for producing a shaper, higher-contrast ultrasound image than that of the fundamental frequency (Ward *et al*., 1997; Hamilton and Blackstock, 1998; Tranquart *et al*., 1999; Desser and Jeffrey, 2001). This is due to its improved conspicuity and border definition hence; it is widely used for detecting subtle lesions (as in thyroid and breast) and visualizing technically challenging patients with high body mass index (Kornbluth, 1998; Shapiro *et al*., 1998; Szopinski *et al*., 2003a; Szopinski *et al*., 2003b). However, compared

to conventional ultrasound imaging, THI suffers from the low signal-to-noise ratio (SNR), resulting in limited penetration depth (Rosenthal *et al*., 2001; Hohl *et al*., 2004; Song *et al*., 2010).

The SNR in THI can be substantially increased by utilizing the recently-developed coded excitation techniques, i.e., coded tissue harmonic imaging (CTHI) (O'Donnell, 1992; Li, 1999; Phillips *et al*., 2001). In CTHI, similar to conventional coded excitation, specially-encoded ultrasound signals (e.g., Barker, Golay and chirp) are transmitted, and then backscattered receive signals containing fundamental and harmonic frequencies are selectively decoded via pulse compression (Takeuchi, 1996; Chiao and Hao, 2005; Song *et al*., 2010). In THI, various methods, such as band pass filtering (BF), power modulation (PM) and pulse inversion (PI), have been proposed to separate the second harmonic signal from the receive signal (Takeuchi, 1996; Averkiou and Hamilton, 1997; Jiang *et al*., 1998; Hwang and Simpson, 1999; Chiao and Hao, 2005). It has been known that CTHI, compared to conventional THI, requires higher rejection of the fundamental and third harmonic components since they may introduce range side lobe artefacts due to incomplete decoding during pulse compression (Kim *et al*., 2000b; Arshadi *et al*., 2007).

2.5.4 Future perspective of contrast-enhanced harmonic EUS

The technique of contrast-enhanced EUS has been used for the evaluation of vascularity (Kitano *et al*., 2011). However, some studies showed this technique can be applied for molecular imaging in the future (Palmowski *et al*.,2008; Willmann *et al*., 2008). The feasibility of new technologies using contrast-enhanced ultrasound with micro bubbles

targeted to vascular endothelial growth factor receptor type 2 are currently being tested (Kitano *et al*., 2011).

2.6 Other Imaging Modalities

There have been significant advances in recent years in the use of diagnostic imaging to evaluate pregnant bitches (Garcia *et al*., 2015). Ultrasound is the modality of choice for pregnancy diagnosis and assessment of foetal viability in small animals (Davidson and Baker, 2009). However, ultrasound has poor accuracy in determining the number of intrauterine foetuses (Root and Spaulding, 1994). Abdominal radiography provides a more accurate assessment of foetal numbers and is thus still the main technique used for counting foetuses (Rendano, 1983; Rendano *et al*., 1984; Toal *et al*., 1986; Lopate,2008; Garcia *et al*., 2015). Once foetal skulls have mineralized they can be counted on radiographs and provide a precise method of determining foetal numbers (Toal *et al*., 1986; Gradial *et al*., 2000). Studies have shown that the best time to perform radiographic examination to assess the number of foetuses in bitch is between 54 and 57 days of gestation when clear visualization of the foetal skull is possible using digital radiography (Meyer-Lindenberg *et al*., 2008; Garcia *et al*., 2015).

Ultrasonography is an important technology for understanding the status of the female reproductive organs (Maeda *et al*., 2016). However, ultrasonography has disadvantages in that imaging accuracy varies with the proficiency of the examiner and not everyone can observe the activity of the ovaries (Leonhardt *et al*., 2014). Moreover, the observable field of ultrasonography is narrow, and it is possible that only limited information is obtained (Leonhardt *et al*., 2014).

2.6.1 Advanced diagnostic imaging (Computed Tomography, Magnetic Resonance Imaging)

With increasing availability, the use of cross-sectional imaging modalities (computed tomography [CT]; magnetic resonance imaging [MRI]) for diagnostic imaging of the abdomen including the lower urinary tract in small animals is likely to increase (Hecht, 2015). Initial applications described include the use of CT excretory urography to evaluate the ureterovesicular junction in patients with suspected ureteral ectopia and the use of either CT or MRI to evaluate intrapelvic lesions (Rozear and Tidwell, 2003; Samii *et al*., 2004; MacLeod and Wisner, 2011; Schwarz, 2011). Both CT and MRI allow excellent anatomic delineation and are useful in assessing origin and extent of intrapelvic masses, identifying lesions associated with the pelvic urethra such as wall thickening or calculi, and evaluating surrounding structures including the spine and regional lymph nodes for the presence of additional lesions like metastatic disease (Hecht, 2015). CT has proven superior to ultrasound in accurately measuring the size of bladder wall masses (Naughton *et al*., 2012). CT and MRI are useful in planning a surgical approach for intrapelvic lesions and may be needed if radiation therapy planning is to be pursued for pelvic canal neoplasia (Hecht, 2015).

Magnetic resonance imaging (MRI) uses the phenomenon of nuclear magnetic resonance, and information taken *in vivo* is converted into images (Maeda *et al*., 2016). Magnetic resonance imaging renders images of soft tissue with excellent contrast (Maeda *et al*., 2016). It has been used for observing normal ovaries and diagnoses of tumors (Nothling *et al*., 2006; Stephenson *et al*., 2012), and can produce images that are not affected by the skill of the examiner and allows observation not only of the ovaries but also of the female

reproductive organs overall (Leonhardt *et al*., 2014; Maeda *et al*., 2016). In MRI examination the ovarian follicles were distinctly seen as hyper intense circular structural images of about 10 mm within the abdominal cavity (Maeda *et al*., 2016). While the ovarian position in the abdominal cavity were confirmed. In addition, it was also possible to estimate the general size of the uterus (Maeda *et al*., 2016).

However, MRI has disadvantages in observing the ovaries, one being that the ovaries cannot be observed in real time (Leonhardt *et al*., 2014), and another is that with the open type MRI system the resolution is not particularly high, hence the detailed ovarian structure cannot be observed (Maeda *et al*., 2016). However, it has been reported that even mouse ovarian follicles can be observed using 11.7-T MRI (Stephenson *et al*., 2012).

2.7 Application of Ultrasound in Pregnant Bitches

2.7.1 Doppler ultrasound scanning evaluation of uterine blood in bitches

In dogs, ultrasonographic evaluation of the uterus provides valuable information to diagnose uterine diseases (Batista *et al*., 2016). The thickness of the uterine walls and the presence of luminal contents can be determined, allowing differentiation between pregnancy and pathologic conditions (Bigliardi *et al*., 2004; Davidson and Baker, 2009). The echogenicity of the uterine contents suggests cellularity, whereas, it is difficult to differentiate the content using ultrasound (Nyland and Mattoon 2002; Bigliardi *et al*., 2004; Davidson and Baker, 2009).

Doppler ultrasonography is a non-invasive technique that provides information about the organs perfusion (Fielder and Baker, 1969). This technique has been found to be a useful tool to diagnose numerous gynaecologic lesions in several species (Emoto *et al*., 2002;

Özbay and Deveci, 2011; Heppelmann *et al*., 2013). In women and cows, uterine pathologic conditions may cause a blood flow increase that can be detected by Doppler ultrasound (Batista *et al*., 2016). In dogs, this technique has been used to evaluate uterine arteries blood flow during oestrus, normal and abnormal pregnancy, and puerperium (Blanco *et al*., 2011; Batista *et al*., 2013; Freeman *et al*., 2013). Although the characterization of uterine blood flows by Doppler ultrasound probably contribute to the precocious diagnosis of cystic endometrial hyperplasia and pyometra (Batista *et al*., 2016).

Although normal bitches presented a significantly higher resistance Doppler wave form of the uterine arteries, studies revealed that Doppler ultrasonography can accurately reflect hemodynamic changes that are present in bitch patients with different uterine pathologic conditions (Batista *et al*., 2016). In pathological condition such as cystic endometrial hyperplasia and pyometra, uterine blood flow was higher than in normal animals. Uterine infection in women was also associated with high uterine blood flow (Alatas *et al*., 1996; Özbay and Deveci, 2011). Resistivity index (RI) is higher in normal diestrous bitches than in pathologic conditions such as cystic endometrial hyperplasia and pyometra (Batista *et al*., 2016) whereas England *et al*. (2012) reported that it was lower in normal diestrous bitches.

During an inflammatory response, there is vasodilation leading to increased blood flow and angiogenesis are present (Özbay and Deveci, 2011). Hence uterine inflammation associated with an infectious process may lead to a diminution of RI values of uterine arteries (Özbay and Deveci, 2011). Furthermore, a previous study in bitches with pyometra reported an increase in the intrauterine concentration of prostaglandin E, which may also

lead to an increase in uterine perfusion (Heap and Poyster, 1975; Still and Greiss, 1978). In addition to prostaglandins, nitric oxide, a potent vasodilator, is also released locally in several mammalian species during endometrial inflammation (Rosselli *et al*., 1998; Fang *et al*., 1999). The low vascular resistance detected by Doppler ultrasonography in pathologic condition can be used to support the diagnosis of canine pyometra (Batista *et al*., 2016).

Doppler ultrasound has been found to be a useful tool to evaluate response to medication (Tinkanen and Kujansuu, 1993). The possibility to follow up the evolution of treated patients could improve the clinical outcome, thus avoiding unnecessary surgery and preserving the reproductive tract in valuable bitches (Batista *et al*., 2016).

2.7.2 Indication for ultrasonography during parturition

Ultrasonographic foetometry has great potential, but the huge morphological and size variability in dogs would require breed-specific foetal growth curves to increase the accuracy of this method (Kutzler *et al*. 2003b; Socha and Janowski 2014). At presentation, in the absence of previous monitoring, ultrasound examination can help the clinician to date pregnancy through measuring some foetal parts such as the biparietal (BP) diameter in the second half of pregnancy (Lopate 2008). This method is relatively simple but breed-specific foetal growth curves are necessary to increase its accuracy (Kutzler *et al*. 2003b; Socha and Janowski 2014).

Even when using a specific equation for a single breed, some variables, such as smaller than average litter size, can greatly reduce the accuracy of the prediction (Groppetti *et al*. 2015b). In small and medium size breeds, approximately 70% of parturition dates were predicted within 1 day and 85% within 2 days using BP (Beccaglia and Luvoni 2006).

Neither of the two methods alone, that is progesterone concentration and ultrasound foetometry appears to be accurate enough to effectively help the clinician, it could be worth to test the association of the two determinations in order to assess whether the diagnostic efficacy becomes higher than with a single one (Rota *et al*., 2015).

2.7.3 Appearance of embryonic/foetal structures using ultrasonographic or radiographic examination

Determination of gestational age has been attempted by both ultrasonographic and radiographic examination through the identification of the degrees of foetal mineralization and the first appearance of different foetal skeletal structures (Haney *et al*., 2003; Rendano, 1983; Rendano *et al*., 1984). The mineralization of structures occurs earlier in cats than in dogs (Haney *et al*., 2003; Lopate, 2008) in which the bone structures characterized by the narrowest period of mineralization are the humerus at 20 ± 1 day (range between 20 and 24 days) and femur at 21 ± 1 day (range between 19 and 23 days) prior to parturition (Boyd, 1971). Some other bones, like fibula, calcaneus and phalanges, mineralize only after birth (Beccaglia *et al*., 2016).

A single lateral radiograph of the abdomen is usually sufficient for pregnancy diagnosis and foetal counting, but a ventro-dorsal radiograph is more suitable to evaluate gestational age although with the aforementioned limitations (Rendano, 1983; Rendano *et al*., 1984., Concannon, 2000;).

2.7.4 Ultrasonographic measurement of extra-foetal or foetal structures

Ultrasonographic measurement of extra-foetal and foetal structures is a common and accurate method for the prediction of delivery date during pregnancy even when the time

of ovulation is unknown (Beccaglia *et al*., 2016). During the first half of gestation (between 19 and 37 days after LH peak), measurements of gestational sac diameter (inner chorionic cavity, ICC or outer uterine diameter, OUD) or foetal crown-rump length (CRL) can easily be obtained. In the second half of pregnancy (after 37 days), foetal measurements of the biparietal diameter (BP) and body diameter (BD) can be used to predict the delivery date (Shille and Gontarek, 1985., England *et al*., 1990; Luvoni and Grioni, 2000; Luvoni and Beccaglia, 2006; Lopate, 2008; Michel *et al*., 2011).

From days 35 to 58, the deep portion of the foetal diencephalo-telencephalic vesicle (DPTV) can be recognized as a symmetric anechoic area viewed on sagittal midline of the foetal head and its measurement could be useful to determine the gestational age (Beccaglia *et al*., 2008; Beccaglia and Luvoni, 2004). An accurate gestational timing in the first and second half of pregnancy is provided by ICC and BP parameters, respectively (Kutzler *et al*., 2003b; Luvoni and Beccaglia, 2006; Lopate, 2008; Michel *et al*., 2011).

2.7.5 Ultrasonographic evaluation of heart rate or foetal flux

In human medicine, it is known that a variation in foetal heart rate (FHR) is connected to antepartum uterine contraction (Nageotte, 2015). In the canine species, it has been reported that FHR should be greater than 220 beats per minute (bpm), with FHR between 180 and 220 bpm indicate moderate foetal distress and values less than 180 bpm indicate severe foetal distress (Zone and Wanke, 2001). It has been proved that when umbilical artery resistivity index (RI) is less than 0.7 in all foetuses, the delivery would occur within 12 hr. As foetal distress could affect this value, the FHR variations should be measured at the same time (Giannico *et al*., 2015; Beccaglia *et al*., 2016).

2.7.6 Use of B-mode ultrasonography for foetal sex determination in dogs

Gestational ultrasonography is used routinely in prenatal monitoring in women and in some animal species (Curran, 1992; Meagher and Davison, 1996; Lopate 2008; Davidson and Baker, 2009; Lamm and Makloski, 2012). It is used to confirm pregnancy, identify congenital foetal defects, assess placental morphology and blood flow, evaluate uterine fluid and endometrial edema or inflammation, identify the presence of cystic endometrial hyperplastic changes, evaluate foetal viability and development, and aid in the identification of foetal distress (Aissi and Slimani, 2008; Lopate 2008; Davidson and Baker, 2009; Lamm and Makloski, 2012). In veterinary medicine, it has been shown that ultrasonographic determination of intrauterine sex is possible in cats, cows, buffaloes, mares, and sheep (Bürstel *et al*., 2002; Mar *et al*., 2002; Holder, 2003; Tainturier and Tainturier, 2004; Zambelli and Prati, 2006; Yotov *et al*., 2011) and this has economic relevance (Gil *et al*., 2015).

Sexual differentiation in canine foetuses starts from the 30th day of gestation and continues until about the 50^{th} day (Pretzer, 2008; Evans and Lahunta, 2013). Examination of the first pregnant bitch every 4 days showed that between 55 and 58 days of gestation, foetuses were larger and foetal organogenesis was complete, making this the ideal age for identification of the genitalia (Evans and Lahunta, 2013; Gil *et al*., 2015).

Ultrasonographic determination of the sex of the female fetus in bitch is similar to that of human in which two or four parallel lines representing the major and minor labia can be identified (Elejalde *et al*., 1985; Whitlow *et al*., 1999; Gil *et al*., 2015). Whereas, the testicles are not seen in male canine foetuses as these migrate into the scrotum after birth

(Baumans *et al*., 1981; Achiron *et al*., 1998). Although the penis develops in the foetal stage, it is not readily visible in gestational ultrasonography in dogs (Gil *et al*., 2015). Again, this characteristic differs from that found in human foetuses because the human foetal penis can be viewed more easily and the testes migrate to scrotum after the 25th week of gestation (Curran *et al*., 1989).

2.7.7 Bidimensional and Doppler ultrasonographic evaluation of postpartum uterine involution

Puerperium is a physiological period after parturition in which uterine involution occurs (Blanco *et al*., 2015). Complete uterine involution is essential for the establishment of future gestations, as failure of normal uterine regression could lead to several diseases such as postpartum metritis and retained placenta in many mammalian species (Hirt *et al*., 2000; Magata *et al*., 2013). Ultrasonographic evaluation of the postpartum uterus is useful for monitoring its normal involution and for the early diagnosis of disorders (Blanco *et al*., 2015). In bitches, postpartum metritis is differentiated from the normal postpartum uterine enlargement by the failure of normal progressive decrease in uterine lumen contents and horn width (Davidson and Baker, 2009). The uterine wall can also be thickened or irregular in these cases (Grundy and Davidson, 2004).

Doppler ultrasound has been used to assess postpartum uterine involution in women, cows, mares, and bitches (Krueger *et al*., 2009; Mulic-Lutvica *et al*., 2009; Mortensen *et al*., 2011; Batista *et al*., 2013). A decrease in the blood flow of uterine arteries throughout puerperium has been reported in these species. However, Doppler uterine blood flow measurements are altered by puerperal uterine diseases in women and cows, contributing

to the diagnosis of postpartum disorders (Mulic-Lutvica *et al*., 2009; Heppelmann *et al*., 2013; Magata *et al*., 2013). A marked decrease in uterine blood flow during the first postpartum week has also been described in bovine and canine puerperium (Krueger *et al*., 2009; Batista *et al*., 2013). However, it has been reported that clinical evidence of involution disappeared before the ultrasonographic evidence of involution in women, cows, mares, and bitches (Krueger *et al*., 2009; Mulic-Lutvica *et al*., 2009; Mortensen *et al*., 2011; Batista *et al*., 2013).

Queen seems to have more rapid postpartum uterine involution than bitch (Walter and Schönkypl, 2006). Study revealed that canine and feline uterine ultrasonographic appearance became indistinguishable from that of anoestrus on Day 52and 18 respectively after parturition (Batista *et al*., 2013). The rapid involution in the feline is due to histologic differences between both species (Dall'Aglio *et al*., 2012a; Dall'Aglio *et al*., 2012b). Postpartum uterine diseases are less frequent in cats than in dogs due to the rapidity of feline uterine involution. More so, uterine wall layers after parturition also seem to differ between dogs and cats (Blanco *et al*., 2015). Up to five ultrasonographically distinct layers have been identified in the postpartum canine uterine wall (Yeager and Concannon, 1990), whereas only three layers were observed in feline (Ferretti *et al*., 2000; Blanco *et al*., 2015). Moreover, uterine wall thickness is markedly thinner in cats than in dogs (Chatdarong *et al*., 2005; Orfanou *et al*., 2009).

2.8 Oestrus Induction in Canine Patients

Both LH and FSH appear to be follicotrophic in the dog, so that administration of either LH or FSH induces oestrus. However, protocols mimicking the natural, gradual increase in

LH and FSH in canine patients have not been successful (Kutzler, 2005). An estrogen peak approximately 30 days before the onset of oestrus is believed to be required to prime the hypothalamuspituitary- ovarian axis, causing release of LH in a pulsating fashion (Shille *et al*., 1984; Jeffcoate, 1993). Successful induction of fertile oestrus in bitches has been accomplished with diethylstilbestrol (DES) in various doses with or without FSH and LH (Bouchard *et al*., 1993; Concannon *et al*., 1997). Other gonadotropins have been evaluated with limited success (Arnold *et al*., 1989; Van Haaften *et al*., 1989; Verstegen *et al*., 1997; Wanke *et al*., 1997). Pregnant mare serum gonadotropin (PMSG) has been effective, but is associated with premature luteal failure, shortened dioestrus, and pregnancy loss (Jones *et al*., 1973; Nakao *et al*., 1985; Weilenmann *et al*., 1993). Protocols using estrogens for oestrus induction in bitches typically also include FSH or PMSG for folliculogenesis and human chorionic gonadotropin (HCG) or LH for induction of ovulation (Kutzler, 2007).

Prolactin inhibition also plays a role in interestrous intervals and is believed to have effects on gonadotropin release and ovarian response to gonadotropins (Wiebe and Howard, 2009). Suppression of prolactin release by dopamine agonists (bromocriptine, cabergoline) shortens the duration of anoestrus and induces oestrus in animals with prolonged anoestrus (Arbeiter *et al*., 1988; Jochle *et al*., 1989). The dopamine agonist, bromocriptine, was the first prolactin inhibitor studied in dogs for oestrus induction, but resulted in significant vomiting in dogs (Van Haaften *et al*., 1989; Zoldag *et al*., 2001).

A newer agent, cabergoline (Dostinex®), has been shown to induce oestrus in most bitches (0.005 mg/kg orally daily, until 3-8 days after onset of proestrus or day 40) with fewer side effects (Kutzler *et al*., 2002). Cabergoline is only available as a 0.5-mg tablet that is

inactivated over time in aqueous solutions containing water (Wiebe and Howard, 2009). For accurate dosing of small animals, tablets can either be compounded into the appropriate- strength capsules or a portion of the tablet can be crushed and diluted with fluid just before dosing (Wiebe and Howard, 2009). McLean and co-workers also report that cabergoline is stable for 28 days if compounded in acidic fluids (1% acetic acid solution) (McLean *et al*., 2007).

GnRHs have also been shown to be very effective if given in pulsated fashion (0.2-0.4 µg/kg every 90-minute intervals intravenously [IV] or subcutaneously [SC] for 3-9 days) to mimic the natural cycle, resulting in LH peaks at the end of proestrus. Subcutaneous administration has been noted to be more practical, but availability of the canine analog is problematic (Vanderlip *et al*., 1987; Cain *et al*., 1988). However, protocols using native GnRH or its agonists via this technique are not practical for clinic purposes because of the expense, time commitment, and lack of availability of analogs (Wiebe and Howard, 2009). GnRH analogs (lutrelin, deslorelin, leuprolide) can also be administered as long-acting dosage forms via subcutaneous injections, mini pumps, or implants. Both expense and side effects may limit the use of these products. Side effects have included premature luteal failure, shortened dioestrus, and pregnancy loss (Concannon, 1989; Kutzler *et al*., 2001). Long-term use is also associated with pituitary overstimulation, down regulation of GnRH receptors, suppression of LH, decreased progesterone secretion, and decreased luteal responsiveness to LH (Kutzler, 2007).

CHAPTER T HREE

3.0 MATERIALS AND METHODS

3.1. Study area

The study was conducted at the Department of Veterinary Surgery and Radiology, Faculty of Veterinary MedicineAhmadu Bello University, Zaria Kaduna Sate. The dogs were housed in the homes of the hunters in Samaru, Zaria which lies between latitude 10 and 11^0North and longitude 7 and 8^0 East, and situated in the Northern boundary of the sub-humid zone.

3.2. Research considerations

Strict adherence to ehical guidelines such as animal welfare and humane practices was observed with the barest minimal stress inflicted on the animal during the course of handling and insonation as approved by the Ahmadu Bello University Committee on Animal Use and Care with clearance number **ABUCAUC/2016/030.**

3.3. Materials

3.3.1 Experimental animals

Nine (9) matured female Nigerian Indigenous dogs that had whelped at least once were used for the study. They were acclimatized for two weeks, during which period they were vaccinated with antirabies and Distemper, Hepatitis, Leptospirosis, Parainfluenza and Parvoviral enteritis (DHLPP) vacines. They were routinely dewormed using membendazole at 7.5 mg/kg body weight orally daily for 3 consecutive days and with praziquantel at the rate of 5.0 mg/kg body weight once orally. They were bathed with Amitrax® against ectopasites. Other Veterinary services were rendered when indicated. All

dogs were clinically examined and confirmed free from any obvious reproductive and systemic disorders. They were fed once a day and water was provided *ad libitum.*

3.3.2 Consumables

The consumables used in the study included, thermal printing paper, aquasonic gel, razor blades, soap and tissue papers. Others were cabergoline (Dostinex®, By Pfizer), methylated spirit, vaccines, dewormers, syringes, sample bottles and antibiotics (Oxytetracycline LA).

3.3.3 Equipment

Ultrasound machine (Sonostar C5 (R) LaptopUltrasound machine by Xuzhou Palmary Electronics Co. Ltd China) with a 6.5MHz transcutaneous probe, a Sony videographic(R) thermal printer, Ultrasound bed. Other equipment used in this study included an electricity generator (Tiger 1.5HP) and a deep freezer (Skyrun 100L).

3.4 Methodology

3.4.1 Experimental Design

The overview of the Experimental design is as shown in the scheme (Fig 3.1) below. The study was divided into four (4) phases during which blood sampling accompanied every other ultrasound examination of the bitches in the various groups. We hope to achieve all outlined and stated objective of this study following this design. The phases are: Phase I (Acclimatization), Phase II (Induction), Phase III (Gestation), and Phase IV (Postpartum).

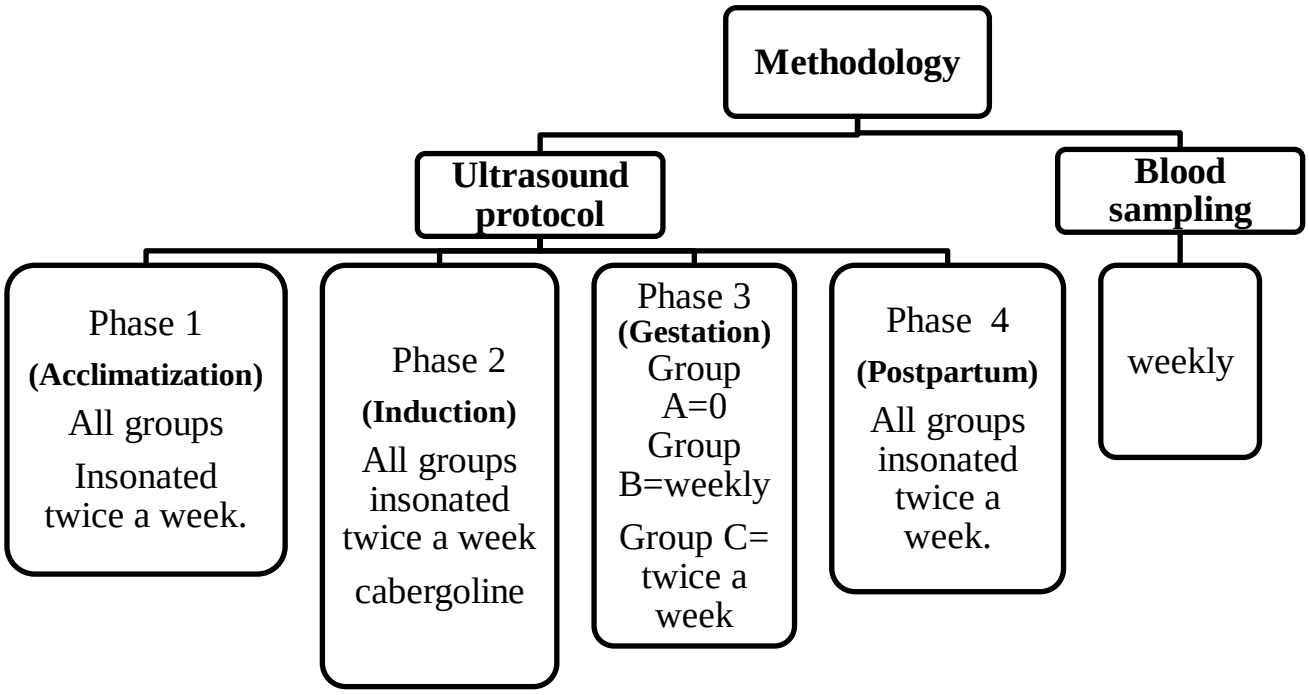


Fig.3.1 shows the overview of the experimental design

3.4.1.1. Phase I (Acclimatization)

This was the 1st phase of the study; it lasted one month and the following activities were carried out:

*a. Sonography***:** During this phase all the bitches had their ventral abdominal areas liberally shaved from the level of the xyphoid cartilage to pubic area. The shaved are were scrubbed, antiseptic applied with the bitch restrained on dorsal recumbency on the examination table. Aquasonic gel applied to the shaved area. Each bitch was then examined sonographically to visualize the internal genitalia of the bitch. This was done to map out the normal sonographic appearance of the reproductive tract. The examination was repeated twice a week for the duration of the phase.

*b. Blood sampling***:** During this phase the bitches were well restrained and blood collected aseptically via cephalic/recurrent tarsal venepuncture. Six millilitre of blood was collected at every sampling period. The first 3 mL were collected in EDTA bottle and was used for haematology and erythrocyte osmotic fragility test, while the remaining 3 mL were placed in anticoagulant free bottles for biochemical analysis. This was done once a week for the duration of the phase.

3.4.1.2 Phase II (Induction and breeding)

This was the 2nd phase of the study; it lasted three weeks and the following activities were carried out:

*a. Sonography***:** During this phase all the bitches had their ventral abdominal areas liberally shaved from the level of the xyphoid cartilage to pubic area. The shaved area was scrubbed, antiseptic applied with the bitch restrained on the examination table. Aquasonic gel was then applied to the shaved area. Each bitch was then examined sonographically to

visualize the internal genitalia. This was to map out the normal sonographic changes of the reproductive tract during the oestrus cycle. The examination was then repeated on each bitch in the study groups twice a week for the duration of the phase.

*b. Blood sampling***:**During this phase the bitches were well restrained and blood collected aseptically via cephalic/recurrent tarsal venepuncture. Six millilitre of blood was collected at every sampling period. The first 3 mL was collected in EDTA bottle and was used for haematology and erythrocyte osmotic fragility test, while the remaining 3 mL was placed in anticoagulant free bottles for biochemical analysis. This was done once a week for the duration of the phase.

c. Induction**:** Each bitch was treated with cabergoline at 5 µg /kg per os for 20 days. This was to induce fertile oestrus in Nigerian indigenous bitches. Five tablets (2500 µg) were placed in a pharmacist's mortar and gently pounded until it had become a powder. Thereafter Vitamin C was added and gently mixed until 100ml of the Vitamin C had been used to dissolve the cabergoline. The solution then contained 25 µg/ml of a stable mixture which was administered to the bitches. They bitches swere observed for signs of oestrus during the treatment period.

*d. Breeding***:**The bitches were bred by natural mating, that was done by taking the bitches to kennels containing adult males in the ratio of three females per male This was modified from the ratio as described by Ajala *et al* 2012 (six: one). This modification was undertaken to accommodate possible decreased fertility, since the oestrus was induced. Breeding was done when signs that the bitches are coming on heat was noticed physically. They were then left with the males for two weeks.

When pregnancy was confirmed by ultrasonography, the bitches were divided into three groups (A,B and C) of three dogs each. Group A dogs had no insonation , Group B had insonation once a week and Group C dogs had insonation twice a week until whelping.

2.4.1.3 Phase III (Gestation)

This was the 3rd phase of the study; it lasted nine weeks and the following activities are expected to be carried out.

*a. Sonography***:** During this phase all the bitches had their ventral abdominal areas liberally shaved from the level of the xyphoid cartilage to pubic area. The shaved area was and scrubbed, antiseptic applied with the bitch adequately restrained on the examination table on dorsal recumbency and aquasonic gel applied to the shaved area. Each bitch was then examined sonographically to visualize the internal genitalia and establish pregnancy. This phase is also designed to map out normal sonographic changes of the female reproductive tract of the bitches during the gestation. The examination was carried out once on all the bitches, and then repeated on each bitch in group B once a week and group C twice a week for the duration of the phase.

*b. Blood sampling***:** During this phase the bitches were restrained and blood collected aseptically via cephalic/recurrent tarsal venepuncture. Six millilitre of blood was collected at every sampling period. The first 3 mL was collected in EDTA bottle and was used for haematology and erythrocyte osmotic fragility test, while the remaining 3 mL was placed in anticoagulant free bottles for biochemical analysis. This was done once a week for the duration of the phase.

Post whelping, the average litter size and other physical parameters were recorded for each group and blood taken from each puppyand evaluated. The average post natal foetal daeth were also recorded.

3.4.1.4 Phase IV (Postpartum)

This was the 4th and last phase of the study; it lasted eight weeks and the following activities were carried out:

*a. Sonography***:** During this phase all the bitches had their ventral abdominal areas liberally shaved from the level of the xyphoid cartilage to pubic area. The shaved area was scrubbed antiseptic applied with the bitch restrained on the examination table. Aquasonic gel was then applied to the shaved area. Each bitch was then examined sonographically to visualize the internal genitalia. This was to map out the normal sonographic changes of the reproductive tract during the oestus cycle. The examination was then repeated on each bitch in the study groups twice a week for the duration of the phase.

*b. Blood sampling***:** During this phase the bitches were restrained and blood collected aseptically via cephalic/recurrent tarsal venipuncture. Six millilitre of blood was collected at every sampling period. The first 3 mL were collected in EDTA bottle and were used for haematology and erythrocyte osmotic fragility test, while the remaining 3 mL would be placed in anticoagulant free bottles for biochemical analysis. This was done twice a week for the duration of the phase.

Ultrasonography of all the bitches was done postpartu twice a week for eight weeks.

3.4.2 Determination of haematological parameters

3.4.2.1. Determination of blood cellular components

Packed cell volume (PCV), erythrocyte count (RBC), thrombocyte count, absolute and differential leucocyte counts was determined as described by Dacie and Lewis (1991). Haemoglobin concentration was determined using cyanmethaemoglobin method. Hb, PCV and RBC count was done as described by Schalm *et al*. (2010).

