UNIVERSITY OF KWAZULU-NATAL ™

INYUVESI YAKWAZULU-NATALI

Investigations into the morphometrics, uterine tissue adaptation, maternal fluid biochemistry, heavy metal offloading and early embryonic teeth development impacting the reproductive strategy of the female Ragged-tooth shark (*Carcharias taurus*)

Kristina Naidoo

B.Sc., B.Med.Sc. (Hons), M. Med.Sci

Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy (Medical Biochemistry), School of Laboratory Medicine and Medical Sciences, College of Health Sciences,
University of KwaZulu-Natal

2018

This project was a collaborative undertaking with the following institutes:

- New South Wales Department of Primary Industries, Port Stephens Fisheries Institute, Taylors Beach, New South Wales, Australia

- KwaZulu-Natal Sharks Board, Umhlanga, South Africa

- Discipline of Medical Biochemistry, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, South Africa

DECLARATION

I, Kristina Naidoo, declare that:

i) The research reported in this thesis (unless otherwise indicated) is my original work, has not been submitted for any degree or examination at any other university, does not contain other persons' data, diagrams, graphs, or other information, unless specifically being acknowledged as sourced from other persons, does not contain other persons' writings unless being acknowledged as being sourced from other researchers. Where other written sources have been quoted then:

a) Their work has been re-written, but the information sourced has been referenced to the authors. Where their exact words have been used, their writing has been placed within quotation marks and referenced.

ii) Where I have reproduced a publication of which I am author, co-author, or editor, I have indicated in detail which part of the publication was written by myself alone and have properly referenced such publication.

iii) This thesis does not contain text, graphics or tables copied and pasted from the internet, unless specifically acknowledged and the source being detailed in the thesis and reference section

Signed

27-AUGUST-2018

Kristina Naidoo — Date

ACKNOWLEDGEMENTS

Prof Chuturgoon: My deepest gratitude to you prof, for your years of mentorship and encouragement and support. Your guidance has been invaluable and strengthening.

Prof Gregory: Your guidance and support during this research endeavour was invaluable. Thank you for all your effort and time.

Mr Cliff: Your enthusiasm for the marine field has never dwindled. Thank you for introducing me to the marine world; you created an environment devoid of boundaries. I thank you for your trust, countless conversations and guidance.

Dr Singh, Dr Ellis and Dr. Otway: Thank you for the opportunity to take on such a project. Thank you for mentoring me.

To my parents and family: Thank you for years of unconditional support, love and unwavering understanding and patience during my studies. Your framework of hard work and commitment engrained in me ensures I go further than I think is humanly possible.

My friends (old and new): A humble thank you to each one of you for your encouragement, laughs and keeping me sane. Special thanks to Dr. M Singh, Dr. S Gounden, Dr. A Phulkdharee, Mrs B Reddy and Ms. P Dudhrajh for the motivation and laughs. To Dr. K Dhuness for your patience and unwavering encouragement to keep pushing forward. To Mr K Singh and Mrs R Sobaren Singh for all your assistance. You all kept me going. Thank you so much.

KZNSB Research Division: Mr P Zungu, Mr E Makhathi, Mr B Zungu, Mrs A Ganesan, Mr T Mlambo, Ms. Naidu, Mrs S Wintner and Dr M Dicken and Mr P von Blerk. It's been an honour working with you all. Thank you for the assistance and advice over the years.

Department of Medical Biochemistry, MMU and Physiology at UKZN: Thank you for the support and encouragement. A special thank you to Mr. S Naidu and Mr. Bharuth, Mr P Christopher and Mrs S Singh for the in between laughs during the work; it made for a wonderful environment to work in.

LIST OF PUBLICATIONS

JOURNAL PUBLICATIONS

(1) Possible maternal offloading of metals in the plasma, uterine and capsule fluid of pregnant Ragged-tooth sharks (*Carcharias taurus)* on the east coast of South Africa

K Naidoo, AA Chuturgoon, G Cliff, SD Singh, M Ellis, N Otway, AVosloo, MA Gregory (2017). Environmental Science and Pollution Research 24 (20): 16798-16805

(2) Dentition facilitates release of encapsulated Ragged-tooth (*Carcharias taurus)* embryos

K Naidoo, AA Chuturgoon, G Cliff, MT Ellis, NM Otway, MA Gregory, SD Singh, SL Naidu (2017). Environmental Biology of Fishes 100:1343-1354

(3) Histology of non-gravid and gravid Ragged-tooth female sharks (*Carcharias taurus*) on the east coast of South Africa

K Naidoo, AA Chuturgoon, G Cliff, MT Ellis, NM Otway, MA Gregory, SD Singh (2018). Prepared for Environmental Biology of Fishes

(4) Biochemistry of non-gravid and gravid Ragged-tooth female sharks (*Carcharias taurus*) on the east coast of South Africa