3.4.3. Determination of Serum Biochemical Parameters

3.4.3.1 Hormonal assay

The serum samples obtained from blood sample collected was analysed for progesterone, using an Enzyme-linked Immunosorbent assay (ELISA) diagnostic kit (Calbiotech Inc. El Cajon, CA. USA).

3.4.6.4 Determination of Oxidant and Antioxidant Biomarkers

The serum samples obtained from blood sample collected was analysed for malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) using a commercial kit (Calbiotech Inc. El Cajon, CA. USA).

3.4.7. Examination of the puppies postpartum

After the bitches' whelped, the average litter size per group was noted, their general physical conditions of the puppies was also be noted. The birth weight for each puppy was recorded and blood samples were collected from the puppies via jugular venupuncture. 6 mls of blood was taken from each puppyand divide into two (3 mls into sample bottles with anticoagulant and the rest in sample bottles without anticoagulant).

3.4.8. Hormonal assay

The serum samples obtained were analysed for triiodothyronine (T3) and Thyroxin (T4) hormones using an Enzyme-linked Immunosorbent assay (ELISA) diagnostic kit (Calbiotech Inc. El Cajon, CA. USA).

3.4.9 Data analysis

All data generated are presented as tables, charts or graphs. Values were expressed as mean± Standard error of mean (SEM). The data was subjected to One-way Analysis of Variance (ANOVA) and Bonferroni post-hoc test using GraphPad Prism version 4.0 for Windows 8 from GraphPad Software, San Diego, California, USA (www. Graphpad.com). Values of $p \leq 0.05$ was considered significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Sonographic features of the reproductive tract of bitches

4.1.1. Sonographic features of the reproductive tract of bitches during phase I

The phase 1 results showed that the bitches were all at various lengths of anoestrus from ten (10) weeks to thirty two (32) weeks (Table 4.1) with mean duration of 26.89 ± 2.29 weeks. The ultrasound examination revealed that the major internal genitalia visualized were the uterus and uterine horns as the absence of follicular activities makes it difficult to visualize the ovaries. The uterus appears as an isoechoic tubular structure with no fluid in the lumen Plate 4.1. The uterine diameter measured less than 1 cm in all the bitches.

Table 4.1 Showing the duration of the anoestrus with reproductive structures visualized on ultrasound examination

Animal	Duration of anoestrus (weeks)	Ovary	Oviduct	Uterus	Cervix
A1	24	-	+	+	+
A2	28	-	+	+	+
A3	10	-	+	+	+
B1	28	-	+	+	+
B2	32	-	+	+	+
B3	28	-	+	+	+
C1	28	-	+	+	+
C2	32	-	+	+	+
C3	32	-	+	+	+
MEAN	**26.89 ± 2.29**				

Key: - = it was not visualized
+ = it was visualized

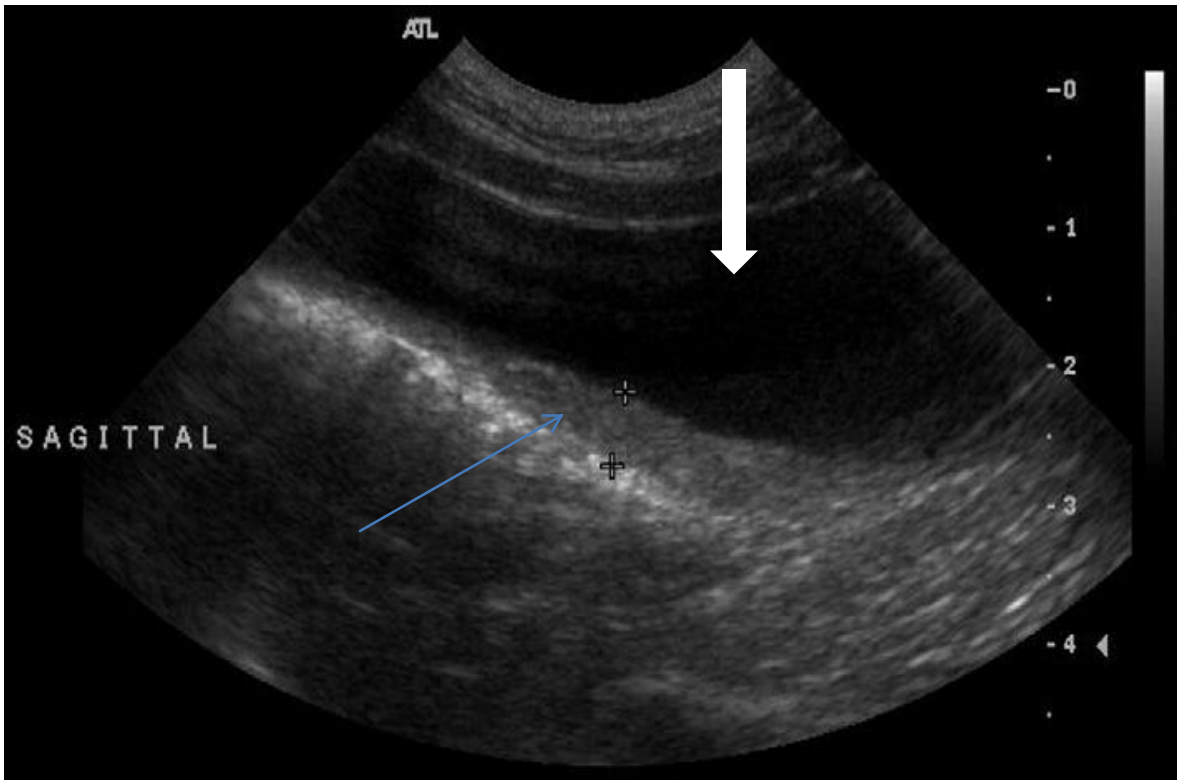


Plate 4.1. Sonographic image of the uterine body which is uniformly echogenic (A-blue arrow) with no fluid within (Arrowed) and the urinary bladder (white broad arrow) containing some volume of urine.

4.1.2. Sonographic features of the reproductive tract of bitches during phase II

We monitored the responses of the various bitches to the reconstituted cabergoline orally administered. The results of this phase showed that the bitches had an uneven response to cabergoline; it took some bitches thirteen (13) days from start of treatment to onset of proestrus. While it took others as late as eighteen (18) days with an average of 15.75 days between start of treatment to onset of proestrus (Table 4.2). The proestrus observed lasted between seven to eleven days in the bitches with an average of 9.38 days between onset of proestrus and the beginning of oestrus. The duration of the induced oestrus was 7-9 days with an average of 8 days. During this period the bitches stood to be mated by the studs we provided. The ultrasound results obtained in this phase of the work, showed that the ovaries which hitherto were not visualized, became visible at the onset of proestrus. The ovaries appeared as roundish structures that had mixed echogenicity. Gradually the follicles became visible and could be differentiated from each other as circular anechoic structures that cluster the ovary. Towards the end of the proestrus, the walls of the follicles became thickened the follicles also became larger and contained more fluid (Plate 4.2). When the bitches manifested oestrus, the follicles were released and the ovaries became relative smaller with more uniform echogenicity. The area where the follicles were became slightly more hyper echoic (from anechoic to isoechoic), they contained the corpora haemorrhagic (Plate 4.3).

Table 4.2. Results of the response of the bitches to Cabergoline treatment.

	Start of treatment to onset of proestrus (Days)	Duration of proestrus (Days)	Duration of oestrus (Days)
Mean ± SEM	15.75 ± 0.68	9.38 ± 0.50	8.0 ± 0.27
Range	13-18	7-11	7-9

Key:
Mean ± SEM = mean with standard error of mean.

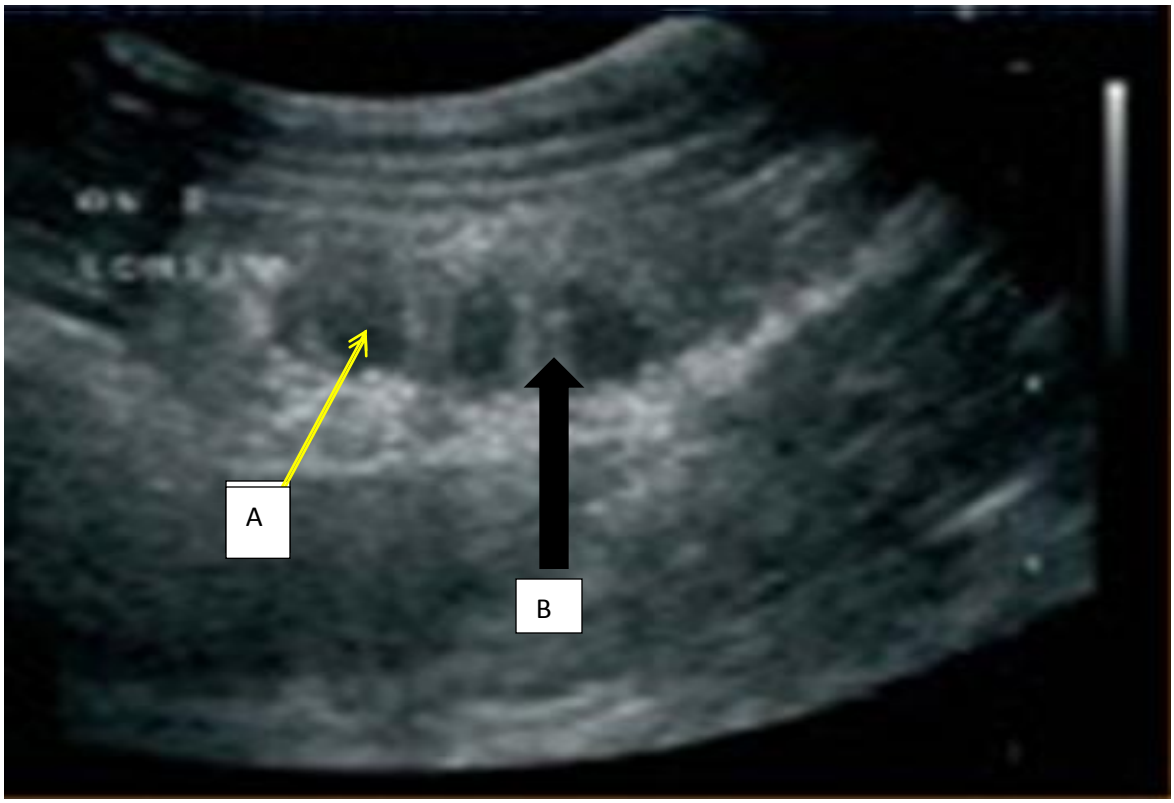


Plate 4.2. Ultrasound appearance of the ovary at late proestrus. There is a large amount of fluid within the follicles (A= yellow arrow). The walls of the follicles are thickened (B=black arrow)

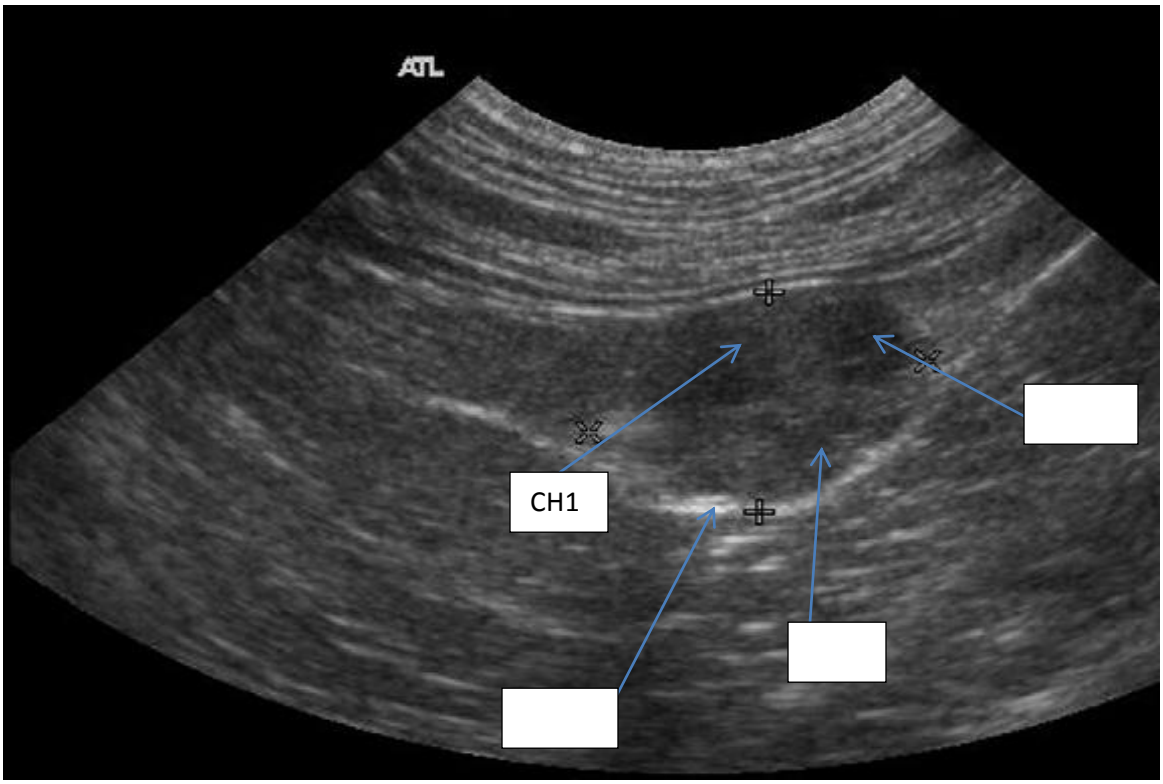


Plate 4.3 Sonograph of ovary post ovulation, the with the copora hemorrhagica are isoechogenic (arrowed, CH1-CH3)

4.1.3. Sonographic features of the reproductive tract of bitches during phase III

The results of ultrasonography in phase 3 showed that the earliest a positive confirmation of pregnancy was made was between days nineteen to twenty-one (19-21 days, Table 4.3) post last service. The sonographic appearance of the gestational sac with anechoic content and a hyper echoic structure at the margins is a definite sign of pregnancy (Plate 4.4). Once pregnancy was confirmed the bitches were then grouped into three A, B and C. Group A was no longer insonated until after whelping while bitches in group B were insonated once a week whiles those of group C were insonated twice a week during this phase. The gestational sac (spherical anechoic structure, with thin and hyper echoic borders) got attached to the wall of the uterus at this point. After which foetal poles then started to develop distinct morphological characteristics (Plate 4.5) by between days 23-25. We started counting the foetal pole at this juncture, the mean maximum foetal poles counted were 8.00 ± 0.58, 9.00 ± 0.58 and 9.00 ± 0.58 for bitches in groups A, B and C respectively (Fig 4.1). It was also at this point that we first visualized foetal cardiac activities. The head became a distinct feature between day 29-31.The head appears as anechoic filled structure just cranial to the forming foetal body.

The ultrasound results of this phase showed the visualization of limb buds which were hyperechoic protrusions from the lateral aspects of both cranial thoracic and caudal abdominal parts. This development was observed between days 34- 37 (with a mean appearance of 35.17 ± 0.48) Plate 4.6. After this was the foetal movement, this was first noticed on day 33 in some bitches while in others it was on day 38 post breeding. The mean period within which this happened was 35.50 ± 0.76. It was observed in most cases

that the urinary bladder becomes visible between days 35- 39 as a spherical anechoic structure at the caudal foetal pole, the mean period was noticed to be 35.50 ± 0.76 days post mating (Plate 4.7, and 4.8). Next structure to become visible was the foetal stomach which appeared as a spherical anechoic organ just caudal to the foetal liver; this happened between day's 35-39 post mating with an average period being 36.83 ± 0.60 days (Table 4.3 and Plate 4.8). The spinal canal became visible as dorsal tubular structure between days 36- 39 with a period mean of 38.00 ± 0.37 days post mating (Plate 4.9). The eyes were visualised as distinct spherical structures with anechoic corpus vitreum, hyper echoic border between sclera and retina and spherical anechoic lens from day 39-40 post mating with a mean of 39.33 ± 0.21 days (Plate 4.10). The foetal heart chambers become visible between day 40- 41 post mating with a mean of 40.33 ± 0.21 day (Plate 4.11). The foetal kidneys appears as a mixed echogenic organ on the lateral aspect of the cranial abdomen of the foetus, This was visible from day 41 -47 post mating with a mean period of 44.83 ± 0.98. The last structure to appear on ultrasound we found was the foetal intestines, they became visible between day 59- 63 post mating and they were the last organ to be visualised before the bitches whelped. The mean period was found to be 60.33 ± 0.61 days. When the bitches whelped we noted each bitches litter size and the birth weight of the puppies. The mean litter size recorded was 7. 33 ± 0.67, 6.67 ± 0.67 and 5.33 ± 0.33 puppies/ litter for bitches in groups A, B and C respectively (Fig 4.2). The mean birth weights for the puppies recorded were 246.4 ± 8.42, 197.5 ± 11.55 and 189.7 ± 10.21 g for puppies whelped by bitches from groups A, B and C respectively (Fig 4.9). The post-partum neonatal deaths were also recorded in the first two weeks post whelping.

4.1.4. Sonographic features of the reproductive tract of bitches during phase IV

The ultrasound results for phase 4 showed that on the long axis ultrasound scanning the post-partum uterus was tubular in shape and initially of mixed echogenicity. In the first week (between days 4-7) postpartum there was a lot of intraluminal fluid which was expelled as involution progressed. There where regions of hyper echogenicity (the uterine walls) which surrounded anechoic substances (Lochia). Measuring the uterine diameter (short axis) was started only after most of the fluid had been expelled the results showed that at the first week postpartum the uterine diameter ranged from 3.510 - 4.510 with a mean of 3.944 ± 0.105 cm this was the highest values obtained during this phase of the study. Involution also involved the reduction in the uterine diameter from the 1st week to means of 3.100 ± 0.116, 2.340 ± 0.114, 1.973 ± 0.058, 1.796 ± 0.042, 1.747 ± 0.025, 1.747 ± 0.027 and 1.727 ± 0.026 cm for weeks 2 to 8 respectively (Fig.4.5). There was also observed a change in the echogenicity from mixed (hyper echoic wall surrounding an echoic fluid) in the first week to almost exclusively isoechoic by the six weeks postpartum.

Table4.3. The summary of the first appearance of various ultrasonographic foetal and extrafoetal features in the bitches post mating

Feature	Days	Average
Gestational sac (spherical anechoic structure, with thin and hyperechoic borders)	19-21	20.11 ± 0.26
Apposition to uterine wall,	23-25	23.5 ± 0.34
Foetal heartbeat	23-25	23.33 ± 0.33
Anechoic area in the foetal head	28-34	29.50 ± 0.96
Limb buds	34-37	35.17 ± 0.48
Foetal movement	33-38	35.50 ± 0.76
Spherical anechoic urinary bladder at the caudal foetal pole	35-39	36.67 ± 0.67
Spherical anechoic stomach caudal to the liver	35-39	36.83 ± 0.60
Spinal canal visible as dorsal tubular structure	36- 39	38.00 ± 0.37
Eyes seen as distinct spherical structures with anechoic corpus vitreum, hyperechoic border.	39-40	39.33 ± 0.21
Lung hyperechoic to liver	39- 42	40.33 ± 0.49
Cardiac chambers	40- 41	40.33 ± 0.21
Kidneys	41- 47	44.83 ± 0.98
Intestines with intestinal layers	59- 63	60.33 ± 0.61

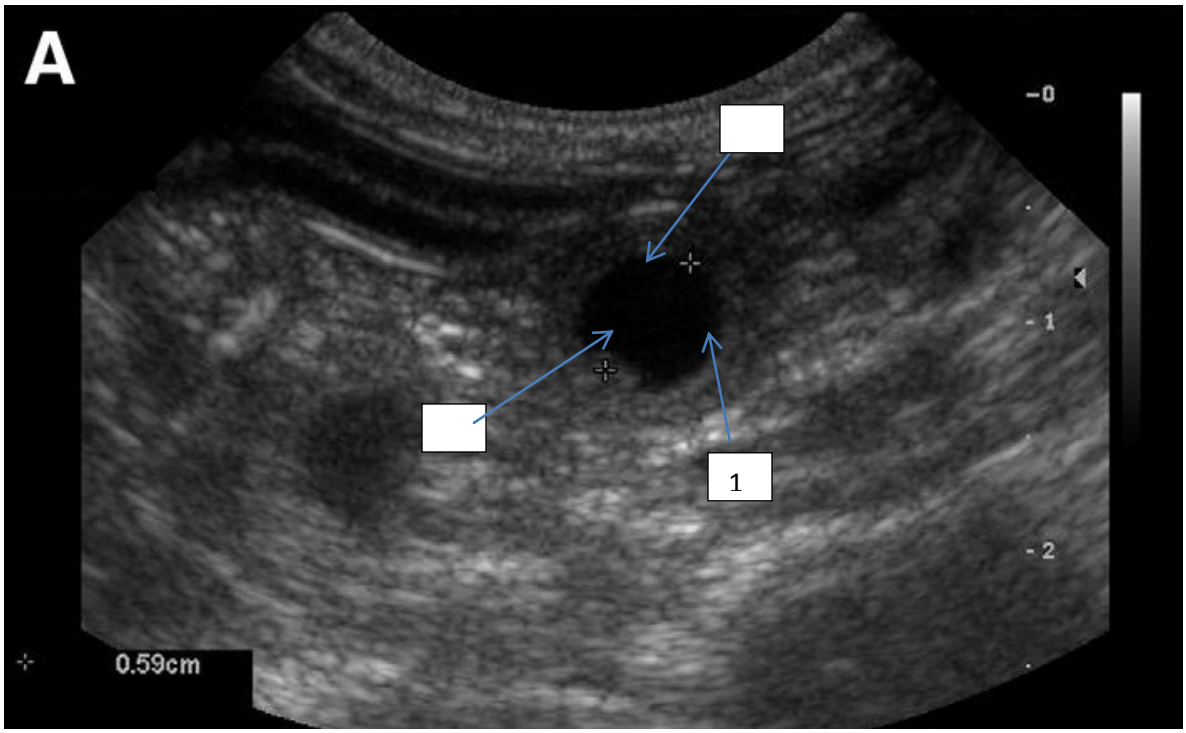


Plate 4.4: Sonograph showing early pregnancy between 19-21 days of gestation. Amniotic sac (1) fluid (2) and foetal pole (3)

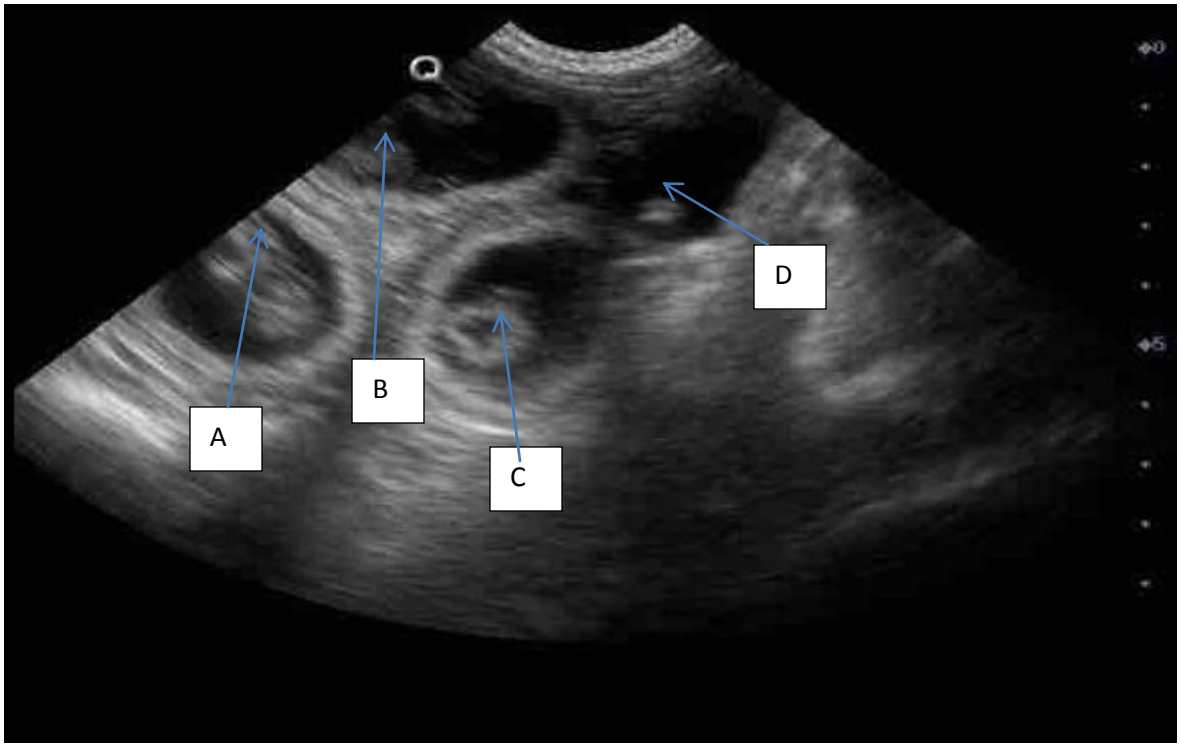


Plate 4.5: Sonograph of foetal poles between days 27-29 . Foetuses have recognizable morphology with their gestational sacs. (Arrows A, B, C and D)

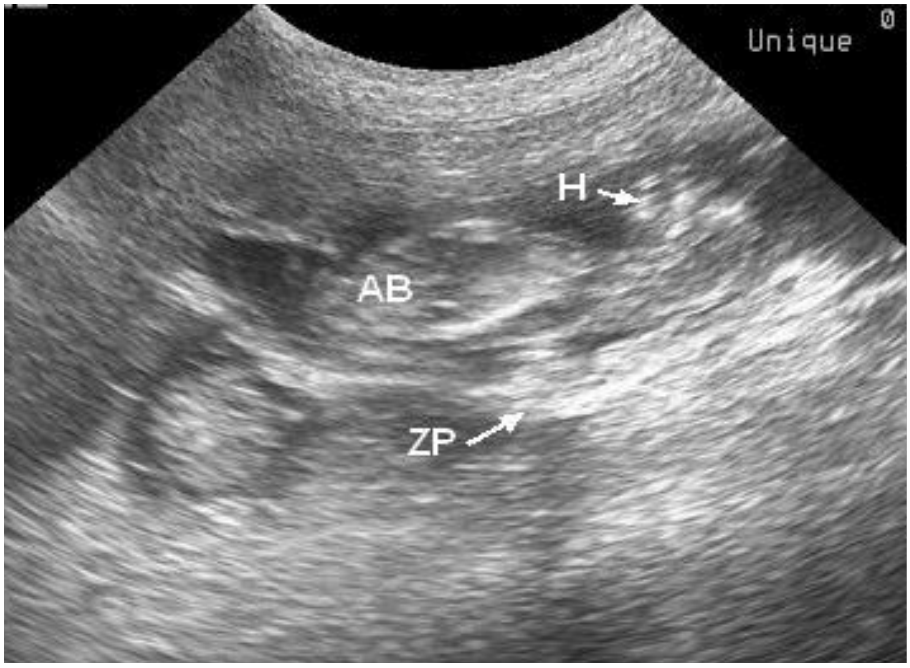


Plate 4.6: Sonogram of the foetal structures in pregnant bitches. Days 28-32 of gestation: Longitudinal image of embryo (H: head, AB, abdomen, ZP: zonary placenta).

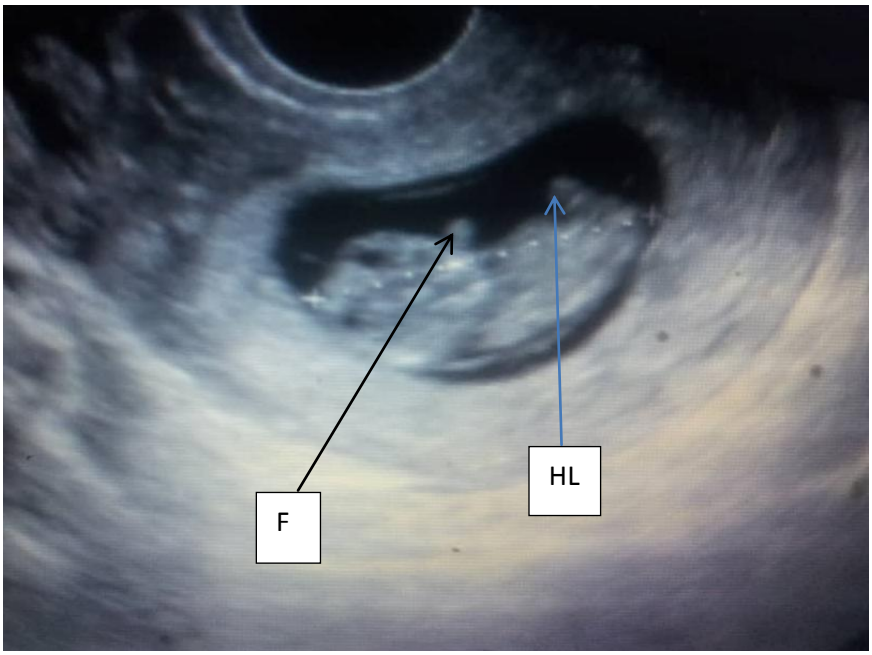


Plate 4.7. Sonogram of the foetal structures in pregnant bitches. between days 34-37 of gestation showing limb bud (FLB= forelimb bud and HLB= Hind limb bud)

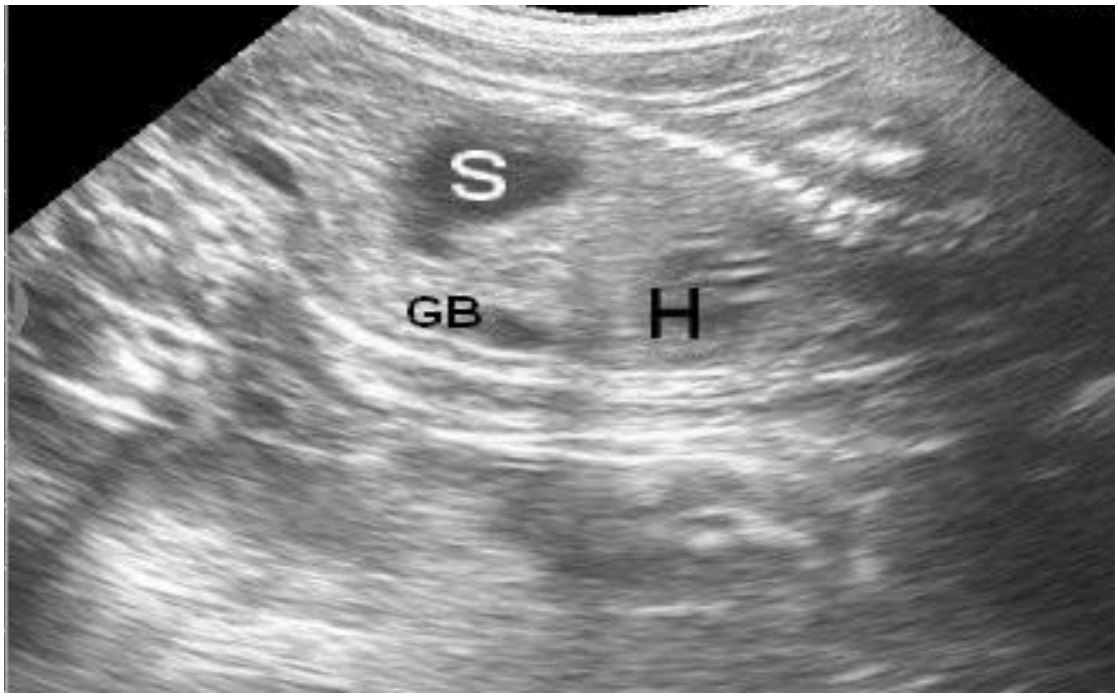


Plate 4.8: Sonogram of the foetal structures in pregnant bitches. Days 35-39 of gestation.Longitudinal image of a foetus with three anechoic area (H: heart, GB: gallbladder, S: stomach).

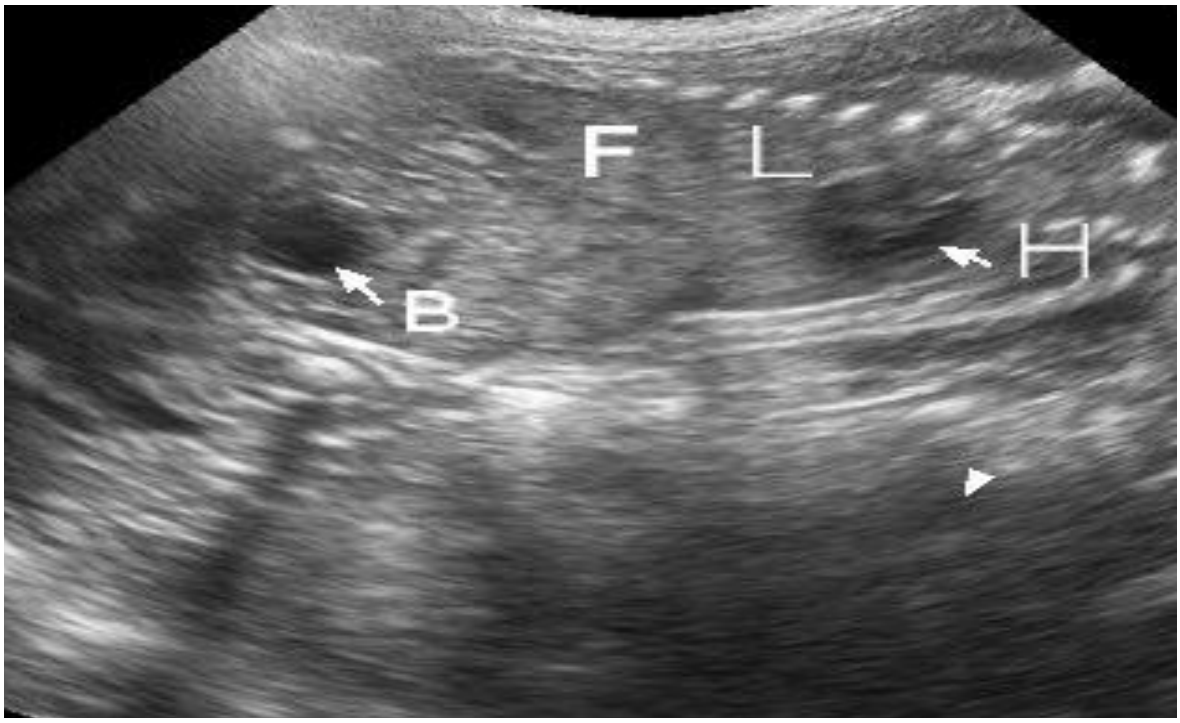


Plate 4.9: Sonograms of the foetal structures in pregnant bitches. Days 39-42 of gestation: Longitudinal image of a foetus (B: bladder, F: liver, L: lung, H: heart).

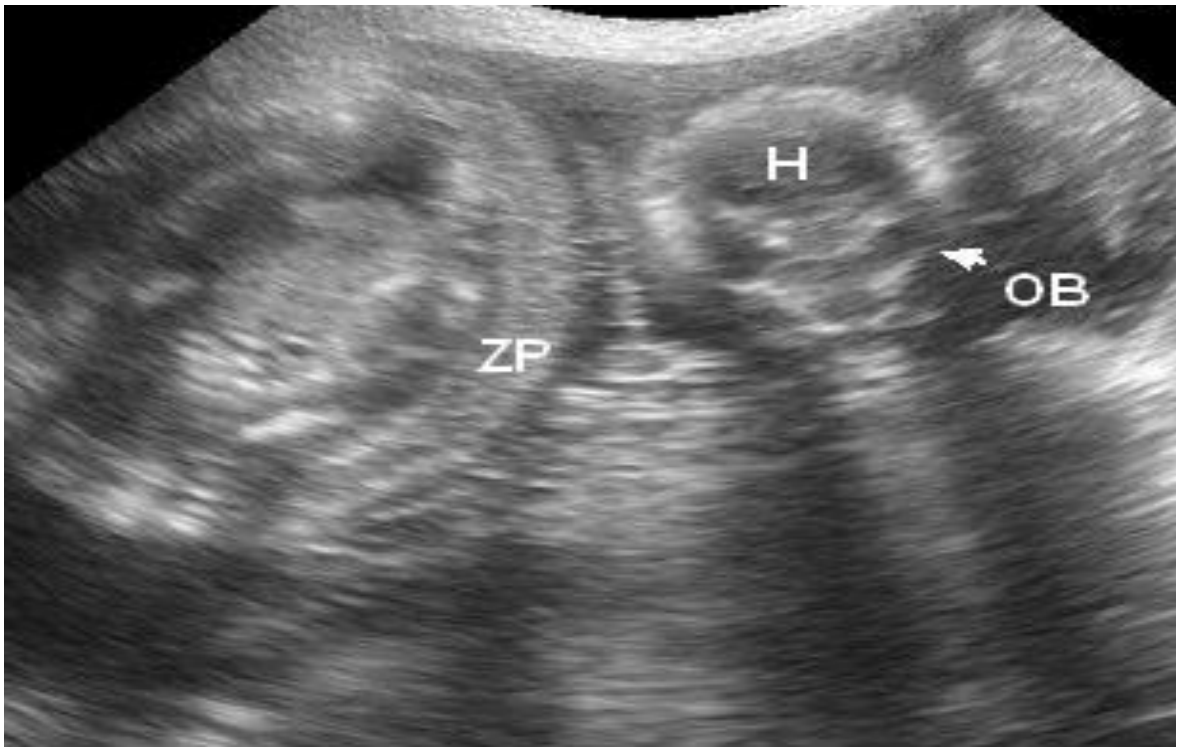


Plate4.10: Sonograms of the foetal structures in pregnant bitches. Days 40-45 of gestation, image of a foetal head (H: head, OB: orbit, ZP: zonary placenta)

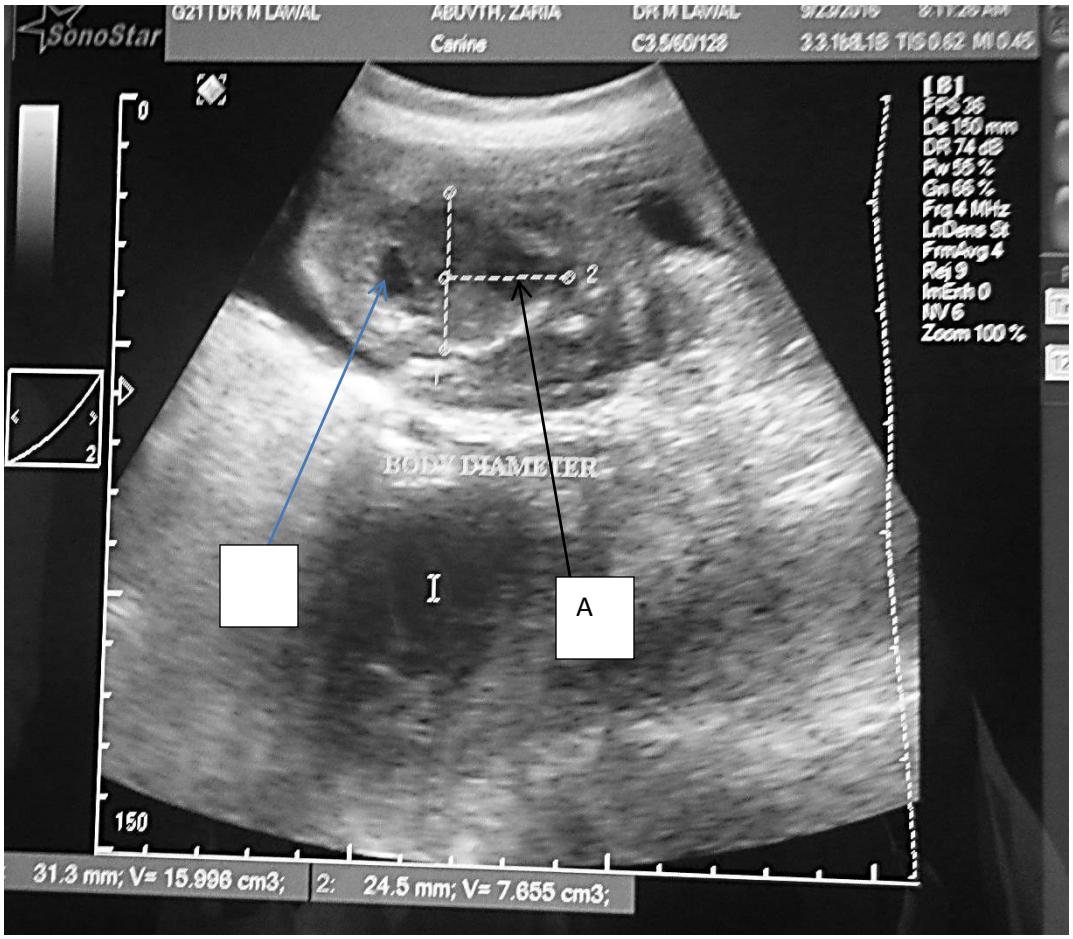


Plate 4.11 Sonogram showing some foetal structures (A= foetal heart with the variouschambers and B = foetal stomach) which was taken between days 41-45 post mating.

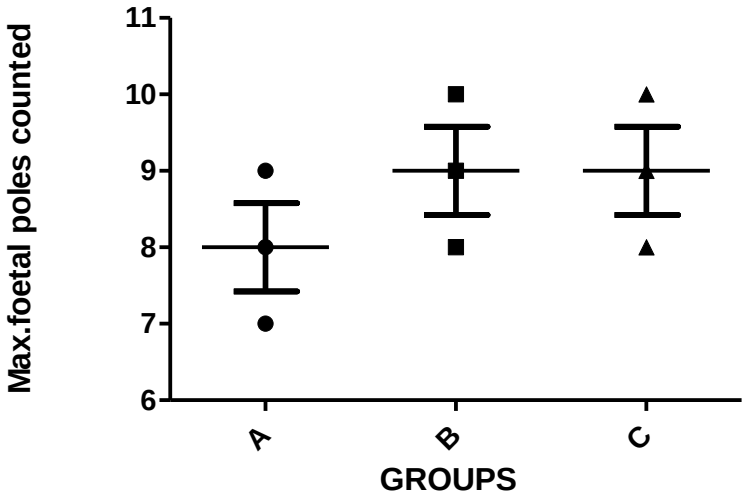


Fig. 4.1. Means of maximum foetal poles visualized at second trimester of gestation

Key: A = not insonated, B= insonated weekly and C = insonated twice a week

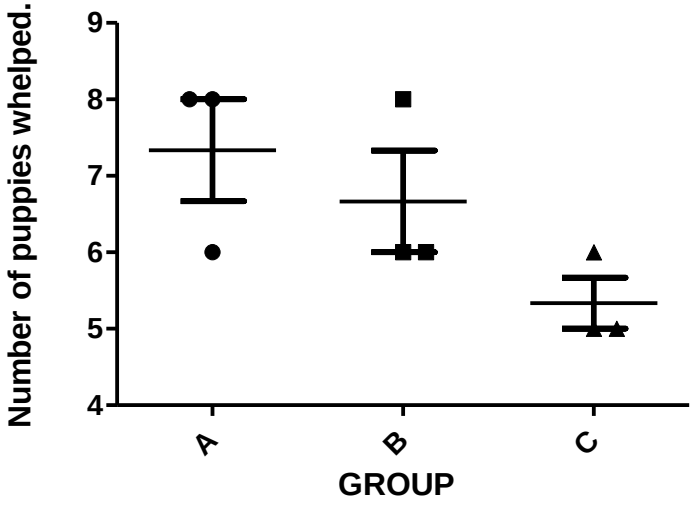


Fig.4. 2. Mean litter size at whelping by the bitches in various groups.

Key: A = not insonated, B= insonated weekly and C = insonated twice a week

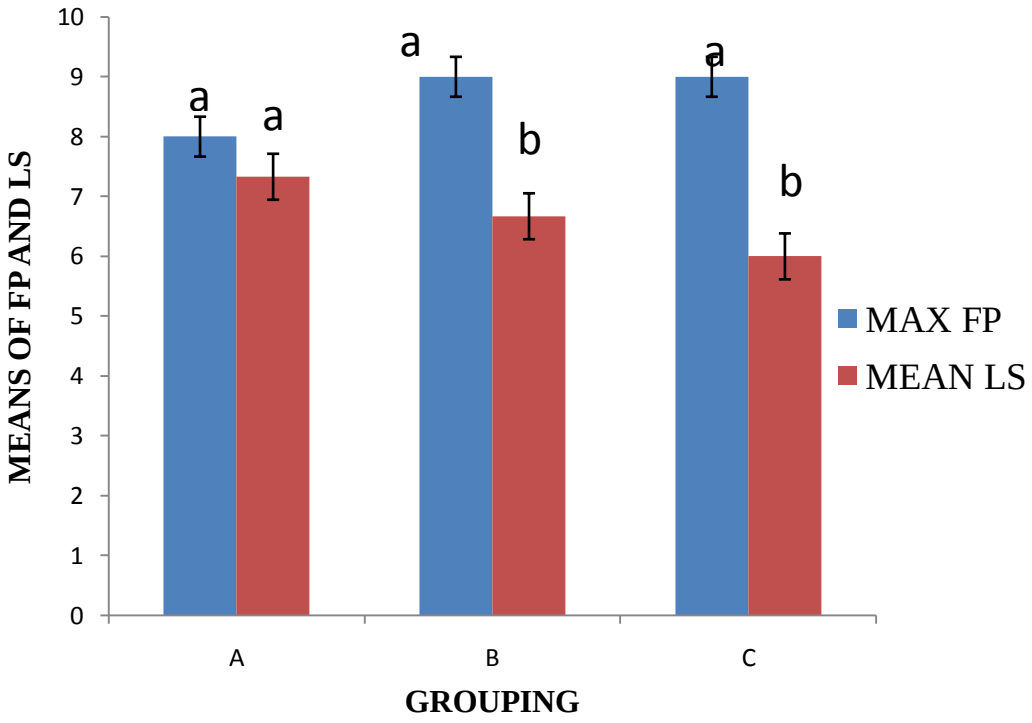


Fig. 4.3. Comparism of the Maximum Fetal poles counted and the mean number of puppies whelped.

Key: A = not insonated, B= insonated weekly and C = insonated twice a week
Max FP= maximum foetal poles counted. LS= litter size

Columns with same alphabets = No statistically significant difference, while those with different alphabets = statistically significant difference.

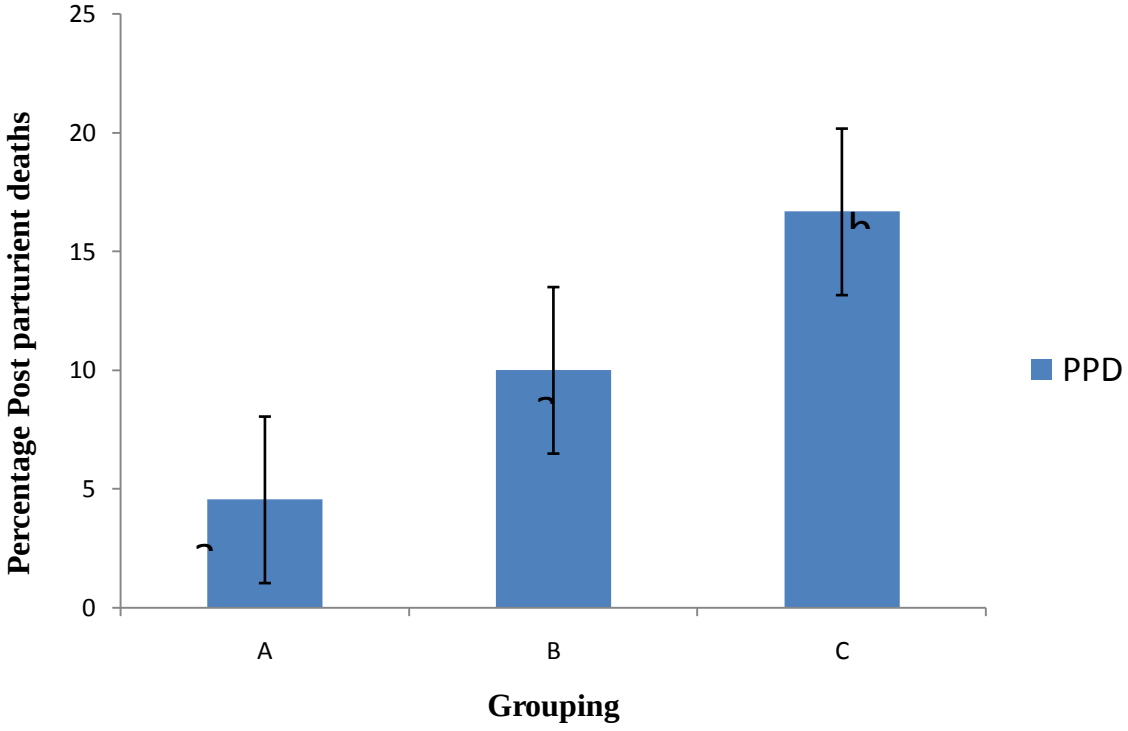


Fig. 4.4. Percentage of post-parturient death recorded two week post whelping

Key: A = not insonated, B= insonated weekly and C = insonated twice a week

PPD= post parturient deaths

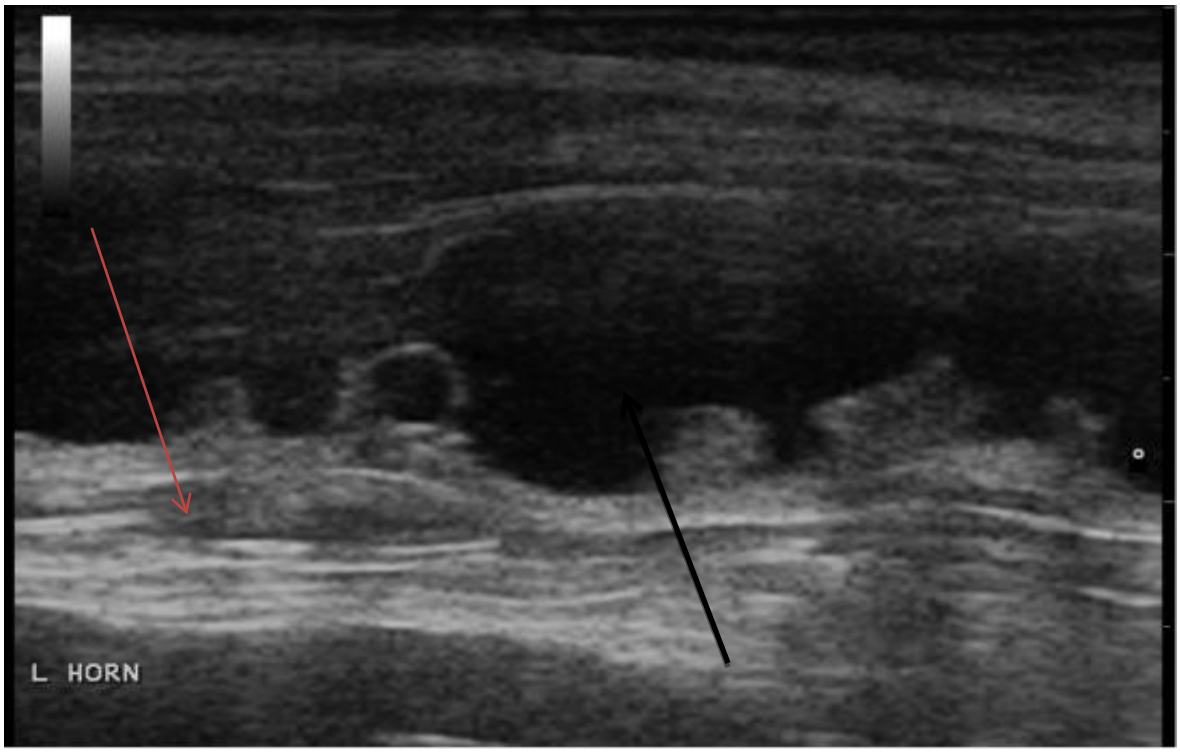


Plate 4.12: Sonographic image of postpartum uterus 3 days after parturition showing thick walls (red arrow) lochia (thick black arrow).

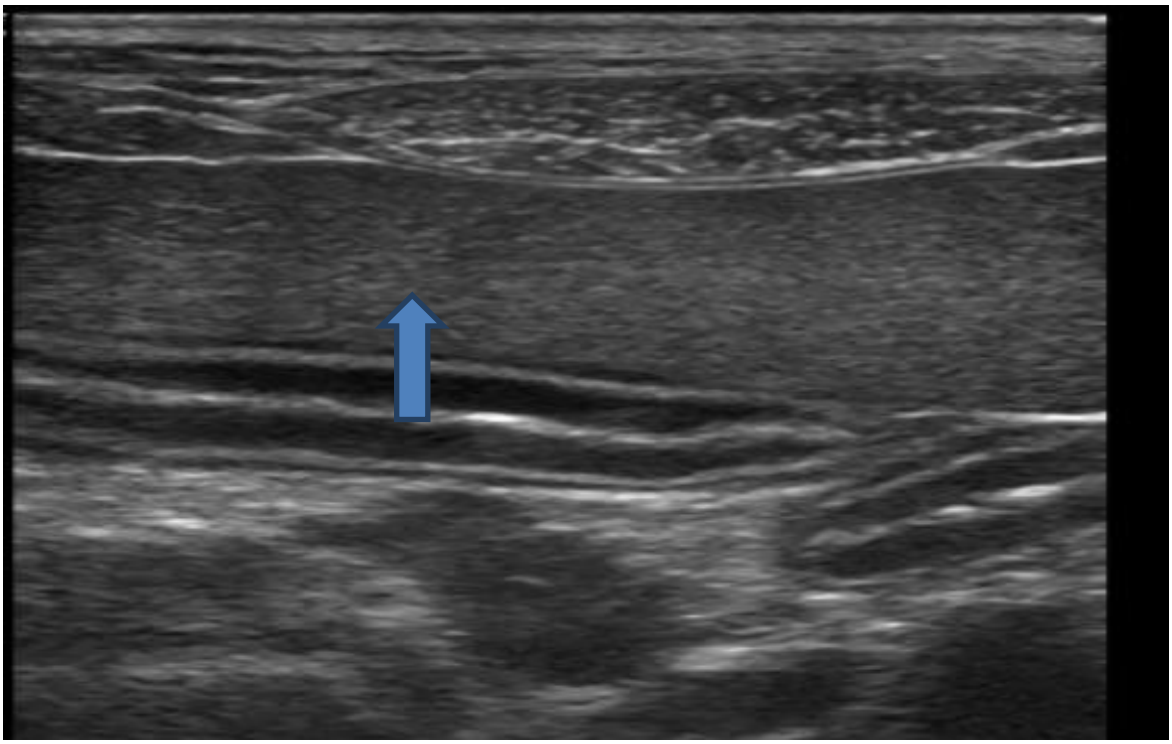


Plate4.13: Sonographic image of postpartum uterus following one month of involution Uterine parenchymal appears isoechogenic (Arrowed)

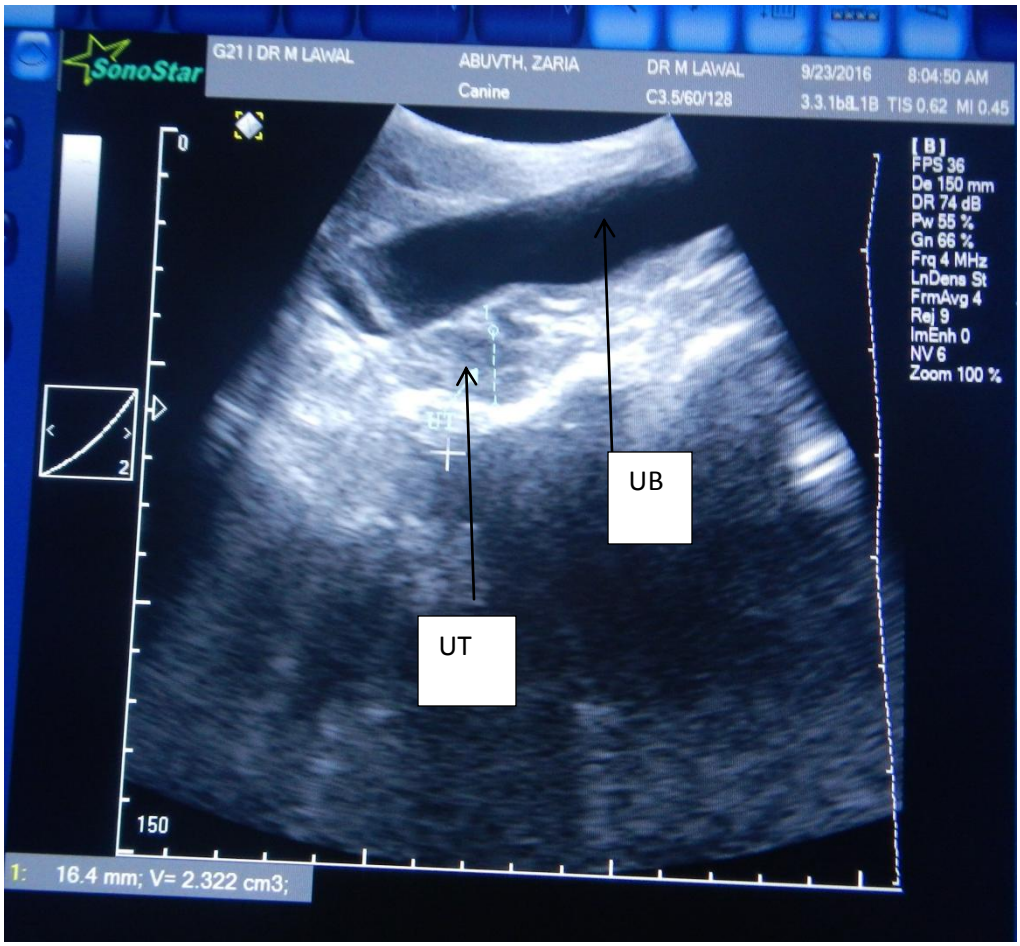


Plate 4.14 Sonograph the urinary bladder (UB) and a mixed echogenic uterus (UT)five weeks postpartum with maximum uterine diameter of 1.64 cm

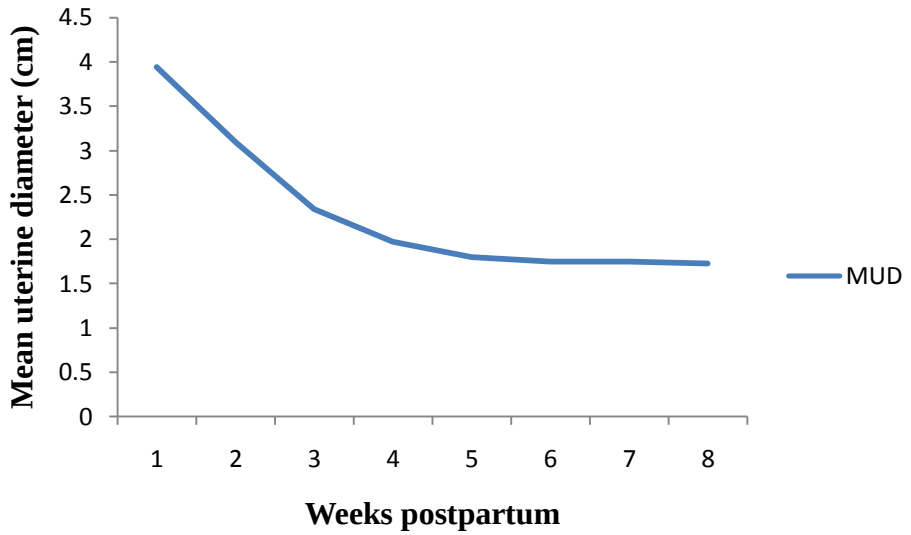


Fig 4.5 The trend in mean diameter of the uterus during part of postpartum (involution) phase of the study plotted against time.

4.2. Haematological parameters of bitches during the reproductive phases

4.2.1. Haematological parameters of bitches during phaseI

The haematology results showed that the mean Packed Cell Volumes for this phase were 38.67 ± 2.81, 39.44 ± 1.72 and 43.00 ± 2.35 for bitches in groups A,B and C respectively (Fig. 4.6). This result was used as baseline for the bitches in this research. Other heamatologic results were: For Haemoglobin (HGB) concentration 12.86 ± .9305, 13.09 ± 0.57 and 14.30 ± 0.78 for groups A, B and C respectively. Leucocyte count was found to be 44.11 ± 4.95, 40.11 ± 4.72 and 44.33 ± 4.61 for groups A, B and C. While the White blood cells counts (WBC) was 9.08 ± 0.83, 12.05 ± 2.20 and 10.98 ± 2.12 for groups A, B and C respectively. The Total protein (TP) result was 9.04 ± 0.58, 7.90 ± 0.21 and 9.27 ± 0.24 A, B and C respectively (Table 4.4).

4.2.2 . Haematological parameters of bitches during phaseII

The haematology results showed that the mean Packed Cell Volumes for thi phase were 36.33 ± 5.64, 49.83 ± 2.37 and 47.33 ± 4.33 % for bitches in groups A, B and C respectively (Fig. 4.6). Other haematological results were: For Haemoglobin (HGB) concentration 12.07 ± 1.88, 16.55 ± 0.80 and 15.72 ± 1.46 for groups A, B and C respectively. Leucocyte count was found to be 23.31 ± 1.45, 39.50 ± 3.02 and 30.17 ± 3.48 for groups A, B and C. While the White blood cells counts (WBC) was 10.68 ± 2.27, 8.90 ± 0.94 and 11.53 ± 1.66 for groups A, B and C respectively. The Total protein (TP) result was 7.37 ± 0.44, 7.15 ± 0.35 and 6.35 ± 0.11 A, B and C respectively (Table 4.4).

4.2.3. Haematological parameters of bitches during phaseIII

The haematology results showed that the mean Packed Cell Volumes for this phase were 47.08 ± 5.64, 45.67 ± 2.37 and 51.42 ± 4.33 % for bitches in groups A, B and C respectively (Fig 4.6). Other heamatologic results were: For Haemoglobin (HGB) concentration 15.69 ± 0.66, 15.18 ± 0.55 and 17.14 ± 0.60 for groups A, B and C respectively. Leucocyte count was found to be 32.83 ± 3.36, 30.25 ± 1.24 and 27.08 ± 2.54 for groups A, B and C. While the White blood cells counts (WBC) was 8.43 ± 0.40, 8.85 ± 0.64 and 10.39 ± 0.78 for groups A, B and C respectively. The Total protein (TP) result was 7.37 ± 0.44, 7.15 ± 0.35 and 6.35 ± 0.11 A, B and C respectively (Table 4.4).

The haematology results in the late gestation phase showed that the mean Packed Cell Volumes for this phase were 42.92 ± 1.70, 40.92 ± 1.46 and 37.25 ± 1.57 % for bitches in groups A, B and C respectively (Fig. 4.6). Other haematological results were: For Haemoglobin (HGB) concentration 14.39 ± 0.55, 13.06 ± 0.80 and 13.68 ± 0.5022 for groups A, B and C respectively. Leucocyte count was found to be 36.50 ± 3.173, 29.75 ± 2.004 and 32.75 ± 2.118 for groups A, B and C. While the White blood cells counts (WBC) was 8.67 ± 0.87, 9.65 ± 1.11 and 9.13 ± 1.40 for groups A, B and C respectively. The Total protein (TP) result was 6.40 ± 0.26, 6.100 ± 0.2339 and 5.900 ± 0.1642 A, B and C respectively (Table 4.4).

4.2.4, Haematological parameters of bitches during phaseIV

The haematology results showed that the mean Packed Cell Volumes for this phase were 34.00 ± 2.51, 43.33 ± 2.34 and 46.89 ± 2.99 % for bitches in groups A, B and C respectively (Fig. 4.6). Other haematological results were: For Haemoglobin (HGB)

concentration 11.33 ± 0.8253, 15.09 ± 0.7804 and 15.59 ± 0.9939 for groups A, B and C respectively. Leucocyte count was found to be 43.11 ± 4.357, 29.22 ± 5.444 and 28.00 ± 4.282 for groups A, B and C. While the White blood cells counts (WBC) was 10.43 ± 1.153, 10.07 ± 1.798 and 7.767 ± 0.6316 for groups A, B and C respectively. The Total protein (TP) result was 8.333 ± 0.51, 8.333 ± 0.5270 and 7.022 ± 0.3968 A, B and C respectively (Table 4.4).

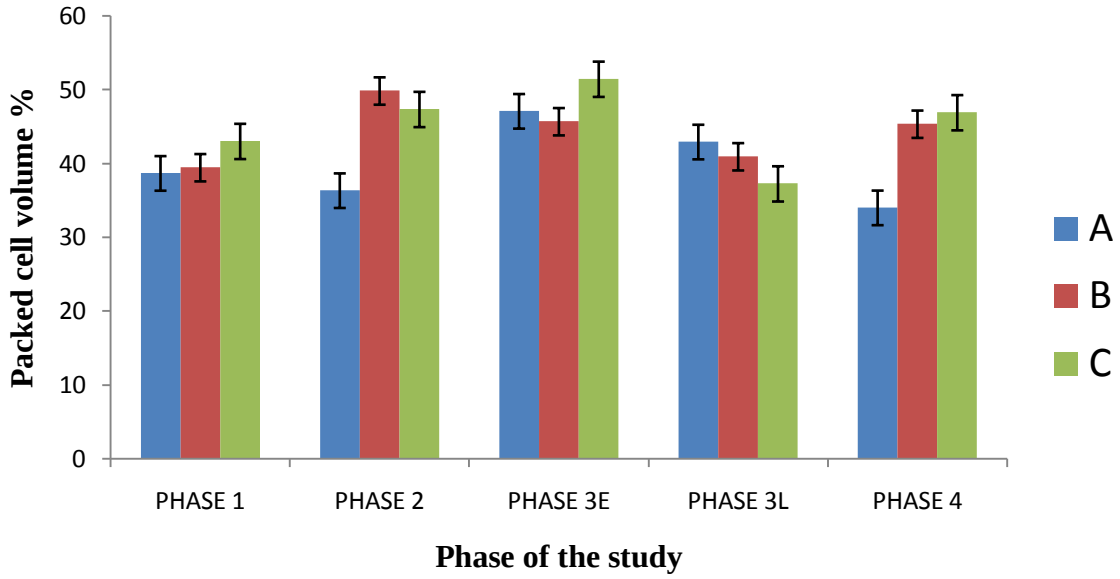


Fig.4 6. Showing the packed cell volumes of the bitches at the various phases of the study.