K Naidoo, AA Chuturgoon, G Cliff, MT Ellis, NM Otway, MA Gregory, SD Singh (2018). Prepared for Environmental Biology of Fishes

LIST OF PRESENTATIONS

(1) Observations of early capsule escape of the Ragged-tooth shark embryos (*Carcharias taurus)*

Naidoo K[1], Chuturgoon AA[1], Cliff G[2], Ellis MT[3], Otway NM[4], Singh SD[5], Gregory MA[6]. [1]Discipline of Medical Biochemistry, [5]Biomedical Resource Unit, [6]School Life Sciences, University of KwaZulu-Natal (UKZN), [2]KwaZulu-Natal Sharks Board (KZNSB), Umhlanga, Durban, South Africa; [3]Gladstone Ports Corporation, Gladstone, Queensland, Australia**, [4]**New South Wales Department of Primary Industries, Port Stephens Fisheries Institute, Taylors Beach NSW, Australia. 2nd Sharks International Conference 2014 [*Poster presentation*]**.**

(2) Uterine changes in the different sexual stages of the Ragged-tooth (*Carcharias taurus)*

Naidoo K[1], Chuturgoon AA[1], Cliff G[2], Ellis MT[3], Otway NM[4], Singh SD[5], Gregory MA[6]. [1]Discipline of Medical Biochemistry, [5]Biomedical Resource Unit, [6]School Life Sciences, University of KwaZulu-Natal (UKZN), [2]KwaZulu-Natal Sharks Board (KZNSB), Umhlanga, Durban, South Africa; [3]Gladstone Ports Corporation, Gladstone, Queensland, Australia, **[4]**New South Wales Department of Primary Industries, Port Stephens Fisheries Institute, Taylors Beach NSW, Australia. 2nd Sharks International Conference 2014 [*Poster presentation*]**.**

(3) Possible early dentition and capsule release Ragged-tooth *(Carcharias taurus)* embryos

Naidoo K[1], Chuturgoon AA[1], Cliff G[2], Ellis MT[3], Otway NM[4], Singh SD[5], Gregory MA[6]. [1]Discipline of Medical Biochemistry, [5]Biomedical Resource Unit, [6]School Life Sciences, University of KwaZulu-Natal (UKZN), [2]KwaZulu-Natal Sharks Board (KZNSB), Umhlanga, Durban, South Africa; [3]Gladstone Ports Corporation, Gladstone, Queensland, Australia, **[4]**New South Wales Department of Primary Industries, Port Stephens Fisheries Institute, Taylors Beach NSW, Australia. 3rd South African Shark and Ray Symposium 2015 [*Oral presentation*].

(4) Possible heavy metal transfer between Ragged-tooth female and embryo

Naidoo K[1], Chuturgoon AA[1], Cliff G[2], Ellis MT[3], Otway NM[4], Singh SD[5], Gregory MA[6]. [1]Discipline of Medical Biochemistry, [5]Biomedical Resource Unit, [6]School Life Sciences, University of KwaZulu-Natal (UKZN), [2]KwaZulu-Natal Sharks Board (KZNSB), Umhlanga, Durban, South Africa; [3]Gladstone Ports Corporation, Gladstone, Queensland, Australia, **[4]**New South Wales Department of Primary Industries, Port Stephens Fisheries Institute, Taylors Beach NSW, Australia. Western Indian Organisation for Marine South Africans (WIOMSA) (2015) [*Oral presentation*].

(5) Observation of early-staged Ragged-tooth (*Carcharias taurus*) embryos

Naidoo K[1], Chuturgoon AA[1], Cliff G[2], Ellis MT[3], Otway NM[4], Singh SD[5], Gregory MA[6]. [1]Discipline of Medical Biochemistry, [5]Biomedical Resource Unit, [6]School Life Sciences, University of KwaZulu-Natal (UKZN), [2]KwaZulu-Natal Sharks Board (KZNSB), Umhlanga, Durban, South Africa; [3]Gladstone Ports Corporation, Gladstone, Queensland, Australia, **[4]**New South Wales Department of Primary Industries, Port Stephens Fisheries Institute, Taylors Beach NSW, Australia. National Association of African-American Studies and Affiliates International Research Congress 2017 [*Oral Presentation*].

(6) Possible maternal offloading of metals in the plasma, uterine and capsule fluid of pregnant Ragged-tooth sharks (*Carcharias taurus)* on the east coast of South Africa.

Naidoo K[1], Chuturgoon AA[1], Cliff G[2], Ellis MT[3], Otway NM[4], Singh SD[5], Gregory MA[6]. [1]Discipline of Medical Biochemistry, [5]Biomedical Resource Unit, [6]School Life Sciences, University of KwaZulu-Natal (UKZN), [2]KwaZulu-Natal Sharks Board (KZNSB), Umhlanga, Durban, South Africa; [3]Gladstone Ports Corporation, Gladstone, Queensland, Australia, **[4]**New South Wales Department of Primary Industries, Port Stephens Fisheries Institute, Taylors Beach NSW, Australia. 4th South African Shark and Ray Symposium 2017 [*Oral Presentation*].

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Supporting Tables for CHAPTER 5

LIST OF ABBREVIATIONS

Species

A. vulpinus	*Alopias vulpinus* (common name: Thresher shark)	*M. mustelus*	*Mustelus mustelus* (Smooth-hound shark)
C. carcharias	*Carcharodon Carcharias* (Great white shark)	*M. antarcticus*	*Mustelus antarcticus* (Gummy shark)
C. obscurus	*Carcharhinus obscurus* (Dusky shark)	*O. ornatus*	*Orectolobus ornatus* (Wobbegong ark)
C. taurus	*Carcharias taurus* (Ragged-tooth shark)	*R. longurio*	*Rhizoprionodon longurio* (Pacific sharpnose shark)
G. galeus	*Galeorhinus galeus* (School shark)	*S. canicula*	*Scyliorhinus canicula* (Small-spotted catfish)
I. oxyrinchus	*Isurus Oxyrinchus* (Shortfin mako shark)	*S. acanthias*	*Squalus acanthias* (Spiny dogfish shark)
L. nasus	*Lamna Nasus* (Porbeagle shark*)*		

Abbreviations

AB^+	Alcian Blue positive	NSW DPI	New South Wales, Department Primary Industries (in Australia)
Al	Aluminium	NW	North-Western
Al	arteriole loops	O. G	oviducal gland
As	Arsenic	O	Oxygen
AU	artificial uterus	OD	oesteodentine layer
BC	basal cells	p	Phosphate
BD	Becton Dickson (Company)	*p*	significant value set at < 0.05
BL	Basal Lamina	PAS	Periodic Acid Schiff
BV	blood vessels	PAS^+	Periodic Acid Schiff positive
Ca	Calcium	PAS&AB	Periodic Acid Schiff and Alcian Blue
Cap_n	capsule sample size	Pb	Lead
Cd	cadmium	PCL	precaudal length
DAAD	Deutscher Akademischer Austauschdienst	ppm	parts per million
ECF	extra capsular fluid	pq	paratoquadrate
EDX	Energy dispersive X-ray	r^2	trendline value
EI	enameloid inner	*r*	correlation value (Spearman/Pearson)
EE	encapsulated embryos	RBC	red blood cell
E1-E6	embryo 1- embryo 6	RT	room temperature
e.g.,	example	S	serosa
ESEM	Environmental SEM	S	serosa
F_n	female sample size	SA	South Africa
FE	field emission	SC	secretory substance
FFE	free-floating embryos	Se	Selenium

g/G	Gram/Gauge
GF	Gravid females
GSI	gonadosomatic index
g/L	grams/litre
H&E	Haematoxylin and Eosin
Hg	Mercury
HSI	Hepatosomatic index
ICF	intracapsular fluid
ICP-MS	Inductively-Coupled Plasma Mass Spectrophotometry
ID	identification number for animal at KZNSB
IQR	interquartile range
IUCN	International Union for Conservation of Nature
in-utero	in the uterus
in vitro	in a glass
kg	kilogram
km	kilometre
KZN	KwaZulu-Natal
KZNSB	KwaZulu-Natal Sharks Board
LM	light microscopy
LP	Lamina Propria
M	male/Mucosa/molar
MBC	mature basal cells
Mc	Meckels cartilage
mg/L	milligram/litre
ml	millilitre
MMU	Microscopy and Microanalysis Unit
MM	Muscularis Mucosa
mmol/L	Millimole/litre
mIU/ml	milli-international units/ millilitre
n	sample size
NA	not applicable
NGF	non-gravid females
NISD	Nikon instrument documentation
nmol/L	nanomole/litre
No	number
RS	Reproductive Stage/s
SEM	scanning electron microscopy
SD	standard deviation
SDD	Silicon drift detector
SL	stratified layer of cells
SLE	shiny layer enameloid
SubM	Submucosa
T	trough/s
TH	tooth height
TL	total length
TEM	Transmission Electron Microscopy
TW	tooth width
µM	micrometre
U	unidentified
UC	upper caudal
UF	uterine fluid
UKZN	University of KwaZulu-Natal
UL	uterine lamella/e
USA	United States of America
U/L	units/litre
UW	uterus width
umol/L	micromole/litre
Vtg	Vitellogenin
VP	variable pressure
v/v	volume/volume
x	mean
Vtg	Vitellogenin
VP	variable pressure
v/v	volume/volume
x	mean
x_d	median
I-VI	Embryology stage one-six of *C. taurus*
^{0}C	celsius degree
%	percentage
Xg	centrifuge rotations per minute
$Ca_5(PO_4)_3$	Fluoroapatite

ABSTRACT

"Vulnerable" status of ragged-tooth sharks (*Carcharias taurus*) in South Africa caused by overexploitation, late maturity and low fecundity suggests an intervention to increase the size of this population is needed. Achieving this will require an understanding of all aspects linked to this species maternal-embryonic relationship.