KEY:

A = group not insonated, B= group insonated once a week in–puppy and C= group insonated twice a week in-pup.

Phase 1 = Acclimatization phase, Phase 2 = Induction phase, Phase 3E = Early gestation phase, Phase 3L = Late gestation phase and Phase 4 = postpartum phase.

Table 4.4. Showing the various means and SEM for the other heamatologic parameters bitches at different phases of the study

	PHASE 1	PHASE 2	PHASE 3E	PHASE 3L	PHASE 4
a	12.86 ± 0.9305[a]	12.07 ± 16.55[a]	15.69 ± 0.6605[a]	14.39 ± 0.5457[a]	11.33 ± 0.8253[a]
b	13.09 ± 0.5738[a]	15.72 ± 0.8007[a]	15.18 ± 0.5546[a]	13.68 ± 0.7965[a]	15.09 ± 0.7804[a]
HGB c	14.30 ± 0.7817[a]	15.72 ± 1.457[a]	17.14 ± 0.5998[a]	13.06 ± 0.5022[a]	15.59 ± 0.9939[a]
a	44.11 ± 4.945[a]	23.33 ± 1.453[a]	32.83 ± 3.355[a]	36.50 ± 3.173[a]	43.11 ± 4.357[a]
b	40.11 ± 4.721[a]	39.50 ± 3.019[a]	30.25 ± 1.238[a]	32.75 ± 2.118[a]	29.22 ± 5.444[a]
LEU c	44.33 ± 4.607[a]	30.17 ± 3.478[a]	27.08 ± 2.536[a]	29.75 ± 2.004[a]	28.00 ± 4.282[a]
a	9.04 ± 0.5819[a]	7.37 ± 0.4364[a]	10.33 ± 3.971[a]	6.40 ± 0.2558[a]	8.33 ± 0.5099[a]
b	7.90 ± 0.2102[a]	7.15 ± 0.3538[a]	6.75 ± 0.1390[a]	6.10 ± 0.2339[a]	8.33 ± 0.5270[a]
TP c	9.27 ± 0.2427[a]	6.35 ± 0.1147[a]	6.35 ± 0.1190[a]	5.90 ± 0.1642[a]	7.02 ± 0.3968[a]
a	9.08 ± 0.8253[a]	10.68 ± 2.265[a]	8.43 ± 0.3966[a]	9.654 ± 1.105[a]	10.43 ± 1.153[a]
b	12.05 ± 2.202[a]	8.90 ± 0.9430[a]	8.85 ± 0.6429[a]	9.13 ± 1.402[a]	10.07 ± 1.798[a]
WBC c	10.98 ± 2.117[a]	11.53 ± 1.661[a]	10.39 ± 0.7762[a]	8.67 ± 0.8737[a]	7.77 ± 0.6316[a]

KEY
HGB = Haemoglobin concentration
LEU = Leucocyte count
TP = Total protein
WBC = White blood cells count

Similar superscripts = no statistically significant difference

4.3. **Serum parameters of theof bitches during the reproductive phases**

4.3.1. Serum parameters of bitches during phaseI

The serum analysis results for this phase showed the progesterone assay was 2.25 ± 0.19, 3.59 ± 0.91 and 2.83 ± 0.79 ng/ml for groups A, B and C respectively. The concentration of Superoxide dismutase was 1.04 ± 0.08, 0.62 ± 0.06 and 0.97 0.07 for Groups A, B and C respectively. Catalase concentration was found to be 6.98 ± 0.12, 6.46 ± 0.03 and 7.78 ± 0.5 µmol/min/ml for groups A, B and C respectively. The concentration of glutathione peroxidase was 28.30 ± 3.04, 21.42 ± 3.15 and 20.09 ± 2.57 µmol/ml for groups A, B and C respectively. While that of malondialdehyde was found to be 3.43 ± 0.50, 4.61 ± 1.58 and 5.57 ± 1.46 µmol/ml for groups A, B and C respectively.

4.3.2. Serum parameters of bitches during phase II

The serology results for this phase showed the progesterone assay was 20.54 ± 4.94, 45.19 ± 24.51 and 70.50 ± 31.50 ng/ml for groups A, B and C respectively. The concentration of Superoxide dismutase was 0.512 ± 0.143, 0.354 ± 0.054 and 0.456 ± 0.14 for Groups A, B and C respectively. Catalase concentration was found to be 5.71 ± 0.717, 5.96 ± 0.324 and 7.123 ± 0.322 µmol/min/ml for groups A, B and C respectively. The concentration of glutathione peroxidase was 12.77 ± 4.493, 9.47 ± 2.17 and 10.5 ± 2.67 µmol/ml for groups A, B and C respectively. While that of malondialdehyde was found to be 0.94 ± 0.27, 1.71 ± 0.51 and 1.876 ± 0.36s µmol/ml for groups A, B and C respectively.

4.3.3. Serum parameters of bitches during phase III

The serum analysis results for early gestation phase showed the progesterone assay was 3.26 ± 0.56, 2.32 ± 0.27 and 3.60 ± 0.33 ng/ml for groups A, B and C respectively (Fig4.7). The concentration of Superoxide dismutase was 1.31 ± 0.04, 1.05 ± 0.05 and

1.47 ± 0.05 for Groups A, B and C respectively (Fig 4.8). Catalase concentration was found to be 10.11 ± 1.41, 11.85 ± 1.20 and 19.12 ± 5.18 µmol/min/ml for groups A, B and C respectively (Fig 4.10). The concentration of glutathione peroxidase was 42.59 ± 2.85, 42.12 ± 3.89 and 41.88 ± 1.72 µmol/ml for groups A, B and C respectively (Fig 4.9). While that of malondialdehyde was found to be 3.43 ± 0.32, 2.31 ± 0.32 and 2.83 ± 0.28 µmol/ml for groups A, B and C respectively (Fig 4.11). The serum analysis results for late gestation phase showed the progesterone assay was 6.54 ± 4.94, 45.19 ± 24.51 and 70.50 ± 31.50 ng/ml for groups A, B and C respectively. The concentration of Superoxide dismutase was 1.50 ± 0.124, 1.86 ± 0.21 and 1.90 ± 0.03 for Groups A, B and C respectively. Catalase concentration was found to be 26.86 ± 4.09, 27.95 ± 4.33 and 33.91 ± 5.83 µmol/min/ml for groups A, B and C respectively. The concentration of glutathione peroxidase was 60.87 ± 3.85, 60.91 ± 5.38 and 76.61 ± 8.98 µmol/ml for groups A, B and C respectively. While that of malondialdehyde was found to be 5.05 ± 0.79, 6.28 ± 2.09 and 7.41 ± 1.62 µmol/ml for groups A, B and C respectively.

4.3.4. Serum parameters of bitches during phase IV

The serology results for this phase showed the progesterone assay was 1.81 ± 0.31, 1.56 ± 0.12 and 1.68 ± 0.13 ng/ml for groups A, B and C respectively (Fig 4.7). The concentration of SOD was 1.53 ± 0.07, 0.898 ± 0.05 and 1.169 ± 0.024 for Groups A, B and C respectively (Fig 4.8). The concentration of GPX was 34.05 ± 3.02, 27.89 ± 2.88 and 32.41 ± 2.04 µmol/ml for groups A, B and C respectively (Fig. 4.9). Catalase concentration was found to be 7.37 ± 4.33, 7.93 ± 0.70 and 12.00 ± 3.24 µmol/min/ml for groups A, B and C respectively (Fig. 4.10). While that of MDAwas found to be 2.049 ± 0.74, 2.83 ± 0.74 and 3.13 ± 0.99 µmol/ml for groups A, B and C respectively (Fig 4.11).

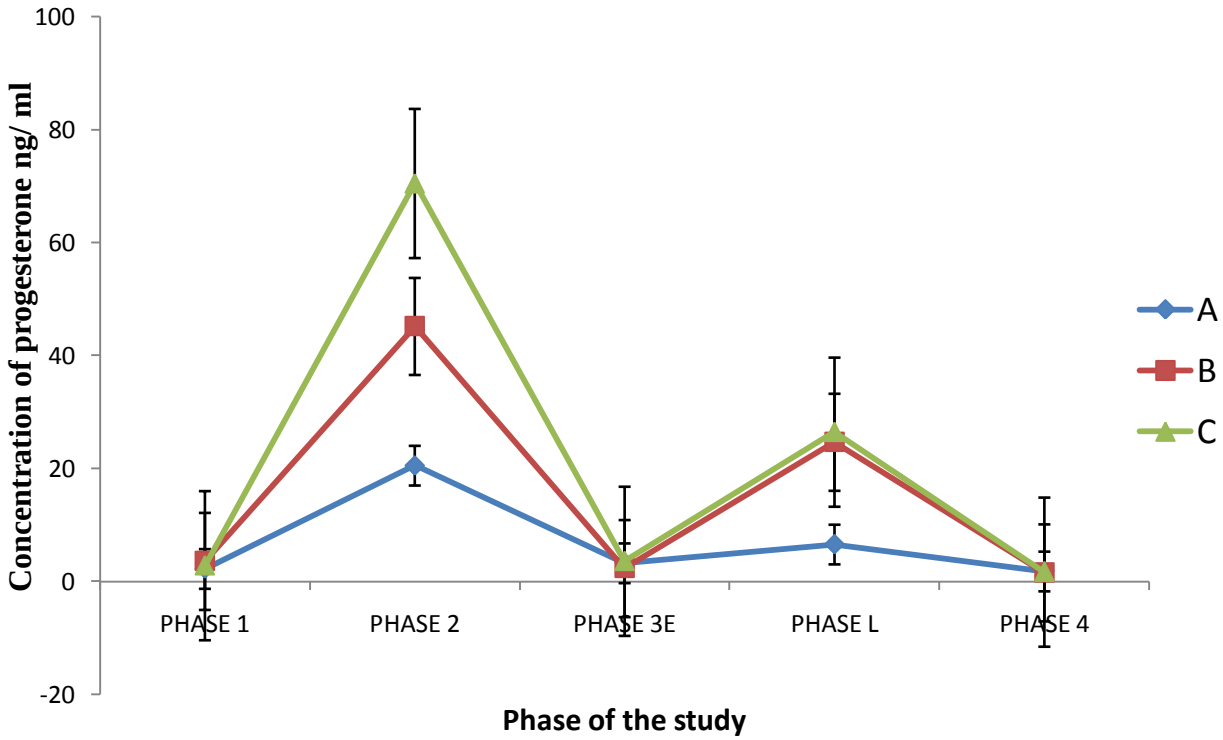


Fig.4.7. Concentration of progesterone of the bitches at the various phases of the study

KEY:

A = group not insonated, B= group insonated once a week in–pup and C= group insonated twice a week in-pup.

Phase 1 = Acclimatization phase, Phase 2 = Induction phase, Phase 3E = Early gestation phase, Phase 3L = Late gestation phase and Phase 4 = postpartum phase.

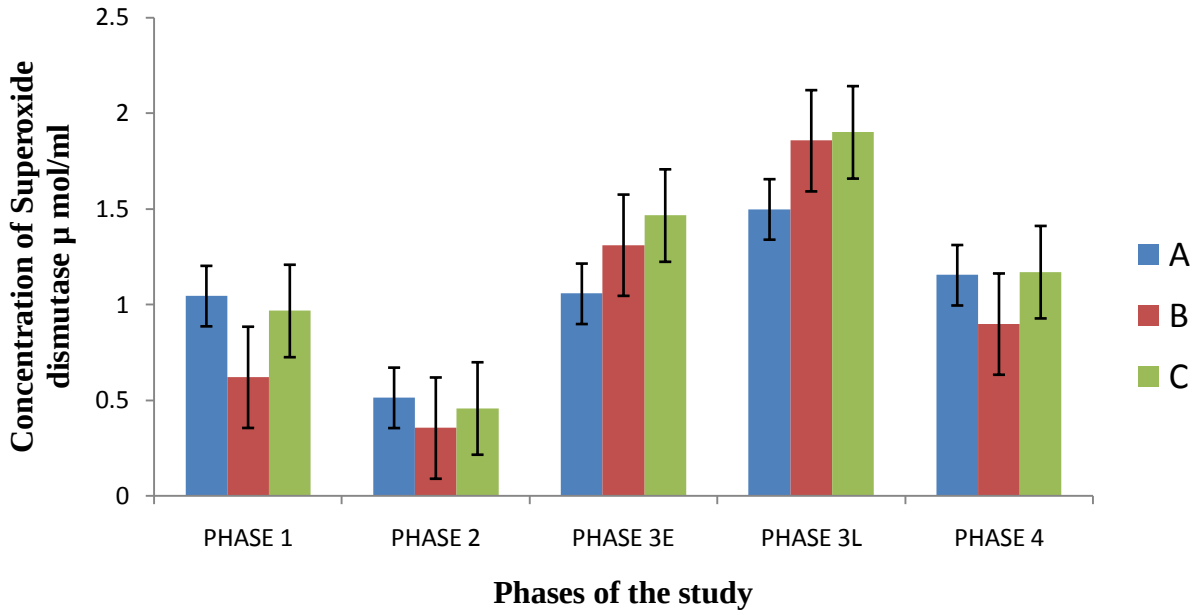


Fig.4.8. Concentration of superoxide dismutase in bitches at various phases of the study

KEY:

A = group not insonated, B= group insonated once a week in–pup and C= group insonated twice a week in-pup.

Phase 1 = Acclimatization phase, Phase 2 = Induction phase, Phase 3E = Early gestation phase, Phase 3L = Late gestation phase and Phase 4 = postpartum phase.

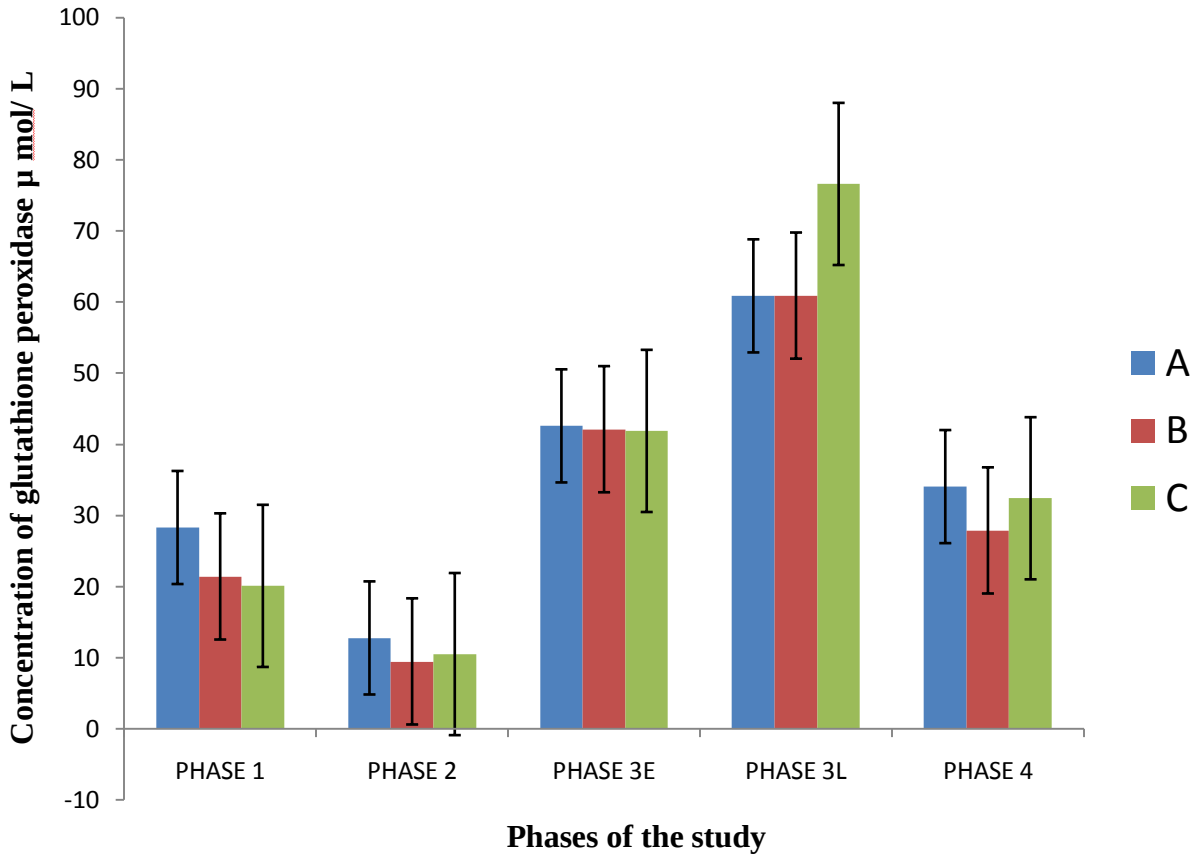


Fig.4.9. Concentration of glutathione peroxidase in the bitches at various phases of the study

KEY:

A = group not insonated, B= group insonated once a week in–pup and C= group insonated twice a week in-pup.

Phase 1 = Acclimatization phase, Phase 2 = Induction phase, Phase 3E = Early gestation phase, Phase 3L = Late gestation phase and Phase 4 = postpartum phase.

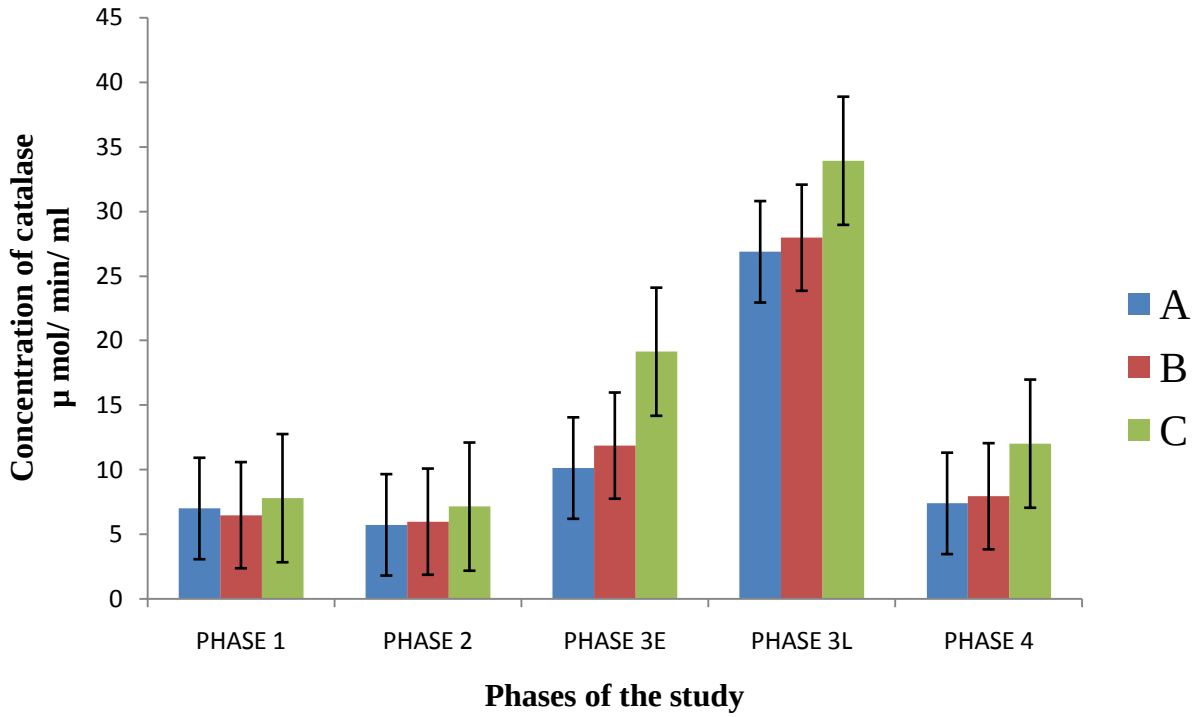


Fig.4.10 Column graph showing concentration of catalase of the bitches at various phases of the study

KEY :

A = group not insonated, B= group insonated once a week in–puppy and C= group insonated twice a week in-puppy.

Phase 1 = Acclimatization phase, Phase 2 = Induction phase, Phase 3E = Early gestation phase, Phase 3L = Late gestation phase and Phase 4 = postpartum phase.

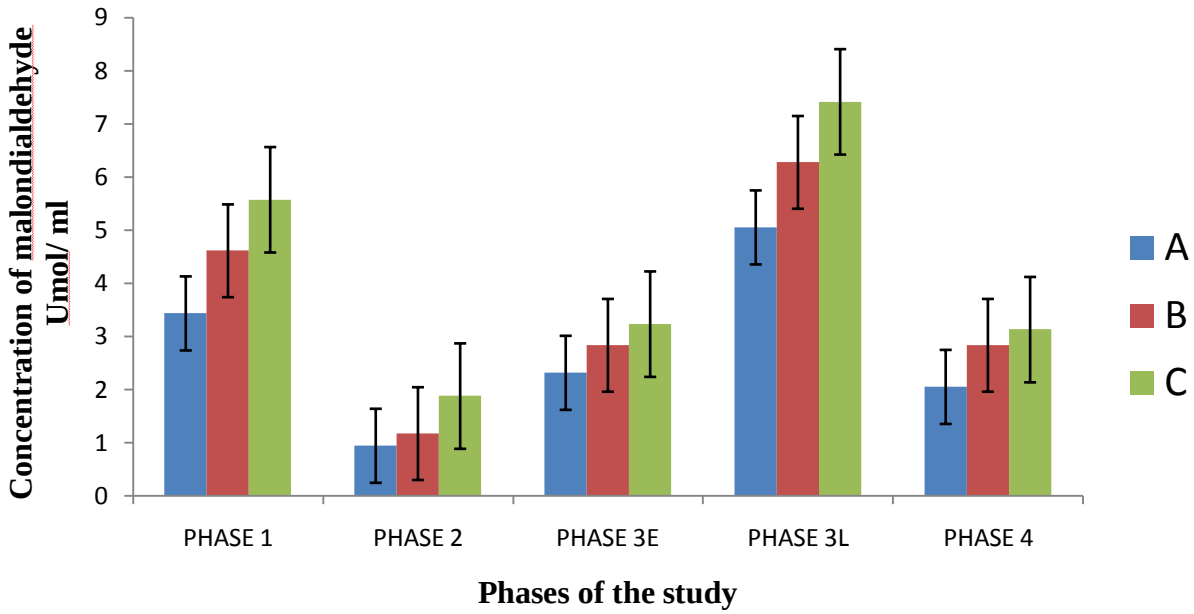


Fig.4.11.Column graph showing concentration of malondialdehyde of the bitches at various phases of the study.

KEY :

A = group not insonated, B= group insonated once a week in–puppy and C= group insonated twice a week in-puppy.

Phase 1 = Acclimatization phase, Phase 2 = Induction phase, Phase 3E = Early gestation phase, Phase 3L = Late gestation phase and Phase 4 = postpartum phase.

4.4. Puppies physical and Serum parameters

4.4.1. Physical parameters postpartum

At whelping the litter size was recorded according to the grouping of the bitches. The total litter size for bitches in group A was 22 (7.33 ± 0.67), group B bitches had a total of 20 puppies (6.67 ± 0.67) while bitches in group C had a litter size of 16 puppies (5.33 ± 0.33). The birth weights of the puppies was also recorded according to the dam's groupings, the mean birth weights for puppies from bitches in group A was 246.4 ± 8.42 g, group B puppies had a mean birth weight of 197.50 ± 11.55 g and those from group C bitches had a mean birth weight of 189.70 ± 10.21 g (Fig. 4.12).

4.4.2. Serum parameters of puppies

The serum analysis results for the puppies showed the mean concentrations of Triiodothyronine were 4.34 ± 0.48, 2.49 ± 0.20 and 1.16 ± 0.20 ng/ml puppies from bitches in groups A, B and C respectively (Fig 4.13). While that of thyroxin was found to be 22.84 ± 6.37, 16.16 ± 1.16 and 14.24 ± 2.19 µg/ml for puppies from bitches in groups A, B and C respectively (Fig 4.14). The concentration of Superoxide dismutase was 0.149 ± 0.045, 0.248 ± 0.0318 and 0.626 ± 0.090 for Groups A, B and C respectively (Fig 4.15). Catalase concentration was found to be 27.03 ± 0.401, 25.11 ± 0.997 and 17.33 ± 5.617 µmol/min/ml for groups A, B and C respectively (Fig 4.16). The concentration of glutathione peroxidase was 74.68 ± 19.33, 71.12 ± 16.66 and 32.03 ± 2.512 µmol/ml for groups A, B and C respectively (Fig 4.17). While that of malondialdehyde was found to be 10.33 ± 0.96, 7.038 ± 0.687 and 4.781 ± 0.853 µmol/ml for groups A, B and C respectively (Fig 4.18).

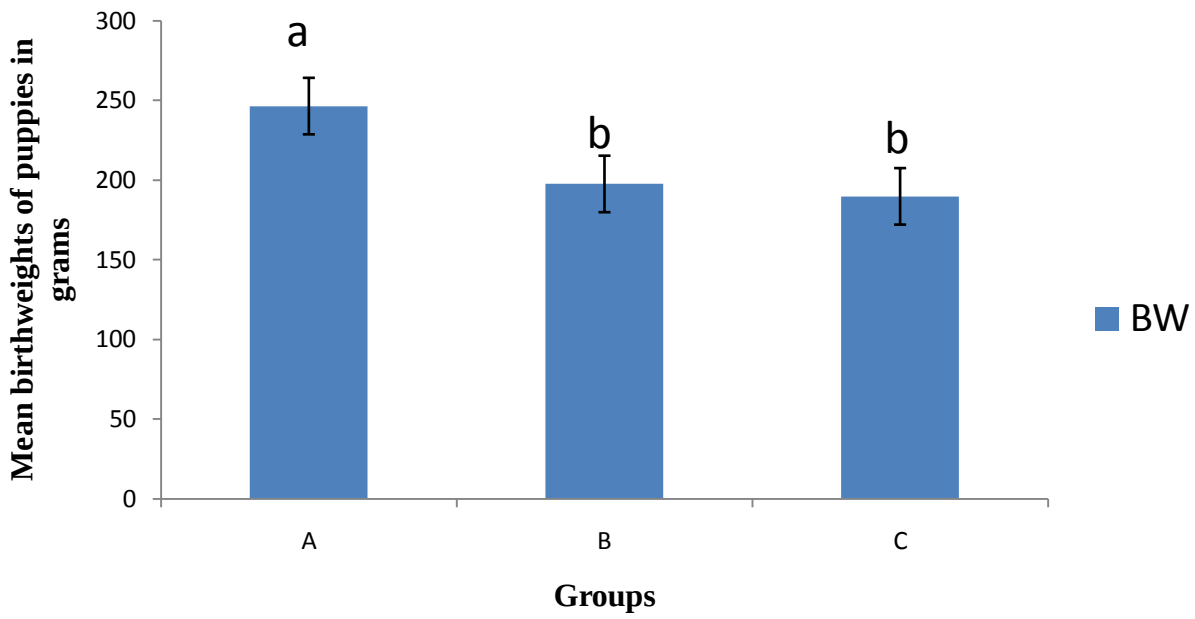


Fig 4.12. Mean birth weights of puppies from the various groups of bitches.

KEY:

A = group not insonated, B= group insonated once a week in –pup and C= group insonated twice a week in-pup

Columns with same alphabets = No statistically significant difference, while those with different alphabets = statistically significant difference.

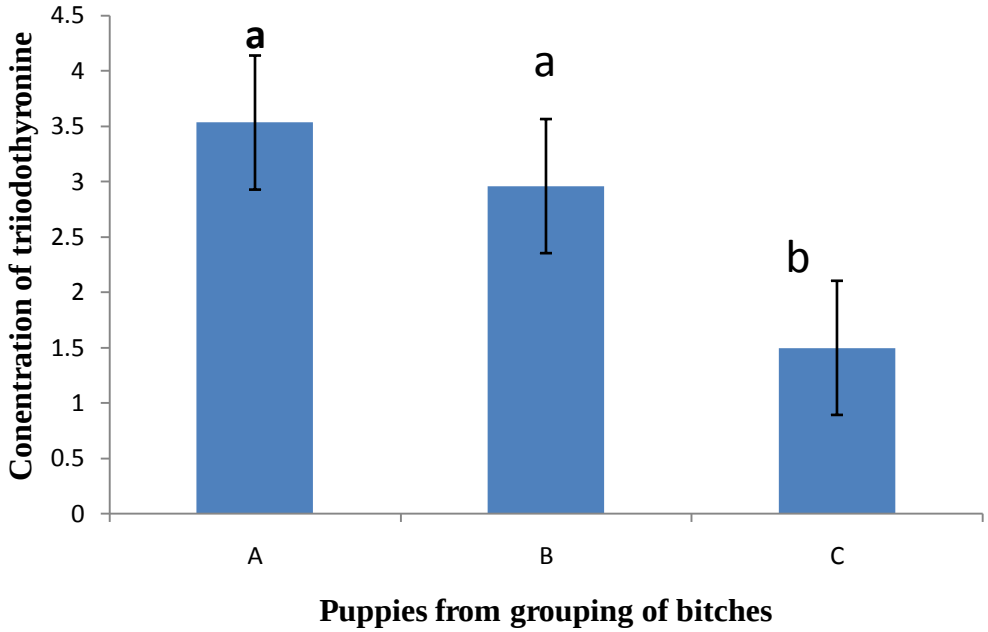


Fig 4.13 Concentration of triiodothyronine of puppies of various bitches

KEY:

A = group not insonated , B= group insonated once a week in–pup
and C= group insonated twice a week in-pup

Columns with same alphabets = No statistically significant difference, while those with different alphabets = statistically significant difference.

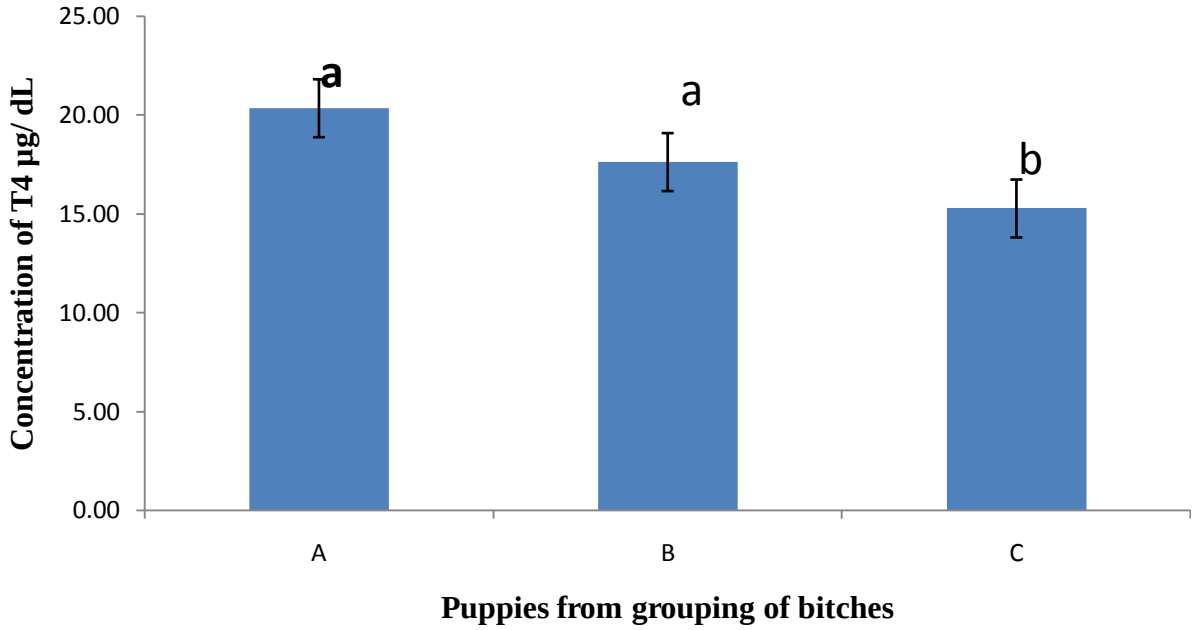


Fig 4.14 Concentration of thyroxin of puppies of various bitches

KEY:

A = group not insonated, B= group insonated once a week in –pup
and C= group insonated twice a week in-pup

Columns with same alphabets = No statistically significant difference, while those with different alphabets = statistically significant difference.