Morphometric relationships, uterine histology and maternal fluid biochemistry were assessed in *C. taurus* through all the respective reproductive stages (RS) from non-gravid (immature to mature-sexually active; RS1-3) to gravid (i.e. only capsules found; RS4 or capsules and pups found; RS5A-5E) females. Examination of metals in the maternal fluids and embryonic dentition were only examined in early-staged gravid females (i.e. RS5A).

Haematoxylin/Eosin and Periodic Acid Schiff-Alcian Blue stains in conjunction with light microscopy was used to assess the uterine epithelium and wall while scanning electron microscopy further evaluated the epithelium. These techniques revealed an increase of the uterine lamellae (folds) protruding into the lumen lined with micro-ridges containing blood vessels. The close proximity of blood vessels to the lumen filled with uterine fluid and the decrease in wall thickness as pregnancy progressed suggests an adaption for the exchange of respiration and osmoregulation in the developing aplacental embryos. Although there is no evidence for uterine secretion through structural adaptations, the female supports the embryos nutritional requirement through embryonic tissue (intrauterine cannibalism) and yolk (oophagy) provisions. The pivotal interplay of the liver and ovary, during vitellogenesis, that impact on yolk formation was evident during the morphometric evaluation of hepatosomatic and gonadosomatic indices. Length, weight, uterine width, capsule production and migration trends of the females as well as length and weight relationship of the embryos were tabulated.

Reproductive hormones, assessed in maternal fluids (i.e. plasma (in RS1-5D females), uterine fluid (in RS4-5D females) and intracapsular fluid (in RS5A females), showed that follicle-stimulating-, progesterone- and oestradiol hormones were responsible for promoting vitellogenesis and encapsulation which led to three main stages where the

rate of ovulation increases in the female. Clinical biochemistry analysers confirmed the composition and concentration of biochemical analytes in same maternal fluids, which were found to be higher in the plasma.

Finally, heavy metals were found to be present in all three fluids, but found highest in the plasma, using inductively coupled mass spectrophotometry. In addition, variable pressure (VP) SEM confirmed the dental composition of the embryonic teeth found in the jaws of some embryos that appeared to escape encapsulation earlier than previously documented. It would appear that the embryos are creating adaptive ways to survive the intracannibalistic stage (RS5C) by escaping encapsulation early. However, the presence of heavy metals in the maternal fluids that surround the embryos could compromise their development over time; creating concern for a species that is Vulnerable.

This study, which serves as the first detailed analysis of the maternal-embryonic relationship, may serve as areas to model in forthcoming programmes aimed at increasing the numbers of this species.

CHAPTER 1

Introduction

Carcharias taurus (*C. taurus*) is a lamnoid shark species that is currently classified as "Vulnerable" globally including South Africa (SA) [1-3]. Sub-populations have been found to be Critically Endangered in Australia (Queensland) [2] and Southwest Atlantic [41] and Near Threatened in Western Australia [2]. The trend of the Critically Endangered *C. taurus* subpopulation is currently in decline [2,4] while other populations remain unknown [3]. This species may be better known by their common-names: Ragged-tooth (i.e., "raggie") (SA), Grey nurse (Australia) and Sand tiger (America). Local [5,6] and international [2,3,7] conservation and management programmes have had minimal success in population recovery. Additional strategies such as the application of breeding programmes [8-10] is required to increase that rate of recovery; which may otherwise take decades to recover [11-15]. Understanding these species biology, migration and reproductive behaviour would be crucial to the latter.

This *C. taurus*, reaches sexual maturity at six years [16], and is known to have one of the lowest fertility rates among chondrichthyans [17,18], due to its unique consistent practice of *in utero* cannibalism (/embryophagy: i.e., "eating siblings in uterus") that occurs biennially, during the early phases of a 9-12 month gestation period [11,19-22]. It has, however, been recently reported that adelphophagy occasionally occurs in another lamnoid *Isurus oxyrinchus* (*I. oxyrinchus*) [23]. Low fecundity, easy accessibility, overfishing (industrial or sport fishing) and anthropogenic activities [12] remain a detriment to their declining population regardless of the great steps to implement protection acts [3], recovery plans [2] and marine protected areas [7]. Their declining number is further impacted by high-levels of heavy metal environmental exposure from anthropogenic activity that creates additional concern to the development of an already limited progeny [24,25]. A naturally low breeding rate with a slow rebound potential to grow indicates that this population may require several decades to recover [11-15,20].

Studies to date have directly or indirectly contributed to the knowledge of *C. taurus* reproductive biology. Some of these studies looked at their reproductive behaviour, migratory distribution [5,12,20], general reproductive endocrinology [26], aplacental

viviparous mode of reproduction that includes oophagy and intrauterine cannibalism [17,21,27-30], extensive intrauterine embryology [17,21,27,31] as well as matrotrophic uterine modifications in elasmobranchs [27,29-33]. Understanding the maternal-embryonic relationship in *C. taurus* requires understanding how the female physiologically supports her progeny until birth. This relationship still requires clarification and assessment.

The current literature shows some ambiguities in Gilmore's (1993) description of epithelium of *C. taurus* as well as an extension of this species tissue presented by Hamlett and Hysell (1999). Gilmore (1993) inferred images from a similar reproductive mode in the lamnoid shark *I. Oxyrinchus*. Hamlett and Hysell (1998) did not examine the uterus from the different reproductive stages of the species. In addition, biochemical analysis has only been done on the plasma of *C. taurus* non gravid females (NGF). No biochemical investigations has been reported on the blood, intracapsular (ICF) and uterine fluid (UF) of *C. taurus* gravid females (GF)[34]. The profile of plasma hormones of both NGF and gravid females (GF) as well the UF and ICF of GF has never been documented in *C. taurus*. This study was undertaken to clarify the existing ambiguity and extending the current information on the uterine tissue [32] and fluid biochemistry [34] of *C. taurus* females through the different NGF and GF reproductive stages. Reproductive stages were determined by maturity indicators (i.e. set of measurements based on the female's biology) that was divided into two main groups i.e. NGF and GF. The NGF reproductive stages included the immature, immature-inactive, and mature-active females. The GF reproductive stages consisted of sharks pregnant with capsules alone or females pregnant with pre-hatched, post-hatched, intrauterine cannibalistic or a single embryo per uterus in addition to the capsules.

The knowledge gained from the histology and fluid biochemistry of this shark, together with additional findings in relation to their embryo survival, may be invested into a possible breeding intervention program postulated by the New South Wales (NSW) Department of Primary Industries (NSW DPI) [9]. This information will also serve as the first documentation for *C. taurus* to better understand the reproductive strategy and better management for both wild and captive species.

1.1 Rationale/Aim

To document the changes in the epithelium and wall of the uterine tissue as well as the biochemical composition (including associated reproductive hormones) of the plasma (and where possible the UF and ICF) through all the reproductive stages (i.e. from immature to pregnant) of the developmentally classified *C. taurus* females in SA.

This information would assist in understanding the maternal-embryonic relationship in *C. taurus* thereby advancing the elasmobranch reproductive and clinical understanding of this species. This information is necessary to institute a breeding intervention program aimed to increase and promote successful breeding by growing *C. taurus* embryos within an artificial uterus (AU) that was previously postulated by the NSW DPI to increase their *C. taurus* sharks [9]. Scientists at NSW DPI have already succeeded in creating AU technology for shark development with the *Orectolobus ornatus* (*O. ornatus*) embryos [8]. The hope is that the knowledge from studies investigating the physiology of *C. taurus* (including this study) can be incorporated into existing AU technology for shark development to assist with *C. taurus* population recovery in Australia. Such an undertaking will require a fundamental knowledge of the maternal environment to create proper *in vitro* AU for the propagation of *C. taurus* embryos. This study provides an insight into the creation of such an AU.

1.2 Hypothesis

This *C. taurus* females support the growth of their young through transformation of the uterine structure and associated fluids (i.e., plasma, UF and ICF).

1.3 Aims

This study aims to document the uterus and fluid (blood, ICF, UF) in the different reproductive stages of *C. taurus* females. To clarify the extent of maternal support to the embryos, the following objectives were investigated:

a) Record and confirm, using KwaZulu-Natal sharks board (KZNSB) records the morphometric measurements (i.e., body, reproductive organs and reproductive-associated structures) to determine any changes between the NGF and GF stage.
b) Use Light and Scanning electron microscope to examine changes in the uterine

epithelium and wall in the NGF and GF stages

c) Document the composition of plasma, with any significant trends in the analytes of the NGF and GF stages

d) Document the composition of the ICF and UF, with any significant trends in the analytes of the GF stages

e) Review any changes in the maternal uterus and fluid biochemistry (blood, ICF, UF) that could be necessary for embryo survival.

f) Document additional findings during the project that may have an impact on the development or survival of *C. taurus* embryos such as presence of heavy metals and the dentition of these embryos

1.4 Summarised study design

This study examined NG and GF *C. taurus* sharks. The studied migration pattern of these females and unfortunate situation of many of them being caught yearly in the KZNSB bather nets (under KZN 2008 Act) [11,35,36], created a unique sampling opportunity at KZNSB (Ethic no 076/10/Animal). This unfortunate opportunity allowed SA to contribute to the physiological information of these sharks [21,37,38]. To the best of our knowledge, the two subpopulations of *C. taurus* are stable along the KwaZulu-Natal provincial coastline of SA [11,39,40].