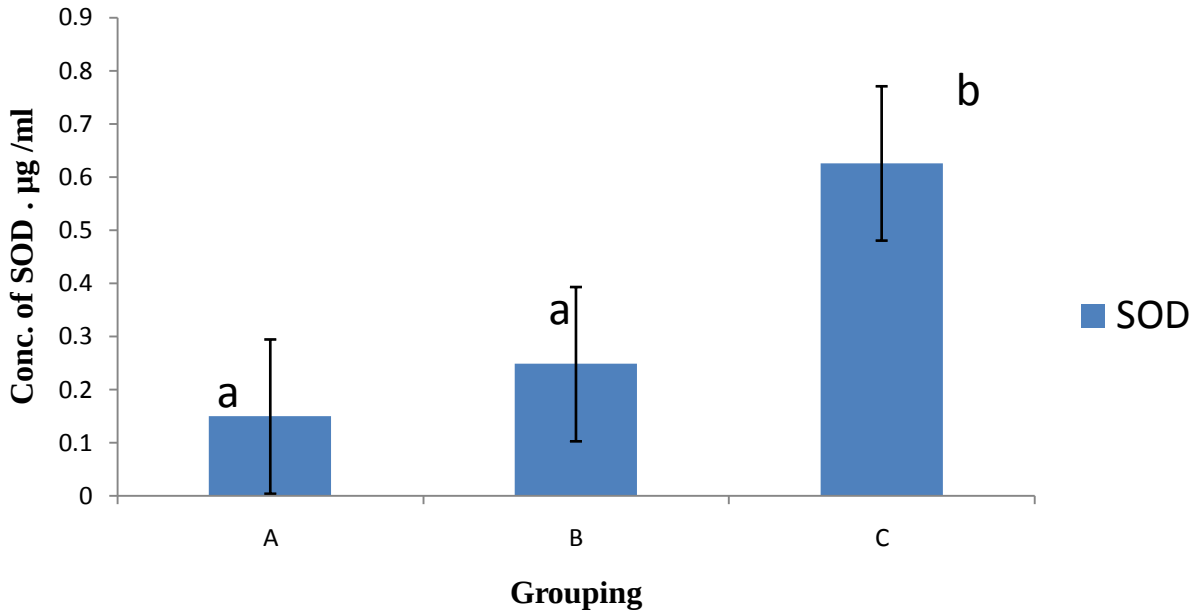


Fig 4.15 Concentration of superoxide dismutase in the puppies from various bitches

KEY:
A = group not insonated, B= group insonated once a week in –pup
and C= group insonated twice a week in-pup

Columns with same alphabets = No statistically significant difference, while those with different alphabets = statistically significant difference.

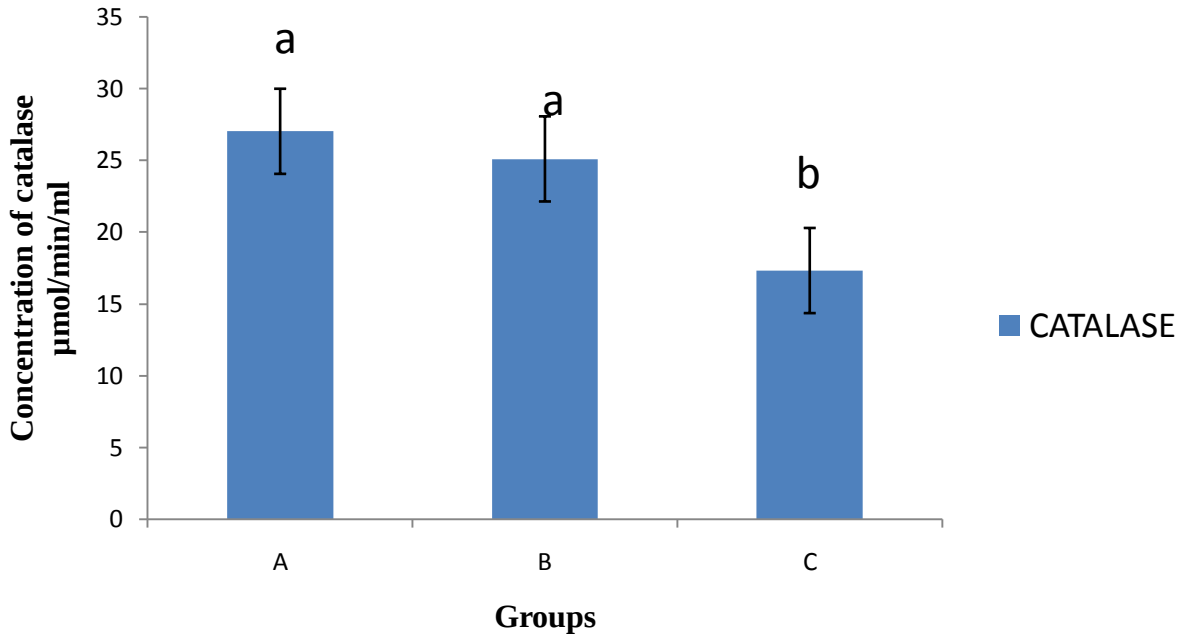


Fig 4.16 Concentration of catalase in the puppies of various bitches

KEY:

A = group not insonated, B= group insonated once a week in –pup
and C= group insonated twice a week in-pup

Columns with same alphabets = No statistically significant difference, while those with different alphabets = statistically significant difference.

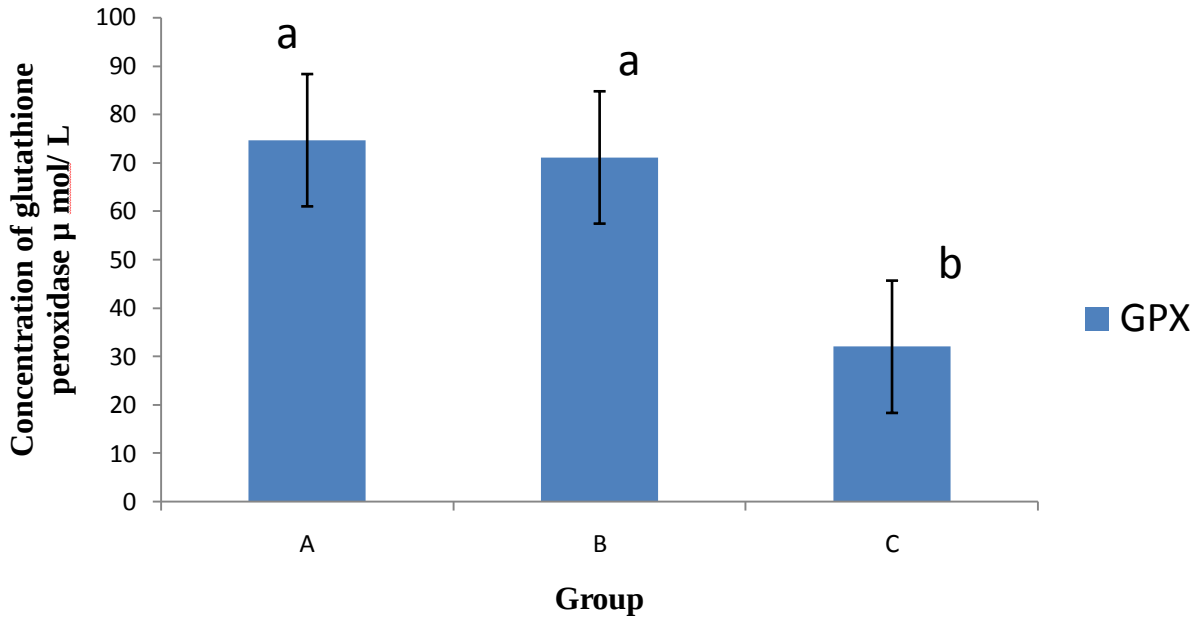


Fig 4.17 Concentration of glutathione peroxidase of puppies of various bitches

KEY:

A = group not insonated, B= group insonated once a week in –pup
and C= group insonated twice a week in-pup

Columns with same alphabets = No statistically significant difference, while those with different alphabets = statistically significant difference.

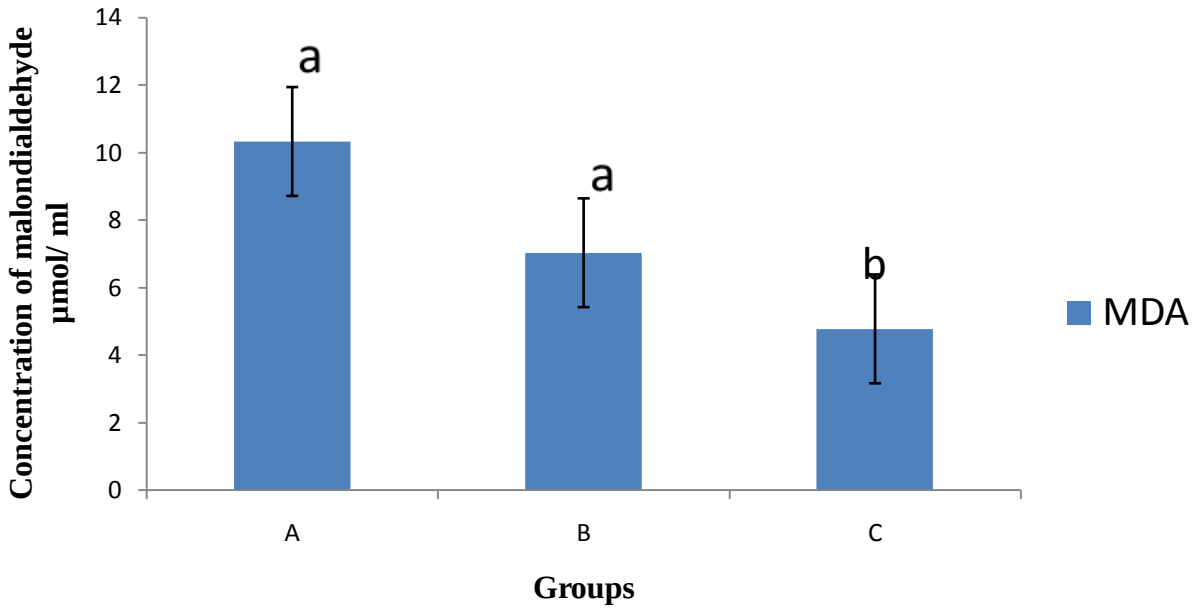


Fig 4.18 Concentration of glutathione peroxidase of puppies of various bitches

KEY:

A = group not insonated, B= group insonated once a week in –pup
and C= group insonated twice a week in-pup

Columns with same alphabets = No statistically significant difference, while those with different alphabets = statistically significant difference

CHAPTER FIVE

5.0 DISCUSSION

Ultrasonography of the internal genitalia in bitches like other mammals revealed that uterine structure are the ones easily visualised when the female is not in season, this is in cornforming with this study. These results agree with that of earlier researcher such as Yeager and Concannon 1990 and Concannon et al 2001, who reported visualising the uterus and uterine horns but not the ovaries or follicles. The failure to visualise the ovaries and follicles was due to their relative small size and absence of follicular activities which would have increased their echogenicity and thereby made them easily visualized.

We monitored the responses of the various bitches to the reconstituted cabergoline orally administered. As early as four (4) days into the treatment, there was evagination of the vulva lips. Followed closely by an increase in the size of the vulva which became larger and turgid initially and this was then followed by serosanguinous vulval discharge. This process took between 13-18 days form start of treatment. These were signs of the onset of proestrus in the bitches. These findings agree with Gobello 2002 and Ajitkumar *et al* 2009. The proestrus response to cabergoline treatment in this study was 100 % which agrees with Verstegen *et al*. (1999) and Ajitkumar *et al*. (2005) who recorded a proestrus response between 75 to 99.33 % in their studies with cabergoline. The mean time taken from initiation of treatment to onset of proestrus was found to be 15.75 ± 0 .675 days though slightly longer than that of, but which agrees with the reports of Ajitkumar *et al*. (2009). The variation seen in the onset of proestrus in our study could be attributed to the variation in the duration of anoestrus which has been known to influence the onset of proestrus in

the bitches when cabergoline was used as an agent of oestrus induction. The proestrus we observed lasted between 7-11 days from onset to the onset of oestrus with a mean period of 9.38 days in the bitches; the duration wass much lower than that reported by England and Yeager 1983 and Concannon 2011, but within the range reported by Ajitkumar *et al* 2009. This difference could be attributed to the fact that the earlier researchers reported natural proestrus duration while our study and that of the latter researchers reported on induced proestrus. Oestrus was considered from the day the bitches stood to be mated by a stud and was considered over when the bitches refused to be mated. The recorded duration for this was found to be between 7-9 days with an average period of 8 days. This was less than that of natural oestrus period but within the induced oestrus as also reported by Ajitkumar *et al* 2009. Initially the ultrasound examination did not differ from the phase 1 part of the study, but by the 10^{th} day the ovaries (right and left) started becoming visible. They appeared as roundish structures with isoechoic tissues. This changed after a few days to have mixed echogenicity due to the development of the follicles which were hyper echoic tissues surrounding an anechoic centre. The anechoic centre increased in size as proestrus advanced and the follicles became visible and could be differentiated from each other as circular anechoic structures that clustered around the ovary. The follicular walls started thickening as the follicles grew larger (and contained more fluid). The follicles were released at oestrus after which the ovaries became smaller and more isoechoic. We observed the area previously occupied by the follicles became first isoechoic and later anechoic due to the release of the follicles which was followed by the development of corpora haemorrhagica. These results agree with the report of previous research finds by Bergeron *et al* (2013), Levy and Fontbonne (2007), Eker and Salmanolu (2006) Fontbonne

and Malandain (2006) and Wallace et al 1992. The ultrasonography in the phaseIII showed that to positively confirm pregnancy diagnosis we needed to closely monitor the serviced bitches for between nineteen to twenty-one days (19-21). The sonographic appearance of the gestational sac was a roundish anechoic structure with a hyper echoic structure at the margins was a definite sign of pregnancy. This structure is usually located around the urinary bladder. The gestational sac (spherical anechoic structure, with thin and hyper echoic borders) got attached to the wall of the uterus at this point; thereafter foetal poles then started to develop distinct morphological characteristics. These morphologic characteristic appeared as hyper echoic non-uniform structures. Then around the period between days 23 to 25, an anechoic part appeared which pulsated (we first visualized foetal cardiac activities). These findings agreed with the reports of Ko, *et. al*; 2004, Concannon *et al* (2001) Kang, *et. al;* 1997, Yeager, *et. al*; 1992 and Yeager et al (1990). We counted the visualised foetal poles and our records show that there was significant difference between the insonated and non-insonated groups. The means of the foetal poles counted were 8.00 ± 0.58, 9.00 ± 0.58 and 9.00 ± 0.58 for bitches in groups A, B and C respectively. The foetuses started to show distinct change in their morphology by separation of the head from the rest of the body a distinct feature between day 29-31.The head appeared as anechoic filled structure just cranial to the forming foetal body. This agreed with the reports of Aissi and Slimani (2008) and Kim and Son (2007). The visualization of limb bud which appeared as short hyper echoic protrusion from the lateral aspects of both cranial thoracic and caudal abdominal parts was first observed between days 34 to 37 (with a mean appearance of 35.17 ± 0.48). The next important occurrence observed on ultrasound examination was foetal movement; this was first noticed on day 33 in some

bitches while in others it was on day 38 post breeding. The mean period within which this happened was 35.50 ± 0.76. These findings agreed with the reports of England and Yeager (1993), Kang et al (1997) and England and Allen (1990). We observed the development of a spherical anechoic structure at the caudal foetal pole between days 35- 39 with the mean period was noticed to be 35.50 ± 0.76 days post mating this was the foetal urinary bladder . Next structure to become visible was the foetal stomach which appeared as a spherical anechoic organ just caudal to the foetal liver; this was visualised between day's 35-39 posts mating with an average period being 36.83 ± 0.60 days. These findings were similar to those of Kim and Son (2007), Ko *et al* (2004) and Yaeger *et al* 1992. The spinal canal became visible as dorsal tubular structure between days 36 – 39g with a period means of 38.00 ± 0.37 days post mating. The eyes were visualised as distinct spherical structures with anechoic corpus vitreum, hyper echoic border between sclera and retina and spherical anechoic lens from day 39-40 post mating with a mean of 39.33 ± 0.21 days. The foetal heart grew larger and the chambers became visible between day 40- 41 post mating with a mean of 40.33 ± 0.21 day. The foetal kidneys appeared as a mixed echogenic structure on the lateral aspect of the cranial abdomen of the foetus. These were visible from day 41 -47 post mating with a mean period of 44.83 ± 0.98. The last structure to appear on ultrasound we found was the foetal intestines; they became visible between day 59- 63 post mating and they were the last organ to be visualised before the bitches whelped. The mean period was found to be 60.33 ± 0.61 days. These findings are in agreement with the reports of Aissi and Slimani (2008) and Kim and Son (2007). Immediately following parturition the uterus appeared tubular on ultrasound with highly thickened walls that were hyper echoic with anechoic intraluminal substances (lochia). The anechoic substances were quickly

expelled four to six postpartum. This agreed with Batista *et al* (2013). In most cases we were able to easily measure the thickness of the walls of the uterus on the short axis. The first week postpartum, the uterine diameter ranged from 3.510 - 4.510 with a mean of 3.944 ± 0.105 cm; this was the highest value obtained during this phase of the study. This was larger than the report of Pharr and Post (1992) who reported uterine diameters sonographically measured in the first week postpartum (2.0-2.8 cm). By second week, we recorded a mean decrease of 21.4 %, to a mean of 3.10 ± 0.116 cm. They uteri were relatively isoechoic. The uterine wall gradually became thinner and less irregular in thickness, and two patterns were noted in the appearance of the uterine contents. In some bitches, the uterine contents became more homogeneous and hypo echoic; while in others, it became more homogeneous and moderately echogenic as fluid components were expelled or resorbed in the uteri of these bitches. By the end of the third week the uteri had reduced by 51 % of the diameter of week 1. This agreed with Ahmed *et al* (2016). Following the third week postpartum uterine involution became more gradual with little or no changes in the echogenicity. The means were 1.973 ± 0.058, 1.796 ± 0.042, 1.747 ± 0.025, 1.747 ± 0.027 and 1.727 ± 0.026 cm for weeks 24 to 8 respectively. These results agree with those of Ahmed *et al* (2016) and Pharr and Post (1992).

The baseline haematological values for this study was obtained at this phase and it showed that the bitches had normal parameters from packed cells volume ranging from 38.67 to 43.00 % , Haemoglobin 12.86 to 14.30, Leucocyte count of between 40.11 to 44.33 with White blood cells counts of 9.08 to 12.05 and Total protein between 7.9 to 9.27. These haematological values served as baseline for the various bitches used in this study. The

haematology results showed an increase from Packed Cell Volume from the baseline data gotten from phase 1, and the values ranged from 36.33 to 49.83 in this phase. There was also a relative increase in other haematological parameters such as Haemoglobin concentration, Leucocyte count, White blood cell count and Total Protein was 12.07 to 16.55, 23.31 to 39.50, 8.90 to 11.53 and 6.35 to 7.37 respectively. These results do not agree with the reports of the findings by Deepthi (2010) who reported a reduction in the haematological parameters during this phase of the study. This could be as a result of the use of cabergoline for the induction of oestrus. The haematology showed that the mean Packed Cell Volumes for this phase increased mildly to between 45.67 to 51.42 % for bitches in groups A, B and C respectively. Other haematological results were: For Haemoglobin (HGB) concentration 15.69 ± 0.66, 15.18 ± 0.55 and 17.14 ± 0.60 for groups A, B and C respectively. Leucocyte count was found to be 32.83 ± 3.36, 30.25 ± 1.24 and 27.08 ± 2.54 for groups A, B and C. While the White blood cells counts (WBC) was 8.43 ± 0.40, 8.85 ± 0.64 and 10.39 ± 0.78 for groups A, B and C respectively. The Total protein (TP) result was 7.37 ± 0.44, 7.15 ± 0.35 and 6.35 ± 0.11 A, B and C respectively. This increment is not in agreement with the reports by Ajala *et al* (2011) and Deepthi (2010) that reported a decrease in the haematological parameters in early pregnancy but agreed with the report of Ahmed *et al* 2015 who reported an increase in the haematological parameters for bitches' pre-treated using cabergoline. The haematology results showed that the mean Packed Cell Volumes for this phase were significantly reduced to 42.92 ± 1.70, 40.92 ± 1.46 and 37.25 ± 1.57 % for bitches in groups A, B and C respectively. This reduction in the PCV is normal in late pregnancy where anaemia is a usual finding. Though the reduction was more in group B than group A bitches which and the most

reduction was observed in group C bitches. This could be attributed to their exposure to ultrasound in-puppy. Ultrasound when introduced to the body was absorbed especially by the bones which led to increase in body temperature which further led to regions of the RBC not containing lipids to melt thereby resulting in haemolysis which was what increased the anaemia we observed in both group B and C bitches. This result agreed with that of Isa and Dom (2016). Other haematological results were: For haemoglobin (HGB) concentration 14.39 ± 0.55, 13.06 ± 0.80 and 13.68 ± 0.5022 for groups A, B and C respectively. Leucocyte count was found to be 36.50 ± 3.173, 29.75 ± 2.004 and 32.75 ± 2.118 for groups A, B and C. While the White blood cells counts (WBC) was 8.67 ± 0.87, 9.65 ± 1.11 and 9.13 ± 1.40 for groups A, B and C respectively. The Total protein (TP) result was 6.40 ± 0.26, 6.100 ± 0.2339 and 5.900 ± 0.1642 A, B and C respectively this result agrees with that of Isa and Dom (2016). There was an increase in the haematological parameters in this phase when compared to the late phase 3. This could be due to the absence of the pregnancy on the haematological values gotten, and the recovery from the pregnancy- induced anaemia. The mean Packed Cell Volumes for this phase were between 34.00 ± 2.51 and 46.89 ± 2.99 %. The Haemoglobin (HGB) concentration ranged from 11.33 ± 0.8253, and 15.59 ± 0.9939, Leucocyte count was found to be between, 28.00 ± 4.282 and 43.11 ± 4.357. While the White blood cells counts (WBC) was 7.767 ± 0.6316 and 10.43 ± 1.153, and finally Total protein (TP) result was between 7.022 ± 0.3968 and 8.333 ± 0.5270.

The serology results which served as the baseline for the bitches showed that progesterone levels at anoestrus ranged from 2.25 to 3.59 ng/ ml, this was higher than those values

reported in other canine by Tsutsui *et al*. (2006) and Concannon 2011 but agrees with the results of Bergeron et al 2013. There are no universally accepted normal ranges for biomarkers of oxidative stress, so the values we got at this phase of served as the baseline for the various bitches. For superoxide dismutase we got a range of 0.62 to 1.04 µ mol/ml that of catalase concentration was found to be 6.46 to 7.78 µmol/min/ml. The concentration of glutathione peroxidase was 20.09 to 28.30 µmol/ml. While that of malondialdehyde was between 3.43 to 5.57 µmol/ml. The serology results showed a statistically significant increase in the serum concentrations of progesterone from that observed at the first phase of the study. The means range from 20.54 to 70.51 ng/ml. This was as a result of the successful induction of oestrus in the bitches using cabergoline. The upwards spike in concentration indicated that the bitches came on heat at this phase of the study. This more than ten (10) folds increment in concentration of progesterone from the initial levels observed in phase 1 of the study agreed with the findings of earlier researchers such as Concannon (2011), England *et al* 2009. The concentrations of all the biomarkers of oxidative stress measured were found to be statistically significantly reduced in this phase of the study. The means of Superoxide dismutase ranged from 0.354 to 0.512 µgm /ml with a p-value of < 0.0001, Catalase concentration was found to be between 5.71 to 7.123 µmol/min/ml with a p-value of < 0.0001. The concentration of glutathione peroxidase was between 9.47 -12.77 µmol/ml had a p-value of 0.013. While concentrations of malondialdehyde was found to be between 0.94 - 1.876 µmol/ml with a p-value of 0.035. These significant reductions in oxidative stress we observed in this phase could be attributed to the antioxidant effects of oral administration of cabergoline which is known to

inhibit lipid peroxidation, activate glutathione peroxidase system and scavenge free radicals. These results complement those of Yoshioka *et al.* (2002).

The serology results for this phase showed the progesterone assay a significant decrease from the previous phase to between 2.32 to 3.60 ng/ml for the bitches. This decrease in progesterone concentration in pregnancy is a unique feature of canine pregnancy. The finding is in agreement with those of Concannon (2011). The concentration of Superoxide dismutase increased significantly from the previous phase to means between 1.05 to 1.47 ± 0.05 bitches at this phase. Mean Catalase concentration was found between 10.11 to 19.12 µmol/ min/ ml for the bitches. The concentration of glutathione peroxidase also significantly increased to between 41.88 to 42.59 µmol/ml. While that of malondialdehyde was found to be from 2.31 to 3.43 µmol/ ml for the various bitches. It is an established fact that pregnancy increases oxygen utilization by the bitches, and this increased demands for oxygen increase production of reactive oxygen species due to increased lipid peroxidation. All of these also led to increase in oxidative stress and an increase in the body's production of antioxidants to try and cope with the stress. These results agree with those of Argarwal *et al* (2008), Vannucci *et al* (2007) Harma *et al* 2004). The serology results for this phase showed the progesterone assay showed an increase in the concentration of progesterone to 6.54 ± 4.94, 45.19 ± 24.51 and 70.50 ± 31.50 ng/ml for groups A, B and C respectively. This phase coincided with the second spike in concentration of progesterone in pregnant bitches. This was a phenomenon only seen in bitches. The increment agrees with the findings of Concannon (2011) and Concannon

(2009). The concentration of Superoxide dismutase was 1.50 ± 0.124, 1.86 ± 0.21 and 1.90 ± 0.03 for Groups A, B and C respectively showed a significant increase as compared to the previous phase. Most especially the bitches in group C, this, we posit could have been due to the greater number of insonation which increased the oxidative stress of the bitches which led to more free radicals being produced and requiring more antioxidants to mop up. This agreed with the finding of Agarwal and Gupta (2014). Catalase concentration was found to be 26.86 ± 4.09, 27.95 ± 4.33 and 33.91 ± 5.83 µmol/min/ml for groups A, B and C respectively. The concentration of glutathione peroxidase was 60.87 ± 3.85, 60.91 ± 5.38 and 76.61 ± 8.98 µmol/ml for groups A, B and C respectively. While that of malondialdehyde was found to be 5.05 ± 0.79, 6.28 ± 2.09 and 7.41 ± 1.62 µmol/ml for groups A, B and C respectively all of these biomarkers showed significant increases in late gestation due to increased oxygen demand and changes in the consumption of the energy substrate by the mother, especially in the foetal–placental unit; this agreed with the finding of Vannucci et al (2007).

The mean progesterone concentration reduced significantly to between 1.56 ± 0.12 to 1.81 ± 0.31 ng/ml. This was the lowest concentration of progesterone we recorded throughout our study. These results agree with the reports of Concannon *et al*, (2006), Concannon (2009) who reported that the concentration of progesterone returns to basal levels postpartum. For biomarkers of oxidative stress we observed a significant reduction in their concentrations as compared to their various levels at the previous phase. With the mean concentration of Superoxide dismutase being between 0.898 ± 0.05 and 1.53 ± 0.07 µmol/ ml. Catalase concentration was found to be between 7.37 ± 4.33, and 12.00 ± 3.24 µmol/min/ml. The concentration of glutathione peroxidase was from 27.89 ± 2.88 and

34.05 ± 3.02, µmol/ml. While that of malondialdehyde was found to be between 2.049 ± 0.74 and 3.13 ± 0.99 µmol/ml.

When the bitches whelped we noted each bitches litter size and the birth weight of the puppies. The mean litter size recorded was 7. 33 ± 0.67, 6.67 ± 0.67 and 5.33 ± 0.33 puppies/ litter for bitches in groups A, B and C respectively. Even though the maximum foetal poles counted showed significant difference between the groups with group A having the lowest means, at parturition the reverse was recorded with group A having the largest litter size. This report agreed with the reports of Idris et al 2017 who reported that insonation had a negative effect on litter size in rabbits. In our case we had three groups with A being non-insonated, B insonated once a week and C insonated twice a week. At parturition the neonatal puppies were weighed and the mean birth weights for the puppies recorded. We recorded a mean birth weight of 246.4 ± 8.42, 197.5 ± 11.55 and 189.7 ± 10.21 g for puppies whelped by bitches from groups A, B and C respectively. This showed that the puppies from dams in group A were the relatively heaviest despite coming from the relatively largest litters. This result did not in agree with the findings of Mila et al (2015), who reported that the larger the litter size the smaller the mean birth weight, but agrees with the finding of Idris et al (2017), who reported a significant difference between the mean birth weights of kittens from dams that were not insonated when in-kitten and dams that were insonated. The post-partum neonatal deaths in the first two weeks showed that the percentage death by group to be 4.55, 10 and 16.67 % for groups A, B and C respectively. We observed that the puppies that died were the mostly the runts of the litter. This agreed with the findings of Mila et al (2015), Devillers et al., (2011) Lawler, (2008);

and Indrebø et al., (2007) who observed that most neonatal deaths occurred in puppies with the least birthweights I n a litter. The increase in the percentage of postpartum deaths in group C could be due to the stress the puppies under went in-utero from multiple insonation when compared to other puppies. This could be due to the fact that in-utero insonation resulted in increased temperature which caused increased oxidative stress which also caused intrauterine growth retardation that led to the puppies being much smaller in birth weight thereby reducing their chances of survival.

The serum collected from the puppies was analysed for Thyroid hormones and biomarkers of oxidative stress, the results showed that both Triiodothyronine (with means of 4.34 ± 0.48, 2.49 ± 0.20 and 1.16 ± 0.20 ng/m for puppies from bitches in groups A,B and C respectively) and thyroxin (with means of 22.84 ± 6.37, 16.16 ± 1.16 and 14.24 ± 2.19 µg/ml for puppies from bitches in groups A, B and C respectively) were significantly different in puppies from the three groups with group A having the highest concentration followed by B and the puppies with the least concentration were those of group C. According to Terranova (2009), formation of thyroid glands begins at the middle of gestation till the end stage of gestation. Hence, external disruption like in-utero heating due to insonation could possibly affect the development of the glands, thus its secretion (Dom *et al*., 2013). These results agree with the findings of Idris *et al* (2017), who reported that there was a difference between the T3 and T4 hormones of the new kittens from dams not insonated and those insonated when pregnant.

The biomarkers of oxidative stress in the puppies showed a significant difference in concentration between the puppies from non-insonated bitches and those of insonated

bitches. The concentration of SOD was 0.149 ± 0.045 µg/ ml for puppies from bitches in group A, 0.248 ± 0.0318 µg/ ml for puppies from group B and 0.626 ± 0.090 µg/ ml for puppies from bitches in group C. Catalase concentration was found to be 27.03 ± 0.401, 25.11 ± 0.997 and 17.33 ± 5.617 µmol/min/ml for groups A, B and C respectively. The concentration of glutathione peroxidase was 74.68 ± 19.33, 71.12 ± 16.66 and 32.03 ± 2.512 µmol/ml for groups A, B and C respectively. While that of malondialdehyde was found to be 10.33 ± 0.96, 7.038 ± 0.687 and 4.781 ± 0.853 µmol/ml for groups A, B and C respectively. These results showed that though the puppies were not directly insonated, they were still exposed to oxidative stress from the in-utero insonation. This result agrees with that of Isa and Dom (2016).

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

12. This study has shown that cabergoline is a very potent agent for inducing oestrus in Nigerian indigenous bitches.

13. There is statistically significant effect of multiple in-utero insonation on maximum fetal poles counted

14. This study has shown that there is statistically significant difference in litter size between the groups

15. There is a significant difference between means of maximum foetal poles visualized and means of litter size in groups with multiple in-utero insonation

16. There is progressive increase of percentage post-parturient deaths from groups A to C.

17. Cabergoline is a very potent antioxidant in our indigenous bitches

18. Multiple in utero insonation significantly affected birth weight of puppies negatively

19. Multiple insonation negatively affected haematological profile of Dams

20. Multiple insonation caused oxidative stress in the dams especially at late gestation

21. In-utero insonation also caused oxidative stress in puppies

22. In-utero insonation negatively affected levels of T3 and T4 hormones in puppies

6.2 Recommendations

5. Further studies should be carried out on a larger population of bitches
6. More reproductive hormones should be studied in the dams
7. More reproductive parameters should be studied
8. More hormone should be studied in the neonatal puppies

6.3 Limitations

5. Funding
6. Lack of adequate electricity supply
7. High cost of laboratory analysis of serum
8. Lack of state of the art laboratory within easy reach and at affordable prices

REFERENCES

Achiron, R., Pinhas-Hamiel, O., Zalel, Y., Rotstein, Z. and Lipitz, S. (1998). Development of foetal male gender: prenatal sonographic measurement of the scrotum and evaluation of testicular descent. *Ultrasound in Obstetrics and Gynecology*, *11*, 242–245.

Adams.G. P. and Ratto, M. H. (2013). Ovulation-inducing factor in seminal plasma: a review.*Animal Reproduction Science, 136*, 148–156.

Agarwal, A., & Gupta, S. (2005). Role of reactive oxygen species in female reproduction. Part I. Oxidative stress: a general overview. Agro Food Industry Hi-Tech, 16(1), 21-25.

Agarwal A, Gupta S, Sekhon L, Shah R.(2008): Redox considerations in female reproductivefunction and assisted reproduction: from molecular mechanisms to health implications. Antioxid Redox Signal;10: 1375–403.

Ahmed. M.N., Ynzeel, J. H and A. F. Majeed. (2016): Ultrasonographic study of uterine involution in of Awassi ewes in Iraq. Al-Anbar J. Vet. Sci., Vol.: 9 No. (1), 16-21

Aissi, A. and Slimani, C. (2008). Time of initial detection of foetal structures and anatomic differentiation by using B-mode ultrasound examination in bitches. *Pakistan Journal of Biological Sciences,11*, 1750–1753.