The study design of investigating the nine female reproductive stages, recruited into the study, was constructed on the reproductive staging (RS) that was assigned to each *C. taurus* female. The staging was based on maturity indicators (measurements associated with the female); a system used at KZNSB for all captures. The reproductive stages (RS) from NGF (immature to mature-sexually active; RS1-3) to GF (i.e. only capsules found; RS4 or capsules and pups found; RS5A-5D) sharks were assessed in this study. Detail of the criteria related to the reproductive staging, freshness evaluation, overall dissection process and sampling requirements are addressed in **CHAPTER 3** for all females. This chapter compared the morphometrics data of all sampled females (i.e., length, weight, uterine width (UW), capsule production, HSI and GSI). The histology of uterus was addressed in **CHAPTER 4** for all reproductive stages of the females. The wall and epithelium of the uterus was stained with Haematoxylin/Eosin and Periodic

Acid Schiff-Alcian Blue and analysed using light microscopy. The epithelium was further analysed using scanning electron microscopy. **CHAPTER 5** addressed the analysis of the female's plasma, using a clinical analyser that determined the concentration and composition of biochemical analytes in all NGF and GF. Analytes were also determined for the UF and ICF in respective GF sharks. The ICF, found in only three early-staged GF (i.e., RS5A), together with the plasma and UF from these females, were also analysed, using inductively coupled plasma mass spectrophotometry to determine the presence of heavy metals in **CHAPTER 6.** Finally, in **CHAPTER 7**, *C. taurus* shark embryos from these three females had their dental composition assessed using Variable Pressure SEM.

1.5 Novelty of the study

The morphological, histological and fluid biochemical analysis of *C. taurus* female is well adapted to provide the nurturing environment to develop her embryos to full term predators; with the possibility that the embryos are creating adaptive ways to survive. A concerning factor, is the presence of heavy metals, which the females appear to be offloading onto her progeny that could lead to devastating consequences over time for an already vulnerable species. It also reveals all aspects of the female's reproduction that needs to be considered when attempting to apply a physiological intervention to increase these species numbers.

The unique contribution of this study to the current literature of *C. taurus* females is as follows:

a) description of all NGF and GF reproductive stages
b) description and confirmation of the changes in the uterine epithelium in all reproductive stages
c) description of the changes in the uterine wall in all reproductive stages
d) composition and trends of the plasma and UF in GF stages
e) composition of the ICF in GF presenting with pre-hatched embryos
f) comparison of reproductive hormone changes through all stages
g) metal offloading/transference into the maternal fluid
h) early development of embryonic teeth in *C. taurus* embryos

1.6 Setup of the thesis

Due to the length of information for each section, it was decided that it would be best to compare the NG and GF in individual chapters dealing with morphometrics, histology and biochemistry themes. References are made to different chapters within the text where necessary. A detailed explanation of the possible maternal supportive role of *C. taurus* female extends throughout this thesis as follows:

- **CHAPTER 1**: a summarised breakdown of literature and flow of the thesis
- **CHAPTER 2**: Literature review
- **CHAPTER 3**: focuses on the morphometrics of the NGF and GF
- **CHAPTER 4**: describes the uterine wall and epithelium changes in the NGF and GF stages
- **CHAPTER 5**: investigates the composition and concentration of the plasma analytes and trends in the biochemistry of the NGF and GF stages. The compositions and concentration of analytes in the ICF and UF from GF with pre-hatched embryos are also reported here.
- **CHAPTER 6**: reports on the presence of heavy metals in the plasma, ICF and UF of GF with pre-hatched embryos.
- **CHAPTER 7**: describes some embryos, found in **CHAPTER 6**, that were found released from their capsules at a far smaller size than previously recorded. Dental structures were observed and reported in these *C. taurus* embryos
- **CHAPTER 8**: Discussion
- **CHAPTER 9**: Conclusion and proposed future work
- **CHAPTER 10**: Appendix
- Bridges appear between the chapters to guide the reader between the chapters

CHAPTER 2

Literature Review

2.1 Taxonomy and background of *Carcharias taurus*

Carcharias taurus (C. taurus) (formerly known as *Eugomphodus taurus*, *Odontaspis taurus*) is one of fifteen shark species in the family Odontaspididae, of the order Lamniformes. This shark species is known as Ragged-tooth (SA), Grey-nurse (Australia) and Sand tiger (United States of America). This elasmobranch species is one of the most widely investigated [11,12,22,26]. *C. taurus* held in captivity lives for 13-16 years, with wild species predicted to live longer. Evidence for longer longevity was provided by tagging data from a mature *C. taurus* female captured in Zinkwazi, (Durban, SA) 20 years after being tagged in St. Lucia (Durban, SA) [41]. It is the most widely kept large shark in aquariums around the world due to its adaptability and tolerance for captive habitats and conditions[42].

2.1.1 Assessment of C. taurus

There has been a huge decline, in some areas, in shark numbers over the years [43,44]. *Cacharias taurus* (in Australia) was the first shark species to be protected by law. Globally, this species has been assessed as Vulnerable by the International Union for Conservation of Nature's (IUCN) Red List of threatened species [3]. The risk status of *C. taurus* population does show variation at different parts of the world. The population along the east coast of Australia and southwest Atlantic are "Critically Endangered" [2,4], the western Australian subpopulation are "Near Threatened" and South African population is listed as "Vulnerable"[1,2]. Critically Endangered *C. taurus* subpopulation trend is currently in decline [2,45], the Western Australian subpopulation remains stable with other populations /subpopulation requiring further investigation [3].

2.1.2 Geographic distribution and diet

This demersal species is known to have an inshore, reef associated distribution mainly in subtropical to warm temperate waters, except for the eastern Pacific, around main continental landmasses [20,38]. The known cosmopolitan ocean distribution includes Western and Eastern Atlantic, Western Indian Ocean (except for Madagascar), Red Sea and the Western Pacific (except for New Zealand) [11,38]. They are often sighted in shallow waters such as shallow bays, surf zones, coral or rocky reefs. Stomach content analyses have shown that these sharks feed largely on teleosts, crabs, lobsters and small elasmobranchs, although cephalopods are also eaten [37,38,46].

2.1.3 Physical description and movement

This species, *C. taurus* shark, is a slow-moving shark that has a stout body, cream to brown in colour with large, irregular darker spots which fade with age **(Figure 2.1).** It has a pointed snout with a mouth that bears razor-like teeth that gives this animal a menacing look and false reputation of being dangerous. Females are generally longer than males. A distinguishing feature of this shark is that the anal and both dorsal fins are the same size. The tail has a long upper lobe and a shorter lower lobe.

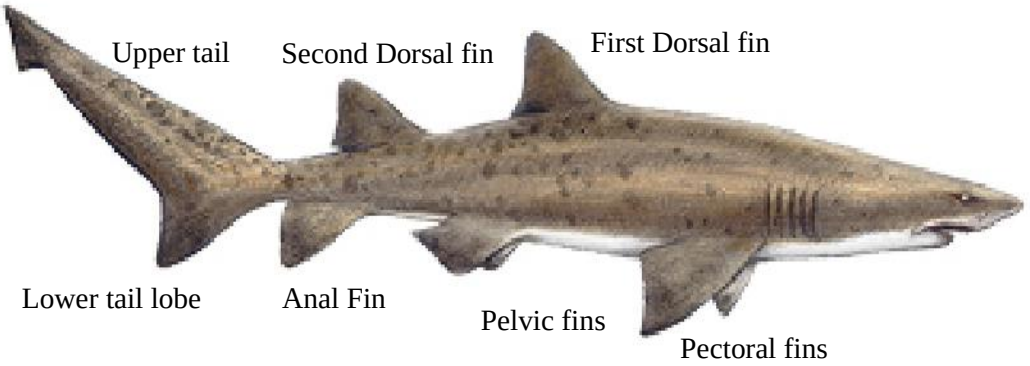


Figure 2.1: Physical Images of *C. taurus*. Image reference: (http://www.oceansafrica.com/ragged-tooth-shark/**)**

2.1.4 Biology

Bass *et al*. (1975) provided the first information on the biology of this species in SA. *C. taurus* reproduces through aplacental viviparity and has one of the lowest fertility rates among chondrichthyans [17,18,37]. This is due to this species' natural history characteristics that encompass late sexual maturity of the females (at 2.2 meters (estimated six years old) [16,45], low fecundity (i.e., only one embryo per uterus) born every two years (i.e., biennial reproductive cycle) [11,19-22] after a long 9-12 month gestation period [21]. This species, however, produces a progeny that is born as a large and fully developed predator that retains the ability to not depend on maternal care for survival.

2.1.5 The importance of C. taurus and protection strategies

Studies show huge declines in shark stocks (depletion of over 90%) due to overfishing (industrial or sport fishing) [47] and various activities locally and internationally [48-52]. Coupled to this is its natural biological characteristics, false fierce appearance and easy accessibility [12], explains why its population is in decline. In addition, the presence of high-levels of contamination from anthropogenic activity e.g. mining, urbanisation, industrial construction, commercial shipping, gas exploration to name but a few [48-52] causes additional concern (e.g. heavy metal toxicity) to the development of an already limited progeny [24,53]. The presence of metals and their associated toxicity have been shown to increase in tissues (most investigated is the liver and muscle) during the lifespan of the animal [54] linked to impaired reproduction, amongst other effects [55]. Metal burden in elasmobranchs has been shown internationally and locally [56-58] as well as in the maternal fluids of *C. taurus* in SA [59]. The overexploitation of this species that has a naturally low breeding rate with a slow rebound potential indicates a lengthy period of time, possibly decades, to recover [3,11-15,20].