Aissi, A., & Slimani, C. (2008). Ultrasonographic appearance of the gestational structures throughout pregnancy in bitches. Am J Anim Vet Sciences, 3(1), 32-35.

Ajala O.O., Fayemi, O.O. and Kolawole, T.O.(2011): Erythrocytic parameters as indicators for differentiating between the pregnant and pseudopregnant bitches in Nigeria. *Nig. J. Physiol. Sci*. 26: 019 – 022

Ajala, Oluwatoyin. O., Fayemi, O.E and Oyeyemi, M. O (2012). Some reproductive indices of the Nigerian local bitches in Ibadan, Nigeria. *Niger. J. Physiol. Sci*. 27.049 – 053.

Ajitkumar, G., Sreekumaran, T., Praseeda, R., Mercy, K. A., & Ghosh, K. A. (2010). Comparative efficacy of bromocriptine, cabergoline and thyroxine in inducing oestrus in bitches. *Veterinary research communications*, 34(1), 65-69.

Alatas, C., Aksoy, E., Akarsu, C., Yakin, K. and Bahçeci, M. (1996). Hemodynamic assessment in pelvic inflammatory disease by transvaginal color Doppler ultrasonography. *European Journal of Obstetrics and Gynecology and Reproductive Biology*, *70*, 75–78.

Albrecht, T., Blomley, M. J. K., Heckemann, R. A., Cosgrove, D. O., Jayaram, V., Butler-Barnes, J., Eckersley, R. J., Hoffmann, C. W., and Bauer, A. (2000). Stimulated acoustic emission with the ultrasound contrast agent Levovist: a clinically useful contrast effect with liver-specific properties. *Rofo-Fortschritte Auf dem Gebiet der Rontgenstrahlen und der Bildgebenden Verfahren*, *172*, 61–67.

Allen, A. K. and Stone, L. R. (1990). Equine diagnostic ultrasonography - equipment selection and use. *Compendium of Continueing Education Practicing Veterinarian*. *12*(9), 1307-1311.

Andersen, A. C. and Simpson, M. E. (1973). The ovary and reproductive cycle of the dog (Beagle). Los Altos, California: Geron-X, Inc.

Arbeiter, K., Brass, W., Ballabio, R. and Jöchle, W. (1988). Estrus induction and fertility with cabergoline, a prolactin inhibitor, in the bitch. *Journal of Small Animal Practices,29*, 781-788.

Arnold, S., Arnold, P., Concannon, P. W., Weilenmann, R., Hubler, M., Casal, M., ... & Rüsch, P. (1989). Effect of duration of PMSG treatment on induction of oestrus, pregnancy rates and the complications of hyper-oestrogenism in dogs. Journal of reproduction and fertility. Supplement, 39, 115-122.

Arshadi, R., Yu, A. C. H. and Cobbold, R. S. C. (2007). Coded excitation methods for ultrasoundharmonic imaging. *Canadian Acoustic,35*, 35–46.

Ascoli, M. (2005). Learning new tricks from an old dog: the processing of the intracellular precursor of the luteinizing hormone receptor (LHR) into the mature cell-surface LHR is a regulated process. Endocrinology, 146(8), 3221-3223.

Ascoli, M., Fanelli, F. and Segaloff, D. L. (2002). The lutropin/choriogonadotropin receptor, a perspective. *Endocrine Reviews*, *23*, 141-174.

Assidi, M., Dieleman, S. J. and Sirard, M. A. (2010). Cumulus cell gene expression following the LH surge in bovine preovulatory follicles: potential early markers of oocyte competence. *Reproduction*, *140*, 835-852.

Averkiou, M. A. and Hamilton, M. F. (1997). Nonlinear distortion of short pulses radiated by plane and focused circular pistons. *The Journal of the Acoustical Society of America,102*, 2539–2548.

Baithalu, R. K., Singh, S. K., Gupta, C., Raja, A. K., Saxena, A., Kumar, Y., Singh, R. and Agarwal, S. K. (2013). Cellular and functional characterization of buffalo (*Bubalus bubalis*) corpus luteum during the estrous cycle and pregnancy.*Animal Reproduction Science*, *140*, 138-146.

Barber, M. R., Lee, S. M., Steffens, W. L., Ard, M., & Fayrer-Hosken, R. A. (2001). Immunolocalization of zona pellucida antigens in the ovarian follicle of dogs, cats, horses and elephants. Theriogenology, 55(8), 1705-1717.

Barrett, E. (2011). Bladder and urethra. In: Barr F, Gaschen L, editors. BSAVA manual of canine and feline ultrasonography. Gloucester (United Kingdom). *British Small Animal Veterinary Association,* 155–164

Barthez, P. Y., Le´ veille´, R. and Scrivani, P. V. (1997). Side lobes and grating lobes artefacts in ultrasound imaging. *Veterinary Radiology and Ultrasound*, *38*(5), 387–393.

Batista, P. R., Gobello, C., Corrada, Y., Pons, E., Arias, D. O. and Blanco, P. G. (2013). Doppler ultrasonographic assessment of uterine arteries during normal canine puerperium. *Animal Reproduction Science,141*, 172–176.

Batista, P. R., Gobello, C., Rube, A., Corrada, Y. A., Tórtora, M. and Blanco., P. G. (2016). Uterine blood flow evaluation in bitches suffering from cystic endometrial hyperplasia (CEH) and CEH-pyometra complex. *Theriogenology,85*, 1258–1261

Batista, P.R. Gobello, C. Corradab, Y. Ponsc, E Ariasa, D.O. Blancoa.P.G (2015) Doppler ultrasonographic assessment of uterine arteries during normal canine puerperium. Animal Reproduction Science. 141.172– 176

Baumans, V., Dijkstra, G. and Wensing, C. J. (1981). Testicular descent in the dog. *Anatomia, Histologia, Embryologia*, *10*, 97–110.

Beccaglia, M., and Luvoni, G. C. (2004). Ultrasonographic study during pregnancy of the growth of an encephalic portion in the canine foetus. *Veterinary Research Communications, 28*, 161-164

Beccaglia, M. and Luvoni, G. C. (2006). Comparison of the accuracy of two ultrasonographic measurements in predicting the parturition date in the bitch. *Journal of Small Animal Practice*, *47*, 670–673.

Beccaglia, M., Faustini, M., and Luvoni, G. C. (2008). Ultrasonographic study of deep portion of diencephalo-telencephalic vesicle for the determination of gestational age of the canine foetus. *Reproduction in Domestic Animals, 43*, 367–370.

Beccaglia, M., Alonge, S., Trovo', C. and Luvoni, G. C. (2016). Determination of gestational time and prediction of parturition in dogs and cats: an update. *Reproduction in Domestic Animals, 51*(S1), 12–17.

Bergeron, L. H., Nykamp, S. G., Brisson, B. A., Madan, P. and Gartley, C. J. (2013). An evaluation of B-mode and color Doppler ultrasonography for detecting periovulatory events in the bitch. *Theriogenology, 79*, 274-283.

Bigliardi, E., Parmigiani, E., Cavirani, S., Luppi, A., Bonati, L., & Corradi, A. (2004). Ultrasonography and cystic hyperplasia–pyometra complex in the bitch. *Reproduction in domestic animals*, *39*(3), 136-140.

Blanco, P. G., Rodríguez, R., Batista, P. R., Barrena, J. P., Arias, D. O. and Gobello, C. (2015). Bidimensional and Doppler ultrasonographic evaluation of postpartum uterine involution in the queen. *Theriogenology*, *84*, 82–85

Blanco, P. G., Tórtora, M., Rodríguez, R., Arias, D. and Gobello, C. (2011). Ultrasonographic assessment of maternal cardiac function and peripheral circulation during normal gestation in dogs. *The Veterinary Journal*, *190*, 154–159.

Bouchard, G. F., Gross, S., Ganjam, V. K., Youngquist, R. S., Concannon, P. W., Krause, G. F. and Reddy, C. S. (1993). Oestrus induction in the bitch with the synthetic oestrogen diethylstilboestrol. *Journal of Reproduction and Fertility*,*47*, 515-516.

Bouchard, G. F., Solorzan, N., Concannon, P. W., Youngquist, R. S. and Bierschwal, C. J. (1991). Determination of ovulation time in bitches based on teasing, vaginal cytology and ELISA for progesterone. *Theriogenology*, *35*, 603-611

Boyd, J. S. (1971). The radiographic identification of the various stages of pregnancy in the domestic cat. *Journal of Small Animal Practice*, *12*, 501–506.

Boyd, J. S., Renton, J. P., Harvey, M. J., Nickson, D. A., Eckersall, P. D. and Ferguson, J. M. (1993). Problems associated with ultrasonography of the canine ovary around the time of ovulation.*Journal of Reproduction and Fertility*, *47*, 101-105.

Brown, J. L. (2011). Female reproductive cycles of wild female felids. Anim.Reprod. Sci. 124, 155–162.Concannon, P.W., 2011. Reproductive cycles of the domestic bitch. *Animal Reproduction Science*, *124*, 200–210.

Brugger, N., Otzdorff, C., Walter, B., Hoffmann, B. and Braun, J. (2011). Quantitative determination of progesterone (P4) in canine blood serum using an Enzyme-linked Fluorescence Assay. *Reproduction in Domestic Animals 46*, 870–873.

Bukovsky, A., Indrapichate, K., Fujiwara, H., Cekanova, M., Ayala, M. E., Dominguez, R., Caudle, **……..** Upadhyaya, N. B. (2003). Multiple luteinizing hormone receptor (LHR) protein variants, interspecies reactivity of anti-LHR mAb clone 3B5, subcellular localization of LHR in human placenta, pelvic floor and brain, and possible role for LHR in the development of abnormal pregnancy, pelvic floor disorders and Alzheimer's disease. *Reproductive Biology and Endocrinology*, *1*, 46.

Bürstel, D., Meinecke-Tillman, S. and Meinecke, B. (2002). Ultrasonographic diagnosis of foetal sex in small ruminants bearing multiple fetuses. *Veterinary Record*, *151*, 635–636.

Bysted, B. V., Dieleman, S. J., Hyttel, P. and Greve, T. (2001). Embryonic developmental stages in relation to the LH peak in dogs. *Journal of Reproduction and Fertility*, *57*, 181-186.

Cain, J. L., Cain, G. R., Feldman, E. C., Lasley, B. L. and Stabenfeldt, G. H. (1988). Use of pulsatile intravenous administration of gonadotropin-release hormone to induce fertile estrus in bitches. *American Journal of Veterinary Research,49*, 1993-1996.

Calliada, F., Campani, R., Bottinelli, O., Bozzini, A. and Sommaruga, M. G. (1998). Ultrasound contrast agents: basic principles. *European Journal of Radiology*,*27*(2), S157-S160.

Casanueva, E ; F.E. Viteri (2003). Iron and oxidative stress in pregnancy**.** *Journal of Nutrition*,133. 1700S–1708S.

Chappell, M. A. and Payne, S. J. (2006). A physiological model of gas pockets in crevices and their behavior under compression. *Respiratory Physiology and Neurobiology*, *152*, 100–114.

Chapwanya, A., Clegg, T., Stanley, P. and Vaughan, L. (2008). Comparison of the Immulite and RIA assay methods for measuring peripheral blood progesterone levels in Greyhound bitches. *Theriogenology*,*70*, 795–799.

Chastant-Maillard, S., Chebrout, M., Thoumire, S., Saint-Dizier, M., Chodkiewicz and M., Reynaud, K. (2010). Embryo biotechnology in the dog: a review. *Reproduction, Fertility, and Development,* 22(7), 1049–1056.

Chastant-Maillard, S., de Lesegno, C. V., Chebrout, M., Thoumire, S., Meylheuc, T., Fontbonne, A., Chodkiewicz, M., Saint-Dizier, M. and Reynaud, K. (2011). The canine oocyte: uncommon features of *in vivo* and *in vitro* maturation. *Reproduction, Fertility, and Development*, *23*, 391-402.

Chastant-Maillard, S., Aggouni, C., Albaret, A., Fournier, A., & Mila, H. (2017). Canine and feline colostrum. *Reproduction in Domestic Animals*, 52, 148-152.

Chatdarong, K., Rungsipipat, A., Axnér, E., Linde and Forsberg. C. (2005). Hysterographic appearance and uterine histology at different stages of the reproductive cycle and after progestagen treatment in the domestic cat. *Theriogenology, 64*, 12–29.

Chatdarong, K., Tummaruk, P., Sirivaid-yapong, S. and Raksil, S. (2007). Seasonal and breed effects on reproductive parameters in bitches in the tropics: a retrospective study. *Journal of Small Animal Practice*,*48*:444–448.

Chiao, R. Y. and Hao, X.(2005). Coded excitation for diagnostic ultrasound: a system developer's perspective, *IEEE Transactions on Ultrasonics, Ferroelectrics, and Frequency Control, 52*, 160–170.

Clarke, P. R. and Hill, C. R. (1970). Physical and chemical aspects of ultrasonic disruption of cells. *The Journal of the Acoustical Society of America*, *47*, 649–653.

Coltera, M. (2010): Ultrasound physics in a nutshell. *Otolaryngologic Clinics of North America*, *43*(6), 1149–1159.

Concannon, P. W. and Hansel, W. (1977). Prostaglandin F2alpha induced luteolysis, hypothermia, and abortions in beagle bitches. *Prostaglandins,13*, 533–542.

Concannon, P. W., Powers, M. E., Holder, W. and Hansel, W. (1977). Pregnancy and parturition in the bitch. *Biology of Reproduction, 16*, 517–526.

Concannon, P. W., Butler, W. R., Hansel, W., Knight, P. J., and Hamilton, J. M. (1978). Parturition and lactation in the bitch: Serum progesterone, cortisol and prolactin. *Biology of Reproduction, 19*, 1113–1118

Concannon, P. W. (1989). Induction of fertile oestrus in anoestrus dogs by constant infusion of GnRH agonist. *Journal of Reproduction and Fertility,39*, 149-160.

Concannon, P. W. (1993). Biology of gonadotrophin secretion in adult and prepubertal female dogs. *Journal of Reproduction and Fertility*, *47*(l), 3-27.

Concannon, P. W. (2000) Canine pregnancy: Predicting parturition and timing events of gestation. In P. W. Concannon, G. England, J. Verstegen III and C. Linde-Forsberg (Eds.), Recent advances in small animal reproduction. Ithaca, NY: International Veterinary Information Service. [A1202.0500 (http://www.ivis.org)].

Concannon, P. W. (2009). Endocrinologic control of normal canine ovarian function*Reproduction in Domestic Animals*, *44* (2), 3-15.

Concannon, P. W. (2011). Reproductive cycles of the domestic bitch. *AnimalReproduction Science*, *124*(3/4), 200–210.

Concannon, P. W. (2012). Research challenges in endocrine aspects of canine ovariancycles *Reproduction in Domestic Animals*, *4* (6), 6-12.

Concannon, P. W., McCann, J. P. and Temple, M. (1989). Biology and endocrinology of ovulation, pregnancy and parturition of dog. *Journal of Reproduction and Fertility*, 39, 3-25.

Concannon, P. W., Whaley, S., Lein, D. and Wissler, R. (1983). Canine gestation length: variation related to time of mating and fertile life of sperm. *American Journal of Veterinary Research*, *44*, 1819–1821.

Concannon, P., Lasley, B. and Vanderlip, S. (1997). LH release, induction of oestrus and fertile ovulations in response to pulsate administration of GnRH to anoestrous dogs. *Journal of Reproduction and Fertility*, *51*, 41-54.

Cools, S., Van den Broeck, W., De Vliegher, S., Piepers, S. and Opsomer, G. (2013). The bovine luteal histological composition: a topographic point of view.*Reproduction in Domestic Animals, 48*(2), 29-32.

Cosgrove, D. (2006). Ultrasound contrast agents: An overview. *European Journal of Radiology*, 60, 324–330.

Coussios, C. C. and Roy, R. A. (2008). Applications of acoustics and cavitation to noninvasive therapy and drug delivery. *Annual Review of Fluid Mechanics, 40*, 395–420.

Coussios, C. C., Farny, C. H., ter Haar, G., and Roy, R. A. (2007). Role of acoustic cavitation in the delivery and monitoring of cancer treatment by high-intensity focused ultrasound (HIFU). *International Journal of Hyperthermia, 23*, 105–120.

Coussios, C. C., Holland, C. K., Jakubowska, L., Huang, S. L., MacDonald, R. C., Nagaraj, A., and McPherson, D. D. (2004). *In vitro* characterization of liposomes and OptisonH by acoustic scattering at 3.5 MHz. *Ultrasound in Medicine and Biology, 30*, 181–190.

Culp, W. C., Porter, T. R., Lowery, J., Xie, F., Roberson, P. K., and Marky, L. (2004). Intracranial clot lysis with intravenous micro bubbles and transcranial ultrasound in swine. *Stroke, 35*, 2407–2411.

Curran, S. (1992). Foetal sex determination in cattle and horses by ultrasonography. *Theriogenology, 37*, 17–21.

Curran, S., Kastelic, J. P. and Ginther, O. J. (1989). Determining sex of the bovine fetus by ultrasonic assessment of the relative location of the genital tubercle. *Animal Reproduction Science, 19*, 217–227.

Corso, J. F. (1963). "Bone-conduction thresholds for sonic and ultrasonic frequencies".*Journal of the Acoustical Society of America* **35** (11): 1738–1743.

Dacie, J.V., Lewis, S.M., 1991. In: Dacie, J.V., Lewis, S.M. (Eds.), BasicHaematological Techniques Practical Haematology. , 7th Ed. ChurchillLivingstone, pp. 1–67

Dall'Aglio, C., Pascucci, L., Mercati, F., Polisca, A., Ceccarelli, P. and Boiti, C. (2012a).Immunohistochemical detection of the orexin system in the placenta of cats. *Research in Veterinary Science, 92*, 362–365.

Dall'Aglio, C., Polisca, A., Boiti, C. and Ceccarelli, P. (2012b). Immunolocalization ofleptin and its receptor in the placenta of cats. *Acta Histochemica, 114*, 719–722.

Daniel, G. (2010). Physical principles: In: *BSAVAManual of Canine and Feline Ultrasonography*. Ed. Barr. F.and Gaschen. Pp 15-16. BSAVA Publication Gloucester.

Datta, S., Coussics, C. C., Ammi, A. Y., Mast, T. D., de Court, and Holland, C. K. (2008). Ultrasoundenhanced thrombolysis using DefinityH as a cavitation nucleation agent. *Ultrasound in Medicine and Biology*, *34*, 1421–1433.

Davidson, A. P. and Baker, T. W. (2009). Reproductive ultrasound of the bitch and queen. *Topics in Companion Animal Medicine, 24*(2), 55-63.

de Gier, J., Kooistra, H. S., Djajadiningrat-Laanen, S. C., Dieleman, S. J. and Okkens A. C. (2006). Temporal relations between plasma concetrations of luteinizing hormone,follicle-stimulating hormone, estradiol-17β, progesterone, prolactin, and α-melanocyte-stimulating hormone during the follicular, ovulatory, and early luteal phase in the bitch*Theriogenology*, *65*, 1346-1359.

De los Reyes, M., Palomino, J., Parraguez, V. H. and Ramirez, F. (2017). Analysis of LH receptor in canine ovarian follicles throughout the estrous cycle. *Theriogenology,93*, 71-77.

Deepthi L. (2010). *Diagnostic and Therapeutic approaches for enhancing reproductive efficiency in female dogs.* (Doctoral Thesis). http://krishikosh.egranth.ac.in/handle/1/5810027207

Desser, T. S. and Jeffrey, R. B. (2001). Tissue harmonic imaging techniques: physicalprinciples and clinical applications. *Seminars in Ultrasound, CT, and MRI*, *22*, 1–10.

Devillers, N., J. Le Dividich, and A. Prunier. (2011). Influence of colostrum intake on piglet survival and immunity. Animal 5: pp1605–1612.

Dom SM, Razak HR, Zaiki FW, Saat NH, Manan KA,Isa IN & Hashim UF (2013). Ultrasoundexposure during pregnancy affects rabbitfoetal parathyroid hormone (PTH) level.Quantitative Imaging in Medicine andSurgery, 3(1): 49-53.

Douglass, J. P. (1993). Bladder wall mass effect caused by the intramural portion of the canine ureter. *Veterinary Radiology and Ultrasound*, *34*(2), 107.

Douglass, J. P. and Kremkau, F. W. (1993). Ultrasound corner—the urinary bladder wall hypoechoic pseudolesion. *Veterinary Radiology and Ultrasound*, *34*(1), 45–46.

Ducker, M. J., Thwaites, C. J. and Bowman, J. C. (1970). Photoperiodism in the ewe: 2. The effects of various patterns of decreasing daylength on the onset of oestrus in clun forest ewes. *Animal Production, 12*, 115–123.

EKER. Kemal, SALMANOLU, Mehmet R›fat. (2006). Ultrasonographic Monitoring of Follicular Development, Ovulation and Corpora lutea Formation in a Bitch. *Turk. J. Vet. Anim. Sci*. 30 589-592

Elder, S. A., Kolb, J., and Nyborg, W. L. (1954). Smallscale acoustic streaming effects in liquids. *The Journal of the Acoustical Society of America*, *26*, 933–933.

Elder, S. and Nyborg, W. L. (1956). Acoustic streaming resulting from a resonant bubble. *The Journal of the Acoustical Society of America*, *28*, 155–155.

Elejalde, B. R., de Elejalde, M. M. and Heitman, T. (1985). Visualization of the foetal genitália by ultrasonography: a review of the literature and analysis of its accuracy and ethical implications. *Journal of Ultrasound in Medicine*, *4*, 633–639.

Emoto, M., Tachibana, K., Iwasaki, H., and Kawarabayashi, T. (2007). Antitumor effect of TNP- 470, an angiogenesis inhibitor, combined with ultrasound irradiation for human uterine sarcoma xenografts evaluated using contrast color Doppler ultrasound. *Cancer Science*, *98*, 929–935.

Emoto, M., Tamura, R., Shirota, K., Hachisuga, T. and Kawarabayashi, T. (2002). Clinical usefulness of color Doppler ultrasound in patients with endometrial hiperplasia and carcinoma. *Cancer*, *94*, 700–706.

England, G. C. W., Allen, W. E., and Porter, D. J. (1990). Studies on canine pregnancy using B mode ultrasound: Development of the conceptus and determination of gestational age. *Journal of Small Animal Practice*, *31*, 324–329.

England G.C.W., Yeager AE (1993). Ultrasonographic appearance of the ovary and uterus of the bitch during oestrus, ovulation and early pregnancy. *Journal of Reproduction and Fertility (Suppl)*47: pp107–117. [PubMed]

England, G., Schäfer-Somi, S., Aurich J., Concannon, P. (Eds.), 2009. Canine and feline reproduction VI: reproductive biology and medicine of domestic and exotic Carnivores. ISSN 0936-6768. Reprod. Domest. Anim. 44 (Suppl. 2), 1–380.

England, G. C. W., Moxon, R. and Freeman, S. L. (2012). Delayed uterine fluid clearance and reduced uterine perfusion in bitches with endometrial hyperplasia and clinical management with post mating antibiotic. *Theriogenology*, *78*, 1611–1617.

England, G. C., Russo, M. and Freeman, S. L. (2009). Follicular dynamics, ovulation and conception rates in bitches. *Reproduction in Domestic Animals*, *44*(2), 53-58.

Evans, H. E. and Lahunta, A. (2013). Prenatal development. In: Evans HE, Lahunta A, editors. Miller's anatomy of the dog. Missouri: Elsevier Saunders. 13–60.

Fang, L., Nowicki, B. J., Dong, Y. L. and Yallampalli, C. (1999). Localized increase in nitric oxide production and the expression on nitric oxide syntethase isoforms in rat uterus with experimental intrauterine infection. *American Journal of Obstetrics and Gynecology*, *181*, 601–609.

Feigenbaum, H., Stone, J. M., Lee, D. A., Nasser, W. K., and Chang, S. (1970). Identification of ultrasound echoes from left ventricle by use of intracardiac injections of indocyanine green. *Circulation, 41,* 615–621.

Feldman E. C. and Nelson, R. W. (2004). Canine and feline endocrinology and reproduction. 3rd edn. USA: Saunders.

Feldman, M. K., Katyal, S. and Blackwood, M. S. (2009). Ultrasound artefacts, *Radiographics, 29*, 1179-1187.

Ferguson, J. M., Renton, J. P., Farstad, W. and Douglas, T. A. (1989). Insemination of beagle bitches with frozen semen. *Journal of Reproduction and Fertility*. *39*, 293-298.

Fernandes, P. A., Bowen, R. A., Kostas, A. C., Sawyer, H. R., Nett, T. M. and Olson, P. N. (1987). Luteal function in the bitch: changes during diestrus in pituitary concentration of and the number of luteal receptors for luteinizing hormone and prolactin. *Biology of Reproduction*, *37*, 804-811.

Ferreira, J. C., Gastal, E. L. and Ginther, O. J. (2008). Uterine blood flow and perfusion in mares with uterine cysts: effect of the size of the cystic area and age. *Reproduction, 135*(4), 541-550.

Ferreira, J. C., Ignácio, F. S. and Meira, C. (2010). Uterine vascular perfusion and spectral-Doppler measurements during early gestation in mares: New concepts of evaluation. *Animal Reproduction Science, 121*(S), 281-283.

Ferreira, J. C., Ignácio, F. S. and Meira, C. (2011). Doppler ultrasonography principles and methods of evaluation of the reproductive tract in mares. *Acta Scientiae Veterinariae, 39*(1), s105 - s111. ISSN 1679-9216 (Online)

Ferretti, L. M., Newell, S. M., Graham, J. P. and Roberts, G. D. (2000). Radiographic and ultrasonographic evaluation of the normal feline postpartum uterus. *Veterinary Radiology and Ultrasound*, *41*, 287–291.

Fielder, F. D. and Baker, R. F. (1969). Diagnostic applications of Doppler ultrasound. *Ultrasonics*, *7*, 36–38.

Fitz, T. A., Mayan, M. H., Sawyer, H. R. and Niswender, G. D. (1982). Characterization of two steroidogenic cell types in the ovine corpus luteum. *Biology of Reproduction, 27*(3), 703-711.

Fontbonne A, Malandain E (2006). Ovarian ultrasonography and follow up of estrus in the bitch and queen. Waltham Focus;16:22–29.

Fontbonne, A. (2010). Infertility in bitches and queens: recent advances. *Revista brasileira de reprodução animal,35*, 202-209.

Forcada, F., Abecia, J. A. and Sierra, I. (1992). Seasonal changes in oestrus activity and ovulation rate in Rasa Aragonesa ewes maintained at two different body condition levels. *Small Ruminant Research, 8*, 313–324.

Freeman, S. L., Russo, M. and England, G. C. (2013). Uterine artery blood flow characteristics assessed during oestrus and the early luteal phase of pregnant and non-pregnant bitches. *The Veterinary Journa*l, *197*, 205–210.

Fukuta, N., Kitano, M., Maekawa, K., Chikugo, T. and Kudo, M. (2005). Estimation of the malignant potential of gastrointestinal stromal tumors: The value of contrast-enhanced coded phase inversion harmonic US. *Journal of Gastroenterology, 40*, 247–255.

Fuller, J. L. (1956). Photoperiodic control of estrus in the Basenji. *Journal of Heredity,47*, 179–180.

Garcia, D. A. A., Rompkovski, M. R., Gil, E. M. U., Giannico, A. T. and Froes, T. R.(2015). Accuracy of abdominal radiography to determine fetus numbers in bitches at two gestational ages. Semina: Ciências Agrárias, *Londrina*, *36*(6), 3769-3776.

Geiser, B., Burfeind, O., Heuwieser, W. and Arlt, S. (2014). Prediction of parturition in bitches utilizing continuous vaginal temperature measurement. *Reproduction in Domestic Animals,49*, 109–114.

Giannico, A. T., Gil, E. M., Garcia, D. A., and Froes, T. R. (2015). The use of Doppler evaluation of the canine umbilical artery in prediction of delivery time and foetal distress. *Animal Reproduction Science*, *154*, 105–112.

Gil, E. M. U., Garcia, D. A. A., Giannico, A. T. and Froes, T. R. (2015). Use of B-mode ultrasonography for foetal sex determination in dogs. *Theriogenology,84*, 875–879

Gil, E. M., Garcia, D. A., Giannico, A. T., and Froes, T. R. (2014). Canine foetal heart rate: Do accelerations or decelerations predict the parturition day in bitches? *Theriogenology, 82*, pp 933-941

Ginther, O. J. (2007). Ultrasonic Imaging and Animal Reproduction: Color-Doppler Ultrasonography, Ginther O.J. (ed). Cross Plains: Equiservices Publishing, 258p.

Ginther, O. J. and Utt, M. D. (2004). Doppler Ultrasound in Equine Reproduction: Principles, Techniques, and Potential. *Journal of Equine Veterinary Science, 24*, 516-526.

Ginther, O. J., Gastal, E. L., Gastal, M. O., Utt, M. D. and Beg, M. A. (2007). Luteal blood flow and progesterone production in mares. *Animal Reproduction Science, 99*(1-2), 213-220.

Ginther, O. J., Rodrigues, B. L., Ferreira, J. C., Araujo, R. R. and Beg, M. A. (2008). Characterization of pulses of 13, 14-dihydro- 15-keto-PGF2alpha (PGFM) and relationships between PGFM pulses and luteal blood flow before, during, and after luteolysis in mares. *Reproduction, Fertility and Development, 20*(6), 684-693.

Gniadecka, M. and Quistorff, B. (1996). Assessment of dermal water by high-frequency ultrasound: comparative studies with nuclear magnetic resonance. *British Journal of Dermatology,135*:218-224.

Gobello, C. (2006). Dopamine agonists, anti-progestins, anti-androgens, long-term-release GnRH agonists and anti-estrogens in canine reproduction: A review. Theriogenology,66(6-7), 1560-1567.

Gradial, C. M., Yeager, A. E. and Concannon, P. W. (2000). Pregnancy diagnosis in the bitch. In: Bonagura, J. D. *Kirk's Current Veterinary Therapy XIII Small Animal Practice*. Philadelphia: W. B. Saunders Company. Pp 918-923.

Gramiak, R. and Shah, P. M. (1968). Echocardiography of the aortic root. *Investigative Radiology, 3*, 356–366.

Groppetti, D., Aralla, M., Bronzo, V., Bosi, G., Pecile, A. and Arrighi, S. (2015a). Periovulatory time in the bitch: What's new to know?: Comparison between ovarian histology and clinical features. *Animal Reproduction Science*, *152*, 108-116.

Groppetti, D., Pecile, A., Arrighi, S., Di Giancamillo, A. and Cremonesi, F. (2010). Endometrial cytology and computerized morphometric analysis of epithelial nuclei: a useful tool for reproductive diagnosis in the bitch. *Theriogenology, 73*, 927-941.

Groppetti, D., Vegetti, F., Bronzo, V. and Pecile, A., (2015b). Breed-specific foetal biometry and factors affecting the prediction of whelping date in the German shepherd dog. Animal *Reproduction Science,152*, 117–122.

Grundy, S. A. and Davidson, A. P. (2004). Theriogenology question of the month. Acute metritis secondary to retained foetal membranes and a retained nonviable foetus. *American Veterinary Medical Association,224*, 844-847.

Gudermann, T., Birnbaumer, M. and Birnbaumer, L. (1992). Evidence for dual coupling of the murine luteinizing hormone receptor to adenylyl cyclase and phosphoinositide breakdown and Ca^{2}+ mobilization. Studies with the cloned murine luteinizing hormone receptor expressed in L cells. *Journal of Biological Chemistry, 267*, 4479-4488.

Gunn, R. G. and Doney, J. M. (1979). Fertility in Cheviot ewes. 1. The effect of body condition at mating on ovulation rate and early embryo mortality in North and South Country Cheviot ewes. *Animal Production, 29*, 11–16.

Hamilton, A., Rabbat, M., Jain, P., Belkind, N., Huang, S. L., Nagaraj, A., Klegerman, M., Mac-Donald, R., and McPherson, D. D. (2002). A physiologic flow chamber model to define intravascular ultrasound enhancement of fibrin using echogenic liposomes. *Investigative Radiology, 37,* 215–221.

Hamilton, M. F. and Blackstock, D. T. (1998). Nonlinear Acoustics, Academic press, San Diego, C. A., 25–39.

Haney, D. R., Levy, J. K., Newell, S. M., Graham, J. P., and Gorman, S. P. (2003). Use of foetal skeletal mineralization for prediction of parturition date in cats. *Journal of the American Veterinary Medical Association*, *223*, 1614–1616

Hangiandreou, N. J. (2003). AAPM/RSNA physics tutorial for residents. Topics in US: B-mode US: basic concepts and new technology, *Radiographics, 23,* 1019-1033.

Hariharan, P., Myers, M. R., and Banerjee, R. K. (2007). HIFU procedures at moderate intensities – effect of large blood vessels. *Physics in Medicine and Biology*, *52*, 3493–3513.

Hase, M., Hori, T., Kawakami, E., and Tsutsui, T. (2000). Plasma LH and progesterone levels before and after ovulation and observation of ovarian follicles by ultrasonographic diagnosis system in dogs. *Journal of Veterinary Medical Science*, *62*, 243–248.

Hayer, P., Günzel-Apel, A. R., Lüerssen, D., and Hoppen, H. O. (1993). Ultrasonographic monitoring of follicular development, ovulation and the early luteal phase in the bitch. *Journal of Reproduction and Fertility, 47*, 93–100.

Heap, R. B. and Poyster, N. L. (1975). Prostaglandins in pyometrial fluid from the cow, bitch and ferret. *British Journal of Pharmacology, 55*, 515–518.

Heap, W. (1900). The "sexual season" of mammals and the relation of the "proestrus" to menstruation. *Quarterly Journal of Microbiological Science*, *44*(1), 61-70.

Hecht, S. (2015). Diagnostic Imaging of Lower Urinary Tract Disease. *Veterinary Clinics in Small Animal,45,* 639–663.

Heng, H. G. and Widmer, W. R. (2010). Appearance of common ultrasound artefacts in conventional vs. spatial compound imaging, *Veterinary Radiology of Ultrasound, 51*,615-621.

Heppelmann, M., Weinert, M., Brömmling, A., Piechotta, M., Hoedemaker, M. and Bollwein, H. (2013). The effect of puerperal uterine disease on uterine involution in cows assessed by Doppler sonography of the uterine arteries. *Animal Reproduction Science*, *143,* 1–7.

Hilgenfeldt, S., Lohse, D., and Zomack, M. (2000). Sound scattering and localized heat deposition of pulsedriven micro bubbles. *The Journal of the Acoustical Society of America*, *107*, 3530–3539.

Hill, C. R., Bamber, J., and ter Haar, G. (Eds) (2004). Physical principles of medical ultrasound, ch. 3 (Wiley, Chichester).

Hindi, A., Paterson, C. and Barr, R. G. (2013). Artefacts in diagnostic ultrasound. *Reports in Medical Imaging*,*6*, 29–48.

Hirooka, Y., Naitoh, Y., Goto, H., Ito, A., Taki, T. and Hayakawa, T. (1997). Usefulness of contrast-enhanced endoscopic ultrasonography with intravenous injection of sonicated serum albumin. *Gastrointestinal Endoscopy*,*46*, 166–169.

Hirt, R. A., Kneissl, S. and Teinfalt, M. (2000). Severe hypercalcemia in a dog with a retained fetus and endometritis. *Journal of the American Veterinary Medical Association*, *216*, 1423–1425.

Hohl, C., Schmidt, T., Haage, P., Honnef, D., Blaum, M., Staatz, G. and Guenther, R. W. (2004). Phase-inversion tissue harmonic imaging compared with conventional Bmode ultrasound in the evaluation of pancreatic lesions. *European Radiology*,*14*, 1109–1117.

Holder, R. D. (2003). Foetal sex determination. *Journal of Equine Veterinary Science*, *23*, 414-419.

Holst, P. A. and Phemister, R. D. (1971). The prenatal development of the dog. Preimplantation events. *Biology of Reproduction*, *5*, 771-779.

Hornbuckle, W. E. and Kleine, L. J. (1980). Medical management of prostatic disease. In: Kirk K.W, editor. Current Veterinary Therapy: Small Animal Practice. VII. Philadelphia, PA: W.B. Saunders Co; 1146–1150.

Hou, X., Arvisais, E. W. and Davis, J. S. (2010). Luteinizing hormone stimulates mammalian target of rapamycin signaling in bovine luteal cells via pathways independent of AKT and mitogen-activated protein kinase: modulation of glycogen synthase kinase 3 and AMP-activated protein kinase. *Endocrinology*, *151*, 2846-2857.

Huang, S. L. (2008). Liposomes in ultrasonic drug and gene delivery. *Advanced Drug Delivery Reviews*, *60*, 1167–1176.

Hwang, J. and Simpson, D. H. (1999). Two pulse technique for ultrasonic harmonic imaging, US Patent No. 5, *951*,478.

Idris, S. Y., Audu, H. A., Fadason, S. T., Lawal, M., Ibinaiye, P. O., Muazu, B. N., & Okafor, R. (2017). Effect of multiple in-utero insonation on rabbit foetal thyroid hormonal level. Sokoto Journal of Veterinary Sciences, 15(4), 94-98.

Indrebø, A., C. Trangerud, and L. Moe. (2007). Canine neonatal mortality in four large breeds. Acta Vet. Scand. 49(Suppl. 1):S2. doi:10.1186/1751-0147-49-S1-S2

Inoue, T., Kitano, M., Kudo, M., Sakamoto, H., Kawasaki, T., Yasuda, C. and Maekawa, K. (2007). Diagnosis of gallbladder diseases by contrast-enhanced phase-inversion harmonic ultrasonography. *Ultrasound in Medicine and Biology,33,* 353–361.

Isa, I. N. C., & Dom, S. M. (2016). Evaluation of the thermal effects of prenatal ultrasound on hematological analysis of young Oryctolagus Cuniculus. Journal of Veterinary Medical Science, 78(9), 1399-1403.

Iwanaga, K., Tominaga, K., Yamamoto, K., Habu, M., Maeda, H., Akifusa, S., Tsujisawa, T., Okinaga, T., Fukuda, J., and Nishihara, T. (2007). Local delivery system of cytotoxic agents to tumors by focused sonoporation. *Physics in Medicine and Biology*, *14*, 354–363.

Jauhiainen, J. (2009). The Physical Principles of Medical Imaging: Sprawls Educational Foundation. *Tekniikan yksikkö Hyvinvointiteknologia.* 10-11. Retrieved from www.sprawls.org.

Jeffcoate, I. A. (1993). Endocrinology of anestrous bitches. *Journal of Reproduction and Fertility,47*, 69-76.

Jemec, G. B., Gniadecka, M. and Ulrich, J. (2000). Ultrasound in dermatology. Part I. High frequency ultrasound. *European Journal of Dermatology,10,* 492-497.

Jeppesen, J. V., Kristensen, S. G., Nielsen, M. E., Peter, H., Dal Canto, M., Fadini, R., Schmidt, K. T., Ernst, E. and Yding, A. C. (2012). LH-receptor gene expression in human granulosa and cumulus cells from antral and preovulatory follicles. *The Journal of Clinical Endocrinology and Metabolism,97*, E1524-1531.

Jiang, P., Mao, Z. and Lazenby, J. C. (1998). A new tissue harmonic imaging scheme with better fundamental frequency cancellation and higher signal-to-noise ratio, in: *Proceedings of IEEE Ultrasononic Symposium*, Sendai, Japan, pp. 1589–1594.

Jöchle, W., Arbeiter, K., Post, K., Ballabio, R. and D'Ver, A. S. (1989). Effects on pseudopregnancy, pregnancy and interoestrous intervals of pharmacological suppression of prolactin secretion in female dogs and cats. *Journal of Reproduction and Fertility,39*, 199-207,

Johnson, A. E., Freeman, E. W., Colgin, M., McDonough, C. and Songsasen, N. (2014). Induction of ovarian activity and ovulation in an inducedovulator, the maned wolf (*Chrysocyon brachyurus*), using GnRH agonist and recombinant LH. *Theriogenology*, *82*, 71–79.

Johnston, S. D., Root Kustritz, M. V. and Olson, P. N. S. (2001a). The canine estrous cycle Canine and Feline Theriogenology, W.B. Saunders Co, Philadelphia, PA, 16-31

Johnston, S. D., Root Kustritz, M. V. and Olson, P. N. S. (2001b). Canine parturition – eutocia and dystocia. In S. D. Johnston, M. V. RootKustritz, and P. N. S. Olson (Eds.), Canine and feline theriogenology (105–128). Philadelphia: WB Saunders.

Jones, G. E., Boyns, E. T., Bell, D. W., Christie, D. W. and Parkes, M. F. (1973). Immunoreactive luteinizing hormone and progesterone during pregnancy and following gonadotropin administration in beagle bitches. *Acta Endocrinologica,72*, 573-581.

Joone, C. J., De Cramer, K. and Nöthling, J. O. (2016). The first case of genetically confirmed monozygotic twinning in the dog. *Reproduction in domestic animals = Zuchthygiene*, *51*, 835-839.

Juffermans, L. J. M., Kamp, O., Dijkmans, P. A., Visser, C. A., and Musters, R. J. P. (2008).Low-intensity ultrasound-exposed micro bubbles provoke local hyperpolarization of the cell membrane via activation of BKCa channels. *Ultrasound in Medicine and Biology*, *34*, 502–508.

Kang BK, Choi HS, Son CH, Shin CR, Seo DH, Park IC (1997). Ultrasonographic appearance of the gestational structures throughout pregnancy in pet dogs. I. Time of initial detection of the foetal and extra-foetal structures. *Korean Journal of Veterinary Clinical Medicine* 14: 279–286.

Kato, T., Tsukamoto, Y., Naitoh, Y., Hirooka, Y., Furukawa, T., Hayakawa, T. (1995). Ultrasonographic and endoscopic ultrasonographic angiography in pancreatic mass lesions. *Acta Radiologica*, *36*, 381–387.

Kersting, S., Konopke, R., Kersting, F., Volk, A., Distler, M., Bergert, H., Saeger, H.D., Grützmann, R. and Bunk, A. (2009). Quantitative perfusion analysis of transabdominal contrast-enhanced ultrasonography of pancreatic masses and carcinomas. *Gastroenterology,137*(6), 1903–1911.

Kim, B. S., & Son, C. H. (2007). Time of initial detection of foetal and extra-foetal structures by ultrasonographic examination in Miniature Schnauzer bitches. Journal of veterinary science, 8(3), 289-293.

Kim, D. H., Klibanov, A. L., and Needham, D. (2000a). The influence of tiered layersof surface-grafted poly- (ethylene glycol) on receptor-ligand-mediated adhesion between phospholipid monolayer-stabilized micro bubbles and coated class beads. *Langmuir*, *16*, 2808–2817.

Kim, D. Y., Lee, J. C., Kwon, S. J. and Song, T. K. (2000b). Ultrasound secondharmonic imaging with a weighted chirp signal, in: *Proceedings of IEEE Ultrasonic Symposium*, San Juan, Puerto Rico, pp. 1689–1694.

Kim, Y. H., Travis, A. J. and Meyers-Wallen, V. N. (2007). Parturition prediction andtiming of canine pregnancy. *Theriogenology*,*68*, 1177–1182.

Kirberger, R. M. (1995). Imaging artefacts in diagnostic ultrasound—a review. *Veterinary Radiology and Ultrasound*, *36*(4), 297–306.

Kitano, M., Kudo, M., Maekawa, K., Suetomi, Y., Sakamoto, H., Fukuta, N., Nakaoka, R. and Kawasaki, T. (2004). Dynamic imaging of pancreatic diseases by contrast enhanced coded phase inversion harmonic ultrasonography. *Gut*,*53*, 854–859.

Kitano, M., Kudo, M., Sakamoto, H., Nakatani, T., Maekawa, K., Mizuguchi, N., Ito, Y., Miki, M., Matsui, U. and von Schrenck, T. (2008a). Preliminary study of contrast-enhanced harmonic endosonography with second-generation contrast agents. *Journal of Medical Ultrasound*,*35*(1), 11–18.

Kitano, M., Sakamoto, H., Komaki, T. and Kudo, M. (2011). New techniques and future perspective of EUS for theDifferential diagnosis of pancreatic malignancies:Contrast harmonic imaging. *Digestive Endoscopy*,*23*(1), 46–50.

Kitano, M., Sakamoto, H., Matsui, U., Ito, Y., Maekawa, K., von Schrenck, T. and Kudo, M. (2008b). A novel perfusion imaging technique of the pancreas: Contrast-enhanced harmonic EUS (with video). *Gastrointestinal Endoscopy*,*67*, 141–150.

Ko JS, Kim BS, Lee SA, Cho YT, Kim JP, Oh KS, Kim SH…… SonCH (2004). Ultrasonographic apperance of the gestational structures throughout pregnancy in Shih-tzu bitches. I. Time of initial detection of the fetal and extra-fetal structures. *Korean Journal of Veterinary Clinics*. 21: 29–34.

Kooistra, H. S., Okkens, A. C., Bevers, M. M., Popp-Snijders, C., van Haaften, B., Dieleman, S. J. and Schoemaker, J. (1999). Concurrent pulsatile secretion of luteinizing hormone and follicle-stimulating hormone during different phases of the estrous cycle and anestrus in beagle bitches. *Biology of Reproduction*, *60*, 65-71.

Kornbluth, M. (1998). Native tissue harmonic imaging improves endocardial border definition and visualization of cardiac structures. *Journal of the American Society of Echocardiography*,*11*, 693–701.

Kremkau, F. W. (1994). Diagnostic Ultrasound: Principles and Instruments (4th edn*). British Journal of Radiology*, 67(799), 746-746.

Kremkau, F. W. (2011). Artefacts. In Kremkau FW, editor: *Sonography:Principles And Instruments*, 8th Ed., St. Louis, Elsevier Saunders, 171-176.

Kremkau, F. W., Gramiak, R., Carstens, E. L., Shah, P. M., and Kramer, D. H. (1970). Ultrasonic detection of cavitation at catheter tips. *The American Journal of Roentgenology Radium Therapy and Nuclear Medicine,*, *110*, 177–183

Krueger, L., Koerte, J., Tsousis, G., Herzog, K., Flachowsky, G. and Bollwein, H. (2009). Transrectal Doppler sonography of uterine blood flow during the first 12 weeks after parturition in healthy dairy cows. *Animal Reproduction Science*, *114*, 23–31.

Kudo, M. (2003). Various contrast-enhanced imaging modes after administration of levovist. In: Kudo, M. (ed.). *Contrast HarmonicImaging in the Diagnosis and Treatment of HepaticTumors*. Tokyo: Springer, 22–30.

Kumar, P. and Sait, S. F. (2011). Luteinizing hormone and its dilemma in ovulation induction. *Journal of Human Reproductive Sciences*, *4*, 2-7.

Kumari, S., Luthra, R. A., Chandolia, R. K.,Kumar, S., Kumar, P., Devender, Kumar, A., Bishnoi, N. and Bishnoi, S.(2016). Study on development of accessory sex glands in prepubertal kids using two-dimensional ultrasonography. *Vet World*, *9*(4), 346–350.

Kutzler, M. A. (2005). Induction and synchronization of estrus in dogs. *Theriogenology64*(3), 766-775.

Kutzler, M. A. (2007). Estrus induction and synchronization in canids and felids. *Theriogenology,68*(3), 354-374.

Kutzler, M. A., Mohammed, H. O., Lamb, S. V. and Meyers-Wallen, V. N. (2003a). Accuracy of canine parturition date prediction from the initial rise in preovulatory progesterone concentration. *Theriogenology,60*, 1187–1196.

Kutzler, M. A., Wheeler, R. and Volkmann, D. H. (2001). Serum deslorelin concentrations in bitches after Ovuplant administration. *Proceedings of the Annual Meeting of the Society ofTheriogenology,13*.

Kutzler, M. A., Wheeler, R., Lamb, S., & Volkmann, D. H. (2002, May). Deslorelin implant administration beneath the vulvar mucosa for the induction of synchronous estrus in bitches. In Proceedings of Third EVSSAR European Congress (p. 96).

Kutzler, M. A., Yeager, A. E., Mohammed, H. O., and Meyers-Wallen, V. N. (2003b). Accuracy of canine parturition date prediction using foetal measurements obtained by ultrasonography. *Theriogenology*, *60*, 1309–1317

Lamm, C. G. and Makloski, C. L. (2012). Current advances in gestation and parturition in cats and dogs. *Veterinary Clinics of North America: Small Animal Practice,42*, 445–456.

Lawler, D. F. 2008. Neonatal and pediatric care of the puppy and kitten. Theriogenology 70: 384–392. doi:10.1016/j.theriogenology. 2008.04.019

Legan, S. J. and Karsch, F. J. (1980). Photoperiodic control of seasonal breeding in ewes: modulation of the negative feedback action of estradiol. *Biology of Reproduction, 23*, 1061–1068.

Lentacker, I., De Geest, B. G., Vandenbroucke, R. E., Peeters, L., Demeester, J., De Smedt, S. C., and Sanders, N. N. (2006). Ultrasound-responsive polymer- coated micro bubbles that bind and protect DNA. *Langmuir, 22*, 7273–7278.

Leong-Poi, H., Christiansen, J., Klibanov, A. L., Kaul, S., and Lindner, J. R. (2003). Noninvasive assessment of angiogenesis by ultrasound and micro bubbles targeted to alpha(v)-integrins. *Circulation, 107*, 455–460.

Leonhardt, H., Gull, B., Stener-Victorin, E. and Hellstrom, M. (2014). Ovarian volume and antral follicle count assessed by MRI and transvaginal ultrasonography: a methodological study. *Acta Radiologica, 55*, 248-256.

Levine, H. III. (2010): *Medical Imaging*. Santa Barbara, California: ABC-CLIO, LLC. 62.

Levy X, Fontbonne A. Determining the optimal time of mating in bitches: particularities. Rev Bras Reprod Anim 2007;31:128–134.

Lighthill, J. (1978). Acoustic streaming. *Journal of Sound and Vibration, 61*, 391–418.

Linde-Forsberg, C. and Wallén, A. (1992). Effects of whelping and season of the year on the interoestrous intervals in dogs. *Journal of Small Animal Practices, 33*, 67–70.

Linde-Forsberg, C., & Persson, G. (2007). Biology of reproduction of the dog and modern reproductive technology. The Genetics of the Dog. USA: CABI Publishing. 401-432.

Lindner, J. R. (2002). Detection of inflamed plaques with contrast ultrasound. *American Journal of Cardiology, 90*, 32L–35L.

Lindner, J. R., Song, J., Xu, F., Klibanov, A. L., Singbartl, K., Ley, K., and Kaul, S. (2000). Noninvasive ultrasound imaging of inflammation using micro bubbles targeted to activated leukocytes. *Circulation, 102*, 2745–2750.

Liu, Y. B., Kon, T., Li, C. Y., and Zhong, P. (2006). High intensity focused ultrasound-induced gene activation in solid tumours. *The Journal of the Acoustical Society of America*, *120*, 492–501.

Lopate, C. (2008). Estimation of gestational age and assessment of canine foetal maturation using radiology and ultrasonography: a review. *Theriogenology*, *70*(3), 397-402.

Louvet, A. (2006). Twinkling artefact in small animal color-Doppler sonography. *Veterinary Radiology and Ultrasound*, *47*(4), 384–390.

Lu, E., Tom, E. M., Felix, M. M., Gretton, J., Varghese, R. P., Wagner, W. R., & Villanueva, F. S. (2004). 1001-33 In vivo microtubule binding to inflammatory endothelium via selectin targeting by Sialyl Lewis X. Journal of the American College of Cardiology, 5(43), A8.

Luvoni, G. C., and Beccaglia, M. (2006). The prediction of parturition date in canine pregnancy. *Reproduction in Domestic Animals*, *41*, 27–32

Luvoni, G. C., and Grioni, A. (2000). Determination of gestational age in medium and small size bitches using ultrasonographic foetal measurements. *Journal of Small Animal Practice*, *41*, 292-294.

Luvoni, G. C., Chigioni, S., Allievi, E. and Macis, D. (2005). Factors involved *in vivo* and in vitro maturation of canine oocytes. *Theriogenology*, *63*, 41-59.

MacLeod, A. G. and Wisner, E. R. (2011). Computed tomography and magnetic resonance imaging of the urinary tract. In: Bartges J, Polzin DJ, editors. Nephrology and urology of small animals. Ames (IA): Wiley-Blackwell, 146–160.

Maeda, M., Takashima, S., Takasu, M., Mori, T., Goto, N., Matsubara, T., Almunia, J., Imaeda, N., Ando, A. and Kitagawa, H. (2016). Magnetic Resonance Imaging of Ovarian Activity in Microminipigs Showing Normal Estrous Cycles. *In vivo*, *30*, 35-40.

Magata, F., Hartmann, D., Ishii, M., Miura, R., Takahashi, H., Matsui, M., Kida, K., Miyamoto, A. and Bollwein, H. (2013). Effects of exogenous oxytocin on uterine blood flow in puerperal dairy cows: the impact of days after parturition and retained foetal membranes. *The Veterinary Journal*, *196*, 76–80.

Mahi, C. A. and Yanagimachi, R. (1976). Maturation of Sperm penetration of canine ovarian oocyte *in vitro*. *Journal of Experimental Zoology*, 196, 189-196.

Mantis, P. (2008). Ultrasonography of the urinary and genital system of the dog and cat. *Iranian Journal of Veterinary Surgery* Supplement for the 2nd ISVS and 7th ISVSAR, 63-71.

Mar, G., Castagnetti, C. and Belluzzi, S. (2002). Equine foetal sex determination using a single ultrasonic examination under farm conditions. *Theriogenology*, *58*, 1237–43.

Marmottant, P. and Hilgenfeldt, S. (2003). Controlled vesicle deformation and lysis by single oscillating bubbles. *Nature*, *423*, 153–156.

Marmottant, P., Biben, T., and Hilgenfeldt, S. (2008). Deformation and rupture of lipid vesicles in the strong shear flow generated by ultrasound-driven micro bubbles. *Proceedings of the Royal Society A: Mathematical, Physical and Engineering Science*, *464*, 1781–1800.

Mattoon, J. S. and Nyland, T. G. (2014). Small Animal Diagnostic Ultrasound. Saunders Elsevier, Missouri.

McDougall, K., Hay, M. A., Goodrowe, K. L., Gartley, C. J. and King, W. A. (1997). Changes in the number of follicles and of oocytes in ovaries of prepubertal, peripubertal and mature bitches. *Journal of Reproduction and Fertility*, *51*, 25-31.

McLean, S., Brandon, S. and Kirkwood, R. (2007). Stability of cabergoline in fox baits in laboratory and field conditions. *Wildlife Research*, *34*, 239- 246.

Meagher, S. and Davison, G. (1996). Early second-trimester determination of foetal gender by ultrasound. *Ultrasound in Obstetrics and Gynecology*, 8:322–324.

Meairs, S. and Alonso, A. (2007). Ultrasound, micro bubbles and the blood–brain barrier. *Progress in Biophysics and Molecular Biology*, *93*, 354–362.

Meinecke-Tillmann, S.(2016). Basicsof ultrasonographic examination in sheep. Small Ruminant Research, 152, 10-21.

Menon, K. M. J., Clouser, C. L. and Nair, A. K. (2005). Gonadotropin receptors: role of posttranslational modifications and post-transcriptional regulation. *Endocrine*, 26, 249-257.

Menon, K. M., Munshi, U. M., Clouser, C. L. and Nair, A. K. (2004). Regulation of luteinizing hormone/ human chorionic gonadotropin receptor expression: a perspective. *Biology of Reproduction*, *70*, 861-866.

Metoki, R., Moriyasu, F., Kamiyama, N., Sugimoto, K., Iijima, H., Xu, H. X., Aoki, T., Miyata, Y., Yamamoto, K., Kudo, K., Shimizu, M., and Yamada, M. (2006). Quantification of hepatic parenchymal blood flow by contrast ultrasonography with flash-replenishment imaging. *Ultrasound in Medicine and Biology*, *32*, 1459–1466.

Meyer-Lindenberg, A., Ebermaier, C., Wolvekamp, P., Tellhelm, B., Meutstege, F. J., Lang, J., Hartung, K., Fehr, M. and Nolte, I. (2008). Comparative evaluation of six different body regions of the dog using analog and digital radiography. *Berliner Und Munchener Tierarztliche Wochenschrift*, *121*(5-6), 216-227.

Michel, E., Spörri, M., Ohlerth, S. and Reichler, I. M. (2011). Prediction of parturition date in the bitch and queen. *Reproduction in Domestic Animals*, *46*, 926-932.

Michiyo Yoshioka, Ken-ichi Tanaka, Ikuko Miyazaki, Naoko Fujita, Youichirou Higashi, Masato Asanuma, Norio Ogawa. (2002). The dopamine agonist cabergoline provides neuroprotection by activation of the glutathione system and scavenging free radicals.*Neuroscience Research*. 43. 259-267

Mila, H., Grellet, A., Feugier, A., & Chastant-Maillard, S. (2015). Differential impact of birth weight and early growth on neonatal mortality in puppies. *Journal of animal science*,93(9), 4436-4442.

Molina, C. A., Ribo, M., Rubiera, M., Montaner, J., Santamarina, E., Gado-Mederos, R., Arenillas, J. F., Huertas, R., Purroy, F., Delgado, P., and Varez-Sabin, J. (2006). Microbubble administration accelerates clot lysis during continuous 2-MHz ultrasoundmonitoring in stroke patients treated with intravenous tissue plasminogen activator. *Stroke, 37,* 425–429.

Moller, O. M. (1973). Progesterone concentrations in the peripheralplasma of the blue fox (*Alopex lagopus*) during pregnancy and the oestrous cycle. *Journal of Endocrinology,59*, 429–438.

Mondain-Monval, M., Dutourne, B., Bonnin-Laffargue, M., Canivenc, R. and Scholler, R. (1977). Ovarian activity during the anoestrus and thereproductive season of the red fox (*Vulpes vulpes* L.). *Journal of Steroid Biochemistry,8*, 761–769.

Mortensen, C. J., Kelley, D. E. and Warren, L. K. (2011). Supplemental L-arginine shortens gestation length and increases mare uterine blood flow before and after parturition. *Journal of Equine Veterinary Science*, *31*, 514–520.

Moxon, R., Batty, H., Irons, G. and England, G. C. W. (2012). Periovulatory changes in the endoscopic appearance of the reproductive tract and teasing behaviour in the bitch *Theriogenology*, *78*, 1907-1916

Mulic-Lutvica, A., Eurenius, K. and Axelsson, O. (2009). Uterine artery Dopplerultrasound in postpartum women with retained placental tissue. *Acta Obstetricia et Gynecologica Scandinavica*, *88*,724–728.

Mutembei, H. M., Mutiga, E. R. and Tsuma, V. T. (2000). A retrospective study onsome reproductive parameters of German shepherd bitches in Kenya. *Journal of the South African Veterinary Association, 71*, 115–117.

Nagashima, J. B., Sylvester, S. R., Nelson, J. L., Cheong, S. H., Mukai, C., Lambo, C.,Flanders, J. A., Meyers-Wallen, V. N., Songsasen, N. and Travis, A. J. (2015). Live Births from Domestic Dog (*Canis familiaris*) Embryos Produced by *In vitro* Fertilization. *PLoS ONE, 10*(12), e0143930.

Nageotte, M. P. (2015). Foetal heart rate monitoring. *Seminars in Foetal and NeonatalMedicine, 20,* 144–148.

Nakao, T., Yukiyo, A., Fukushima, S., Moriyoshi, M. and Kawata, K. (1985). Inductionof estrus in bitches with exogenous gonadotropins and pregnancy rate and blood progesterone profiles. *The Japanese Journal of Veterinary Science, 47,* 17-24.

Naughton, J. E., Widmer, W. R., Constable, P. D. and Knapp, D. W. (2012). Accuracyof three-dimensional and two dimensional ultrasonography for measurement of tumor volume in dogs with transitional cell carcinoma of the urinary bladder. *American Journal of Veterinary Research, 73*(12), 1919–1924.

Nett, T. M., Akbar, A. M., Phemister, R. D., Holst, P. A., Reichert, L. E., Niswender, G. D.(1975). Levels of luteinizing hormone, estradiol and progesterone in serum during the estrous cycle and pregnancy in the beagle bitch. *Proceedings of the Society for Experimental Biology and Medicine, 148,* 134–139.

Newnham, R. E., Xu, Q. C., & Yoshikawa, S. (1991). U.S. Patent No. 4,999,819. Washington, DC: U.S. Patent and Trademark Office.Reynaud, 2012

Nothling, J. O., De Cramer, K. G. M., Gerber, D. and Kammer, V. R. (2006). Lutealand follicular count in bitches: assessment by means of magnetic resonance imaging.*Theriogenology,66***,** 1343-1354**.**

Novelline, Robert (1997). Squire's Fundamentals ofRadiology (5th ed.). Harvard University Press. 34–35. ISBN 0-674-83339-2.

Noviana, D., Aliambar, S. H., Ulum, M. F. and Siswandi, R. (2012). Small AnimalDiagnostic Ultrasound [Diagnosis Ultrasonografi pada Hewan Kecil]. Bogor: IPB Press.

Nyland, T. G. and Matton, J. S. (1995). Veterinary Diagnostic Ultrasound. Philadelphia:WB Saunders.143-265.

Nyland, T. G. and Mattoon, J. S. (2002). Ovaries and uterus. In: Nyland, T. G.,Matton, J. S., editors. Small animal diagnostic ultrasound. Second edition. Philadelphia: WB Saunders. 231–249.

O'Donnell, M. (1992). Coded excitation system for improving the penetration of realtime phased array imaging systems, *IEEE Transactions on Ultrasonics, Ferroelectrics, and Frequency Control,39,* 341–351.

Okkens, A. C., Teunissen, J. M., Van Osch, W., van den Brom, W. E., Dieleman, S. J. and Kooistra, H. S. (2001). Influence of litter size and breed on the duration of gestation in dogs. *Journal of Reproduction and Fertility, 57*, 193-197.

Oktay, K., Newton, H., Mullan, J., Gosden, R. G. (1998). Development of human primordial follicles to antral stages in SCID/hpg mice stimulated with follicle stimulating hormone. *Human Reproduction, 13*, 1133-1138**.**

Olson, P. N. and Nett, T. M. (1986). Reproductive endocrinology and physiology of the bitch In: Marrow DA, ed. Current therapy in theriogenology: diagnosis, treatment and prevention of reproductive diseases in small and large animals. Philadelphia: Saunders, 453–7.

Olson, P. N., Bowen, R. A., Behrendt, M. D., Olson, J. D. and Nett, T. M. (1982). Concentrations of reproductive hormones in canine serum throughout late anestrus, proestrus and estrus. *Biology of Reproduction,27*, 1196–1206.

Onclin, K., Murphy, B. and Verstegen, J. P. (2002). Comparisons of estradiol, LH and FSH patterns in pregnant and nonpregnant beagle bitches. *Theriogenology, 57*, 1957-1972.

Orfanou, D. C., Ververidis, H. N., Pourlis, A., Fragkou, I. A., Kokoli, A. N., Boscos, C. M., Taitzoglou, A. and Tzora, C. M. N. (2009). Post partum involution of the canine uterus gross anatomical and histological features. *Reproduction in Domestic Animals, 44*, 152–155.

Ortega-Pacheco, A., Rodriguez-Buenfil, *J. C.*, *Segura, C. J. C.*, Bolio-Gonzalez, M. E., *Jiménez-Coello, M. and* Linde- Forsberg, C. (2007), Reproductive patterns and reproductive pathologies of stray bitches in the tropics. *Theriogenology, 67*, 382–390.

Özbay, K. and Deveci, S. (2011). Relationships between transvaginal colour Doppler findings, infectious parameters and visual analogue scale scores in patients with mild acute pelvic inflammatory disease. *European Journal of Obstetrics and Gynecology and Reproductive Biology, 156*, 105–108.

Pakarainen, T., Zhang, F. P., Nurmi, L., Poutanen, M. and Huhtaniemi, I. (2005). Knockout of luteinizing hormone receptor abolishes the effects of follicle-stimulating hormone on preovulatory maturation and ovulation of mouse graafian follicles. *Molecular Endocrinology, 9*, 2591-2602.

Palmowski, M., Huppert, J., Ladewig, G. Hauff, P., Reinhardt, M., Mueller, M. M., Woenne, E. C., ……. and Kiessling, F. (2008). Molecular profiling of angiogenesis with targeted ultrasound imaging: Early assessment of antiangiogenic therapy effects. *Molecular Cancer Therapeutics,7* (1), 101–109.

Park, R. D., Nyland, T. G., Lattimer, J. C., Miller, C. W., and Lebel, J. L. (1981). B-mode grayscale ultrasound: imaging artefacts and interpretation principles, *Veterinary Radiology*. *22*, 204-210.

Patrick, R. K. (2010). Equipment: In: *BSAVAManual of Canine and Feline Ultrasonography*. Ed. Barr. F. and Gaschen. 15-16. BSAVA Publication Gloucester.

Pavlin, C. J. and Foster, F. S. (1998). Ultrasound biomicroscopy: High-frequency ultrasound imaging of the eye at microscopic resolution. *Radiologic Clinics of North America*, 36, 1047 1058.

Payan-Carreira, R. and Pires, M. A. (2008). Multioocyte follicles in domestic dogs: a survey of frequency of occurrence. *Theriogenology*, *69*, 977-982.

Penninck, D. G. (2002). Artefacts. In Nyland TG, Mattoon, J.S, editors: *Small animal diagnostic ultrasound*, ed 2, Philadelphia, Saunders, p 19.

Peter, A. T. (2001). The reproductive system In: Hoskins JD, ed. Veterinary pediatrics: dogs and cats from birth to six months. 3rd edn. Philadelphia, USA: Saunders, 463–475.

Peter, A. T., Pierson, R. A. and Jakovljevic, S. (1992). Use of real-time ultrasonography in bovine and equine reproduction. *Compendium on Continuing Education for the Practicing Veterinarian*, *14*(8), 1116–1124.

Pharr, J. W., and Post, K. (1992). Ultrasonography and radiography of the canine postpartum uterus. Veterinary Radiology & Ultrasound, 33(1), 35-40.

Phillips, P., Holley, G. L., Napolitano, D. J. and Ustuner, K. F. (2001). Medical ultrasound diagnostic imaging method and system with nonlinear phase modulation pulse compression, US Patent No. 6, *241*,674.

Pidoux, G., Gerbaud, P., Tsatsaris, V., Marpeau, O., Ferreira, F., Meduri, G., Guibourdenche, J., Badet, J., Evain-Brion, D. and Frendo, J. L. (2007). Biochemical characterization and modulation of LH/CG receptor during human trophoblast differentiation. *Journal of Cellular Physiology*, *212*(1), 26-35.

Pierson, R. A. and Adams, G. P. (1995). Computer-assisted image analysis, diagnostic ultrasonography and ovulation induction: Strange bed fellows. *Theriogenology*, 43, 105–112.

Piyasena, P., Mohareb, E. and Mckellar, R. C. (2003). Inactivation of microbes using ultrasound: a review. *International Journal of Food Microbiology*, *87*, 207-216.

Porter, M. (1991). Therapeutic ultrasound. *Journal of Equine Veterinary Science*, *11*, 357-362.

Powis, R. L. (1986). Ultrasound science for the veterinarian. *Veterinay Clinic for North American Equine Practice, 2*(1), 3-27.

Powis, R. L. (1993). The saga of the lingering transducer. *Jounal of Equine Veterinary Science*. *13*(6), 323-324.

Prentice, P., Cuschierp, A., Dholakia, K., Prausnitz, M., and Campbell, P. (2005). Membrane disruption by optically controlled microbubble cavitation. *NaturePhysics, 1*, 107–110.

Pretzer, S. D. (2008). Canine embryonic and foetal development: a review. *Theriogenology, 70*, 300–305

Prokop, A. F., Soltani, A., and Roy, R. A. (2007). Cavitational mechanisms in ultrasound accelerated fibrinolysis. *Ultrasound in Medicine and Biology, 33*, 924–933.

Rapoport, N., Gao, Z. G., and Kennedy, A. (2007). Multifunctional nanoparticles for combining ultrasonic tumor imaging and targeted chemotherapy. *Journal of the National Cancer Institute, 99*, 1095–1106.

Reddy, N. K., Ioncicaˇ, A. M., Saˇ ftoiu, A.,Vilmann, P. and Bhutani, M. S. (2011). Contrast-enhanced endoscopic ultrasonography. *World Journal of Gastroenterology,17,* 42–48.

Rehm, S., Stanislaus, D. J. and Williams, A. M. (2007). Estrous cycle-dependent histology and review of sex steroid receptor expression in dog reproductive tissues and mammary gland and associated hormone levels. Birth Defects Res. B Dev. *Reproductive Toxicology, 80*, 233-245.

Rendano, V. T. (1983). Radiographic evaluation of foetal development in the bitch and foetal death in the bitch and queen. In: Kirk, R. W. *Current veterinary therapy*. Philadelphia: WB Saunders Co., v. VIII, 1440 p. 947-952

Rendano, V. T., Lein, D. H. and Concannon, P. W. (1984). Radiographic evaluation of prenatal development in the Beagle: correlation with time of breeding, LH release, and parturition. *Veterinary Radiology, 25*(3), p. 132-141.

Reynaud, K., de Lesegno, C. V., Chebrout, M., Thoumire, S. and Chastant-Maillard, S. (2009). Follicle population, cumulus mucification, and oocyte chromatin configuration during the periovulatory period in the female dog. *Theriogenology, 72*, 1120-1131.

Reynaud, K., Fontbonne, A., Marseloo, N., Thoumire, S., Chebrout, M., de Lesegno, C. V.and Chastant-Maillard S. (2005). *In vivo* meiotic resumption, fertilization and early embryonic development in the bitch. *Reproduction,130*(2), 193–201. doi: 10.1530/rep.1.00500 PMID: 16049157 11.

Reynaud, K., Fontbonne, A., Marseloo, N., Viaris de Lesegno, C., Saint-Dizie, M. and Chastant Maillard, S. (2006). *In vivo* canine oocyte maturation, fertilization and early embryogenesis: a review. *Theriogenology*, *66*, 1685-1693.

Rhind, S. M. and McNeilly, A. S. (1986). Follicle populations, ovulation rates and plasma profiles of LH, FSH and prolactin in Scottish Blackface ewes in high and low levels of body condition. *Animal Reproduction Science, 10*, 105–15.

Richards, R. G., Gadsby, J. E. and Almond, G. W. (1994). Differential effects of LH and PGE2 on progesterone secretion by small and large porcine luteal cells. *Journal of Reproduction and Fertility*, *102*(1), 27-34.

Rooney, J. A. (1970). Hemolysis near an ultrasonically pulsating gas bubble. *Science, 169*, 869–871.

Root, C. R. and Spaulding, K. A. (1994). Diagnostic imaging companion animal theriogenology. *Seminars in Veterinary Medicine and Surgery*, *9*(1), 7-27.

Rosenthal, S. J., Jones, P. H. and Wetzel, L. H. (2001). Phase inversion tissue harmonic sonographic imaging: a clinical utility study. *American Journal of Roentgenology,176*, 1393–1398.

Rosselli, M., Keller, P. J. and Rubey, R. K. (1998). Role of nitric oxide in the biology, physiology and pathophysiology of reproduction. *Human Reproduction Update*, *4*, 3–24.

Rota, A., Charles, C., Cucuzza, A. S. and Pregel, P. (2015) Diagnostic Efficacy of a Single Progesterone Determination to Assess Full-Term Pregnancy in the Bitch. *Reproduction in Domestic Animals,50*, 1028–1031. doi: 10.1111/rda.12631. ISSN 0936–6768.

Rota, A., Milani, C., Romagnoli, S., Zucchini, P. and Mollo, A. (2010). Pregnancy and conception rate after two intravaginal inseminations with dog semen frozen either with 5% glycerol or 5% ethylene glycol. *Animal Reproduction Science*, *118*, 94-97.

Rowlands, I. W. (1950). Some observations on the breeding of dogs. *Proceedings Society for the Study of Fertility, 2*, 40–55.

Rozear, L. and Tidwell, A. S. (2003). Evaluation of the ureter and ureterovesicular junction using helical computed tomographic excretory urography in healthy dogs. *Veterinary Radiology and Ultrasound*, *44*(2), 155–64.

Saint-Dizier, M., Jaffre, N., Reynaud, K., Remy, B., Thoumire, S. and Chastant-Maillard, S. (2008). Expression of follicle-stimulating hormone and luteinising hormone binding sites in the bitch ovary during the follicular phase. *Reproduction, Fertility, and Development*, *20*, 925-934.

Scampini, J. (2017). Overview of Ultrasound Imaging System and Electrical components required formain Subfunction. Available at http://www.maximintegrated.com/en/app-notes/index.mvp/id/4696. Accessed on 30/1/2017@ 12:51 GMT.

Schalm. S.S. Ballif. B.A., Buchanan. S.M., Phillips. G.R., Maniatis. T. (2010)Phosphorylation of protocadherin proteins by the receptor tyrosine kinase RetProc. Natl. Acad. Sci. U. S. A., 107 (31). 13894-13899

Schlief, R. (1991). Ultrasound contrast agents. *Current Opinion in Radiology*, *3*, 198–207.

Schlosser, T., Pohl, C., Veltmann, C., Lohmaier, S., Goenechea, J., Ehlgen, A., Koster, J., Bimmel, D., Kuntz-Hehner, S., Becher, H., and Tiemann, K. (2001). Feasibility of the flash replenishment concept in renal tissue: which parameters affect the assessment of the contrast replenishment?. *Ultrasound in Medicine and Biology*, *27*, 937–944.

Schwarz, T. (2011). Urinary system. In: Schwarz T, Saunders J, editors.Veterinary computed tomography. Chichester (United Kingdom): John Wiley and Sons ltd. Pp 331–338.

Shapiro, R. S., Wagreich, J., Parsons, R. B., Stancato-Pasik, A., Yeh, H. and Lao, R. (1998). Tissue harmonic imaging sonography: evaluation of image quality compared with conventional sonography. *American Journal of Roentgenology*,*171*, 1203–1206**.**

Shille, V. M., and Gontarek, J. (1985). The use of ultrasonography for pregnancy diagnosis in the bitch. *Journal of the American Veterinary Medical Association*, *187*, 1021–1025.

Shille, V. M., Thatcher, M. J. and Simmons, K. J. (1984). Efforts to induce estrus in the bitch, using pituitary gonadotropins. *Journal of the American Veterinary Medical Association, 184,* 1469-1473.

Shille, V., Thatcher, M. J., Lloyd, M., Miller, D., Seyfert, D. and Sherrod, J. (1986). Gondotropin control of follicular development and the use of exogenous gonadotropins for induction of oestrus and ovulation in the bitch. *Journal of Reproduction and Fertility, 39*, 103-113.

Siddharth, S. and Goyal, A. (2007): The origin of echocardiography. *Texas Heart Institute Journal*,*34*(4), 431-438

Siddiqui, M. A., Ferreira, J. C., Gastal, E. L., Beg, M. A., Cooper, D. A. and Ginther, O. J. (2010). Temporal relationships of the LH surge and ovulation to echotexture and powerDoppler signals of blood flow in the wall of the preovulatory follicle in heifers. *Reproduction, Fertility and Development, 22*(7), 1110-1117.

Silva, L. A. and Ginther, O. J. (2006). An early endometrial vascular indicator of completed orientation of the embryo and the role of dorsal endometrial encroachment in mares. *Biology of Reproduction, 74*(2), 337-343.

Silva, L. A., Gastal, E. L., Beg, M. A. and Ginther, O. J. (2005). Changes in vascular perfusion of the endometrium in association with changes in location of the embryonic vesicle in mares. *Biology of Reproduction, 72*(3), 755-761.

Smith, D. A. B., Porter, T. M., Martinez, J., Huang, S. L., MacDonald, R. C., McPherson, D. D., and Holland, C. K. (2007). Destruction thresholds of echogenic liposomes with clinical diagnostic ultrasound. *Ultrasound in Medicine and Biology, 33*, 797– 809.

Smith, F. O. (2007). Challenges in small animal parturition-Timing elective and emergency cesarian sections. *Theriogenology,68*, 348–353.

Socha, P. and Janowski, T. (2014). Predicting the parturition date in bitches of different body weight by ultrasonographic measurements of Inner Chorionic Cavity Diameter and Biparietal Diameter. *Reproduction in Domestic Animals*, *49*, 292–296.

Sokolowski, J. H., Stover, D. G. and VanRavenswaay, F. (1977). Seasonal incidence of estrus and interestrous interval for bitches of seven breeds. *Journal of the American Veterinary Medical Association,171*, 271–273

Song, J., Christiansen, J., Klibanov, A. L., Ley, K., Kaul, S., and Lindner, J. R. (2001). Adhesion of Pselectin- targeted micro bubbles to venules: implications for imaging inflammation. *Journal of the American College of Cardiology*, *37*, 383A–383A.

Song, J., Kim, S., Sohn, H., Song, T. and Yoo, Y. M. (2010). Coded excitation for ultrasound tissue harmonic imaging. *Ultrasonics,50*, 613–619

Songsasen, N., Woodruff, T. K. and Wildt, D. E. (2011). *In vitro* growth and steroidogenesis of dog follicles are influenced by the physical and hormonal microenvironment. *Reproduction*, *142*, 113-122

Steckler, D., De Cramer, K. G. M. and Nöthling, J. O. (2017). Estimated impact of multiple conceptuses per follicle on fecundity in the bitch. *Theriogenology,102,* 108-115

Steckler, D., Nöthling, J. O. and Harper, C. (2013). Prediction of the optimal time for insemination using frozen-thawed semen in a multi-sire insemination trial in bitches. *Animal Reproduction Science*, *142*, 191-197.

Steinetz, B. G., Goldsmith, L. T., Hasan, S. H. and Lust, G. (1990). Diurnal variation of serum progesterone, but not relaxin, prolactin, or estradiol-17 beta in the pregnant bitch. *Endocrinology,127*, 1057–1063.

Steiss, J. E. (2000). Physical therapy modalities: therapeutic ultrasound and phonophoresis. *Compendium on Continuing Education for the Practicing Veterinarian, 22*, 690-693

Stephenson, A. P., Tyler, D. J., Carr, C. A. and Williams, S. A. (2012). Magnetic resonance imaging for the study of ovarian follicles in the mouse. *Theriogenology,78*, 1190-1198.

Stewart, R. A., Pelican, K. M., Crosier, A. E., Pukazhenthi, B. S., Wildt, D. E., Ottinger, M. A. and Howard, J. (2012). Oral progestin priming increases ovarian sensitivity to gonadotropin stimulation and improves luteal function in the cat. *Biology of Reproduction*, *87*, 1–11.

Still, J. G. and Greiss Jr, F. C. (1978). The effect of prostaglandins and other vasoactive substances on uterine blood flow and myometrial activity. *American Journal of Obstetrics and Gynecology,130*, 1–8.

Stride, E. P. and Coussio, C. C. (2009). Cavitation and contrast: the use of bubbles inultrasound imaging and therapy. *Journal of Engineering in Medicine*, *224*, 171-191. Doi: 10.1243/09544119JEIM622

Suslick, K. S. (1990). Sonochemistry. *Science, 247*, 1439–1445.

Szopinski, K. T., Pajk, A. M., Wysocki, M., Amy, D., Szopinska, M. and Jakubowski, W. (2003a). Tissue harmonic imaging: utility in breast sonography. *Ultrasound in Medicine and Biology*, *22*, 479–487.

Szopinski, K. T., Wysocki, M., Pajk, A. M., Slapa, R. Z., Jakubowski, W. and Szopinski, M. (2003b). Tissue harmonic imaging of thyroid nodules: initial experience. *Ultrasound in Medicine and Biology,22,* 5–12.

Tainturier, B. and Tainturier, D. (2004). Bencharif foetal sex determination in cattle by ultrasonography. *Journal of Animal and Veterinary Advances, 3:*136–41.

Takalkar, A. M., Klibanov, A. L., Rychak, J. J., Lindner, J. R., and Ley, K. (2004). Binding and detachment dynamics of micro bubbles targeted to Pselectin under controlled shear flow. *Journal of Controlled Release,96*, 473–482.

Takeuchi, Y. (1996). Coded excitation for harmonic imaging, in: Proceedings. IEEE Ultrasonics Symposium San Antonio, TX, pp. 1433–1436.

Tarantal, A. F., O'Brien, W. D., & Hendrickx, A. G. (1993). Evaluation of the bioeffects of prenatal ultrasound exposure in the cynomolgus macaque (Macaca fascicularis): III. Developmental and hematologic studies. Teratology, 47(2), 159-170.

Tedor, J. B. and Reif, J. S. (1978). Natal patterns among registered dogs in the United States. *Journal of the American Veterinary Medical Association, 172*, 1179–85.

Telfer, E. and Gosden, R. G. (1987). A quantitative cytological study of polyovular follicles in mammalian ovaries with particular reference to the domestic bitch (Canis familiaris). *Journal of Reproduction and Fertility*, *81*, 137-147.

Terranova PF (2009). Fertilization, Pregnancy, andFetal Development: Medical Physiology,Part X, Reproductive Physiology Vol.XXXIX. Saunders, USA. 684-704.

Themmen, A. P. N. and Huhtaniemi, I. T. (2000). Mutations of gonadotropins and gonadotropin receptors: elucidating the physiology and pathophysiology of pituitarygonadal function. *Endocrine Reviews*, *21*, 551-583.

Thomassen, R., Farstad, W., Krogenaes, A., Fougner, J. A. and Berg, K. A. (2001). Artificial insemination with frozen semen in dogs: a retrospective study. *Journal of Reproduction and Fertility*, *57*, 341-346.

Tinkanen, H. and Kujansuu, E. (1993). Doppler ultrasound findings in tuboovarian infectious complex. *Journal of Clinical Ultrasound*, *21*, 175–178.

Tiukinhoy, S. D., Khan, A. A., Huang, S. L., Klegerman, M. E., MacDonald, R. C., and McPherson, D. D. (2004). Novel echogenic drug-immunoliposomes for drug delivery. *Investigative Radiology*, *39*, 104–110.

Toal, R. L., Walker, M. A. and Henry, G. A. (1986). A comparison of real-time ultrasound, palpation and radiography in pregnancy detection and litter size determination in the bitch. *Veterinary Radiology*,*27*(4), 102-108,

Tranquart, F., Grenier, N., Eder, V. and Pourcelot. L. (1999). Clinical use of ultrasound tissue harmonic imaging. *Ultrasound in Medicine and Biology*,*25*, 889–894**.**

Tsang, B. K., Moon, Y. S. and Simpson, C. W. (1979). Androgen biosynthesis in human ovarian follicles: cellular source, gonadotropic control, and adenosine 3959monophosphate mediation. *The Journal of Clinical Endocrinology and Metabolism*, *48*, 153-158.

Tsutsui, T. (1989). Gamete physiology and timing of ovulation and fertilization in dogs. *Journal of Reproduction and Fertility*, *39*, 269–275.

Ulum, M. F., Maharani, A. D. K., Kurniawan, R. E., Sariningrum, A., Frastantie, D., Erwin, Tumbelaka, L. I. and Noviana, D. (2017). Ultrasonography of the external reproductive organs in Tom. *Jurnal Kedokteran Hewan*. *11*(3), 94-100. DOI: https://doi.org/10.21157/j.ked.hewan.v11i3.5700

Unger, E. C., McCreery, T., Sweitzer, R., Vielhauer, G., Wu, G., Shen, D., and Yellowhair, D.(1998). MRX 501: a novel ultrasound contrast agent with therapeutic properties *Academic Radiology*, *5*(1), S247–S249.

Van Haaften, B., Dieleman, S. J., Okkens, A. C., Bevers, M. M. and Willemse, A. H. (1989). Induction of estrus and ovulation in dogs by treatment with PMSG and/or bromocriptine. *Journal of Reproduction and Fertility, 39,* 330-331

van Wamel, A., Kooiman, K., Harteveld, M., Emmer, M., ten Cate, F. J., Versluis, M., and de Jong, N. (2006). Vibrating micro bubbles poking individual cells: drug transfer into cells via sonoporation. *Journal of Controlled Release, 112,* 149–155.

Vanderlip, S. L., Wing, A. E., Felt, P., Linkie, D., Rivier, J., Concannon, P. W. and Lasley, B. L. (1987). Ovulation induction in anestrous bitches by pulsatile administration of GnRH. *Laboratory Animal Science, 27,* 459-468.

Vannucchi, C. I., Jordao, A. A., & Vannucchi, H. (2007). Antioxidant compounds and oxidative stress in female dogs during pregnancy. *Research in veterinary science,* 83(2), 188-193.

Veronesi, M. C., Battocchio, M., Marinelli, L., Faustini, M., Kindahl, H. and, Cairoli, F. (2002).Correlations among body temperature, plasma progesterone, cortisol and prostaglandin F2a of the periparturient bitch. *Journal of veterinary medicine. A, Physiology, pathology, clinical medicine, 49,* 264–268.

Verstegen, M.W.A. and Close, W.H. (1994). The environmental and the growing pigs. In: Principles of Pig Science, Cole, D.J.A., Wiseman, J. and Varley, M.A. (eds). NottinghamUniversity Press, Nottingham, England, pp. 333-353.

Verstegen, J., Onclin, K., Silva, L. and Concannon, P. (1997). Termination of obligate anestrus and induction of fertile ovarian cycles by administration of purified pig. *Journal of Reproduction and Fertility,111,* 35-40.

Verstegen, J.P. K. Onclin, L.D.M. Silva and P.W. Concannon (1999). Effect of stage of anestrus on the induction of estrus by the dopamine agonist cabergoline in dogs. *Theriogenology.* 51: 597-611

Vesely, J. A. (1978). Application of light control to shorten the production cycle in two breeds of sheep. *Animal Production, 26,* 169–176.

Vignoli, M., and Saunders, J. (2011): Image-guided interventional procedures in the dog and cat. *The Veterinary Journal, 187,* 297–303.

Wallace SS, Mahaffey MB, Miller DM, Thompson FN, Chakraborty PK. Ultrasonographic appearance of the ovaries of dogs during the follicular and luteal phases of the estrous cycle. Am J Vet Res 1992; 53: 209–215.

Walter, I. and Schönkyp, S. (2006). Extracellular matrix components and matrix degrading enzymes in the feline placenta during gestation. *Placenta, 27,* 291–306.

Wanke, M., Loza, M., Monachesi, N., and Concannon, P. (1997). Clinical use of dexamethasone for termination of unwanted pregnancy in dogs. *Journal of Reproduction and Fertility*, *51*, 233-238.

Ward, B., Baker, A. C. and Humphrey, V. F. (1997). Nonlinear propagation applied to the improvement of resolution in diagnostic medical ultrasound. *The Journal of the Acoustical Society of America, 101*, 143–154.

Ward, M., Wu, J. R., and Chiu, J. F. (1999). Ultrasound induced cell lysis andsonoporation enhanced by contrast agents. *The Journal of the Acoustical Society of America*, *105*, 2951–2957.

Weilenmann, R., Arnold, S., Dobeli, M., Rüsch, P. and Zerobin, K. (1993). Estrusinduction in bitches by the administration of PMSG and HCG. *Schweizer Archiv Fur Tierheilkunde,135*(8), 236-241.

Whitlow, B. J., Lazanakis, M. S. and Economides, D. L. (1999). The sonographicidentification of foetal gender from 11 to 14 weeks of gestation. *Ultrasound in Obstetrics and Gynecology*, *13*, 301–304.

Whittingham, T. A. (2005). Contrast-specific imaging techniques; technicalperspective. In: Quaia E (ed.). *Contrast Media inUltrasonography. Basic Principles and Clinical Applications*. Berlin: Springer, 43–84.

Wiebe, V. J. and Howard, J. P. (2009). Pharmacologic Advances in Canine and FelineReproduction: Topical Review.*Topics in Companion Animal Medicine*, 24(2), 71-99. doi:10.1053/j.tcam.2008.12.004

Wigham, E. E., Moxon, R. S., England, G. C. W., Wood, J. L. N. and Morters, M. K.(2017). Seasonality in oestrus and litter size in an assistance dog breeding colony in the United Kingdom. *Veterinary Record*, 5-6. Doi: 10.1136/vr.104217

Wildt, D. E., Chakraborty, P. K., Panko, W. B. and Seager, S. W. J. (1978). Relationship of reproductive behavior, serum luteinizing hormone and time of ovulation in the bitch. *Biology of Reproduction*, *18*, 561-570.

Willmann, J. K., Lutz, A. M., Paulmurugan, R., Patel, M. R., Chu, P., Rosenberg, J. and Gambhir, S. S. (2008). Dual-targeted contrast agent for US assessment of tumor angiogenesis *in vivo*. *Radiology,248*, 936–944.

Wright, P. J. (1990). Application of vaginal cytology and plasma progesterone determinations to the management of reproduction in the bitch. *Journal of Small Animal Practices*, *31*, 335-340.

Xu, B. and Feng, H. L. (2016). Ovulation, fertilization and preimplantation embryonic development in raccoon dogs (*Nyctereutes procyonoides*). *Animal Reproduction Science*, xxx. http://dx.doi.org/10.1016/j.anireprosci.2016.12.001

Yeager, A. E. and Concannon, P. W. (1990). Serial ultrasonographic appearance of postpartum uterine involution in beagle dogs. *Theriogenology, 34*, 523–525.

Yeager AE, Mohammed HO, Meyers-Wallen V, Vannerson L, Concannon PW. (1992) Ultrasonographic appearance of the uterus, placenta, fetus, and fetal membranes throughout accurately timed pregnancy in beagels. *Am J Vet Res.;*53: 342–351.

Yoshioka, M., Tanaka, K. I., Miyazaki, I., Fujita, N., Higashi, Y., Asanuma, M.,& Ogawa, N. (2002). The dopamine agonist cabergoline provides neuroprotection by activation of the glutathione system and scavenging free radicals. Neuroscience research, 43(3), 259-267.

Yotov, S. A., Atanasov, A. and Georgiev, P. (2011). Determination of foetal sex in buffaloes through a single sonographic examination. *Bulgarian Journal of Veterinary Medicine*, *14*, 39–44.

Zagzebski, J. A. (2005). Physics and instrumentation in Doppler and B-Mode ultrasonography. Philadelphia: Elsiever Saunders, 752p.

Zambelli, D. and Prati, F. (2006). Ultrasonography for pregnancy diagnosis and evaluation in queens. *Theriogenology, 66*, 135–144.

Zhou, Y. C., Wang, J., Zhang, B. and Su, Y. Q. (2002). Ultrasonic immunization of sea bream, *Pagrus major*, with a mixed vaccine against *Vibrio alginolyticus* and *V. anguillarum*. *Journal of Fish Diseases, 25*, 325-331.

Zoldag, L., Fekete, S., Csaky, I. and Bersényi, A. (2001). Fertile estrus induced in bitches by bromocryptine a dopamine agonist; a clinical trial. *Theriogenology,55* (8), 1657-1666,

Zone, M. A., and Wanke, M. M. (2001). Diagnosis of canine foetal health by ultrasonography. *Journal of Reproduction and Fertility*, *57*, 215–219.

APPENDICES

Appendix 1. Concentration of Superoxide dismutase at various phases for the bitches

PHASE 1			PHASE 2			PHASE 3E		
A	B	C	A	B	C	A	B	C
0.417284	1.152789	0.685231	0.197517	0.150659	0.059278	1.134078	1.294227	1.411546
0.469274	1.197393	0.886406	0.219739	0.620112	0.249383	1.145251	1.305469	1.499069
0.824953	1.197393	1.009311	0.364991	1.022222	0.258845	1.165736	1.307263	1.649907
0.612663	1.147114	0.95717	0.357542	0.091358	0.445065	1.199255	1.318436	1.724395
0.631285	1.266294	1.106145	0.43203	0.901304	0.77037	1.216015	1.364991	1.312849
0.759777	1.288641	1.1527	0.55121	1.111732	0.955307	0.849162	1.366853	1.325885
	0.616387			0.069136		0.865922	1.407821	1.392924
	0.726257			0.088889		0.931099	1.428305	1.402235
	0.804469			0.553073			1.556797	
							1.579143	
							0.998138	
							1.126629	
							1.156425	
							1.171322	
							1.264432	

PHASE 3L			PHASE 4		
A	B	C	A	B	C
1.348231	1.366853	1.979516	0.895717	1.223464	1.055866
1.566108	1.495345	1.979516	1.001862	1.240223	1.206704
1.56797	1.506518	1.988827	1.080074	1.264432	1.214153
1.843575	1.564246	1.996276	0.769088	1.314711	1.165736
1.977654	1.953445	1.769088	0.817505	1.316574	1.165736
1.054321	1.648045	1.785847	0.821229	1.357542	1.208566
1.07635	1.700186	1.839851		0.83054	
1.098696	1.98324	1.86406		0.839851	
1.944134	1.990689			0.992551	
	4.577778				
	1.348231				
	1.379888				
	1.38175				
	1.506518				
	2.437037				

Appendix 2. Concentration of Malondialdehyde at various phases for the bitches

PHASE 1			PHASE 2			PHASE 3E		
A	B	C	A	B	C	A	B	C
7.715	2.203	10.103	1.924	1.205	0.98	2.345	3.722	3.273
2.111	5.991	1.69	0.377	0.038	0.049	4.037	1.228	4.057
10.098	5.159	11.187	1.318	1.945	0.23	2.418	1.637	3.886
4.499	3.461	2.007	2.458	2.609	1.258	5.853	1.639	2.528
3.433	2.405	1.563	2.646	0.053	1.492	2.05	1.652	2.086
	2.891	2.848	2.531		1.637	2.775	2.671	2.214
	3.086	2.878				4.28	3.286	2.294
	6.353					0.117	3.397	2.32
	2.99					4.797	1.594	
	1.648					2.241		
	1.569					4.233		
						3.663		
						2.269		
						6.012		
						1.36		

PHASE 3L			PHASE 4		
A	B	C	A	B	C
0.837	7.406	2.558	0.075	1.034	1.598
2.795	6.959	4.184	0.158	2.703	2.439
8.164	1.492	4.652	3.035	2.89	0.104
2.935	19.939	6.713	4.697	7.655	4.606
11.085	2.165	10.509	2.328	3.407	1.498
3.329	7.602	1.903	3.931	1.073	
4.476	4.463	3.939	6.891		
2.767	6.404	4.146	1.043		
3.061	10.162	4.627	3.327		
33.421	7.545	7.28			
5.272					
2.32					
1.556					
2.358					
9.754					

Appendix 3. Concentration of Catalase at various phases for the bitches

PHASE 1			PHASE 2			PHASE 3E		
A	B	C	A	B	C	A	B	C
7.198205	6.393635	8.34908	6.530334	6.174916	7.1943	8.244928	7.204715	27.69523
7.471603	6.396238	8.533949	6.777693	6.341559	7.840039	8.446722	8.096512	35.61335
7.588773	6.449616	9.581973	6.956053	6.341559	8.250135	9.416633	8.563892	37.00507
6.601938	6.456126	6.685259	0.074128	4.343039	6.260841	18.50906	9.812408	7.646057
6.785505	6.460031	6.725618	5.590366	6.228294	6.510805	23.86234	12.10363	7.721567
6.793316	6.608447	6.805033	6.180124	6.324634	6.682655	7.077129	13.9277	8.944045
6.694372			6.323332			7.398697	14.14772	9.226557
6.792014			6.336351			7.894719	15.5134	
6.877939			6.625372			8.25274	17.28268	
						8.343872		
						7.698133		
						7.985851		
						8.270966		

PHASE 3L			PHASE 4		
A	B	C	A	B	C
27.2733	12.58154	43.36743	7.651264	6.502994	11.30828
36.87749	16.30484	49.17388	7.807492	6.611051	11.97225
40.98887	19.06107	50.71141	7.920756	6.834977	27.35923
45.82931	21.24044	50.76089	6.890958	7.575755	6.900072
51.07464	38.28484	12.6219	6.920902	9.321594	7.006827
8.379024	18.07944	13.41215	6.980789	10.70941	7.45598
8.932328	20.55304	23.8989	7.173469		
26.61205	27.57534	27.35792	7.385678		
27.12098	47.31738		7.61351		
49.85997	47.55302				
8.742251					
8.915403					
12.31986					
38.32259					

Appendix 4. Concentration of Glutathione peroxidase at various phases for the bitches

PHASE 1			PHASE 2			PHASE 3E		
A	B	C	A	B	C	A	B	C
20.36853	11.25333	24.3072	3.0384	5.176533	1.3504	34.4352	41.74987	48.2768
21.38133	12.71627	26.89547	8.1024	5.7392	14.1792	37.24854	42.98773	50.64
21.944	23.85707	32.18453	9.677867	6.864533	15.5296	45.46347	46.47626	52.21547
21.71893	23.85707	11.36587	0.900267	8.327467	5.626667	55.47893	36.01067	56.7168
23.632	24.3072	16.2048	5.176533	15.41707	9.4528	60.65547	38.03627	28.80853
24.6448	24.53227	17.5552	18.2304	21.49387	10.69067	30.3848	46.02613	30.83413
40.1744			1.0128			33.30987		32.7472
40.28693			30.7216			33.98507		36.68587
40.512			38.03627			34.88533		
						46.81387		
						48.72693		
						49.73973		

PHASE 3L			PHASE 4		
A	B	C	A	B	C
64.36906	48.72693	57.16693	28.0208	33.87253	32.63467
70.896	57.50453	62.456	29.0336	34.21013	33.53493
87.4384	82.59946	65.1568	29.93387	39.4992	36.1232
108.8197	46.2512	66.73227	25.6576	25.32	18.9056
136.2779	55.3664	79.11093	27.79573	28.3584	22.84427
38.03627	57.0544	44.11307	27.90827	33.19733	23.2944
41.97493	78.88586	48.952	44.9008		
63.8064		63.24373	46.36374		
89.57653			46.81387		
147.3061					
50.75253					
54.57867					
57.392					
61.33067					

Appendix.5 Showing the uterine diameters of the bitches from week 1 to week 8 postpartum

Dog	WEEK 1	WEEK 2	WEEK 3	WEEK 4	WEEK 5	WEEK 6	WEEK 7	WEEK 8
A1	4.51	3.71	2.85	2.19	2.02	1.85	1.85	1.84
A2	3.9	3.13	2.36	2.03	1.8	1.76	1.76	1.74
A3	3.51	2.54	1.81	1.69	1.62	1.61	1.59	1.58
B1	4.21	3.4	2.68	2.17	1.88	1.82	1.83	1.79
B2	4.04	3.14	2.51	2.04	1.86	1.78	1.78	1.76
B3	3.66	2.8	1.98	1.78	1.64	1.7	1.69	1.68
C1	4.14	3.28	2.56	2.1	1.86	1.79	1.81	1.78
C2	3.86	3.06	2.16	1.89	1.74	1.71	1.71	1.69
C3	3.67	2.84	2.15	1.87	1.74	1.7	1.7	1.68