Sharks provide stability and biodiversity to the marine ecosystem that has long-term benefits on communities [43,44] therefore implementing conservation programmes is

crucial. However, regardless of the protection acts implemented [3], establishments of recovery plans [2] and marine protected areas [7], a solution to increase this species population size over and above their natural breeding ability is still urgently needed. Breeding programmes have been successfully used previously to sustain species such as Brown-banded bamboo sharks, Pacific white sided dolphins and Bottlenose dolphins [10]. There have been attempts to gain knowledge to breed *C. taurus*. An artificial uterus (AU), created by the NSW DPI, showed success in growing *O. ornatus* embryos, a species that also gives birth to live embryos. The technical skills are intended to be applied to their *C. taurus* species (i.e., Grey nurse shark) in an attempt to increase their sharks declining numbers [8,9]. In addition, an artificial insemination programme to improve the breeding of *C. taurus* sharks) was launched in the Dubai aquarium (2015) [10].

2.2 Reproduction

Studies on elasmobranch reproductive modes, reproductive cycles (i.e., ovulatory and endocrine cycles) and migratory behaviour have played vital roles in understanding their reproductive patterns [15,22,26].

2.2.1 Migration

Variation exists in female migration (movement patterns) of sharks [15,17,22,60-62]. *C. taurus* are located mainly along the eastern and southern coasts from Cape Town to northern KwaZulu-Natal (KZN) in SA (**Figure 2.2)**. Mating is believed to occur off the south coast of KZN. This is followed by pregnant females moving northward to gestate in warmer waters of northern KZN and southern Mozambique [6]. The near-term, pregnant females then migrate south to cooler waters of the Eastern Cape which serve as their pupping ground [11,19,63-65]. Bass *et al*. (1975) was the first to record migration of pregnant females to the Eastern Cape where embryos are born.

2.2.2 Reproduction and ovulatory cycles

The reproductive cycle is the annual gestation (and resting pattern) elasmobranchs

follow which do not segregate species on reproductive mode. There are three types of reproductive cycles: 1) A continuous breeder is a female that has no rest between pregnancies, 2) a seasonal breeder is a female that is pregnant for only a portion of the year, 3) while a punctuated breeder is a female that has a one-two-year interval between pregnancies [26]. *C. taurus* is a punctuated breeder [26]. These females have a biennial reproductive cycle (i.e., a year of rest between pregnancies) [11,66] reported in the south west Atlantic population [22,27]. There has also been reports of an annual cycle for the north west Atlantic population [17,61] and south eastern coast of Australia [16,62].

Ovulatory cycles are dependent on follicle growth. Follicle development, occurring in the ovary, can develop-continuously throughout a reproductive cycle or could be restricted to some portion of the cycle [26]. Continuous breeders can have follicle development occurring continuously during pregnancy or it could be restricted to a phase of the pregnancy stage. Seasonal/Punctuated breeders have ovulatory cycles restricted between pregnancies, during the months preceding the next ovulation. The female, however, exhibits oophagy which would suggest the ovulatory cycle will be superimposed on the pregnancy stages to allow eggs to be ovulated to provide nutrients to the developing embryos [17,26]**.**

The lengthy resting phase, after a long gestation, reflects the time required for the female to acquire and store sufficient nutrients (energy) in her liver that will be required for the next pregnancy and new progeny [66]. The changes in indices of the ovary (gonadosomatic index: GSI (%) = ovary weight/total body weight (kg) *100) and liver (hepatosomatic index: HSI(%) = liver weight (kg)/ total body weight (kg) *100) are valuable tools in evaluating female and embryos to indicate the nutritional and energy reserve [18,21,27].

2.2.3 Reproductive tract

The ova travel from a single function right ovary through a pair of tube-like structures called the oviducts, which comprise four sections, consistent with most modern chondrichthyans i.e., ostium/anterior tube, oviducal gland (O.G), isthmus and uterus on

the left and right sides of the female (**Figure 2.3**). Ova moves from the ovary into both the left and right anterior oviducts, where fertilisation occurs (near the ostium). Fertilised and unfertilised ova then move into the respective O.G (the tissue that encapsulates the ova in a membranous collagenous sheath). This is where ova (1-23) are encased in to forms 6 distinct types of capsules (membranous collagen sheath) [17,21]. Some contain only ovalbumin and/or mucus while others contain several fertilized ova [21]. These capsules leave the O.G and pass through the isthmus to enter and reside in the uterus, wherein the ova develop into functional embryos until birth [29,30,32] . A maximum of three embryos have been reported to develop from one capsule [27].

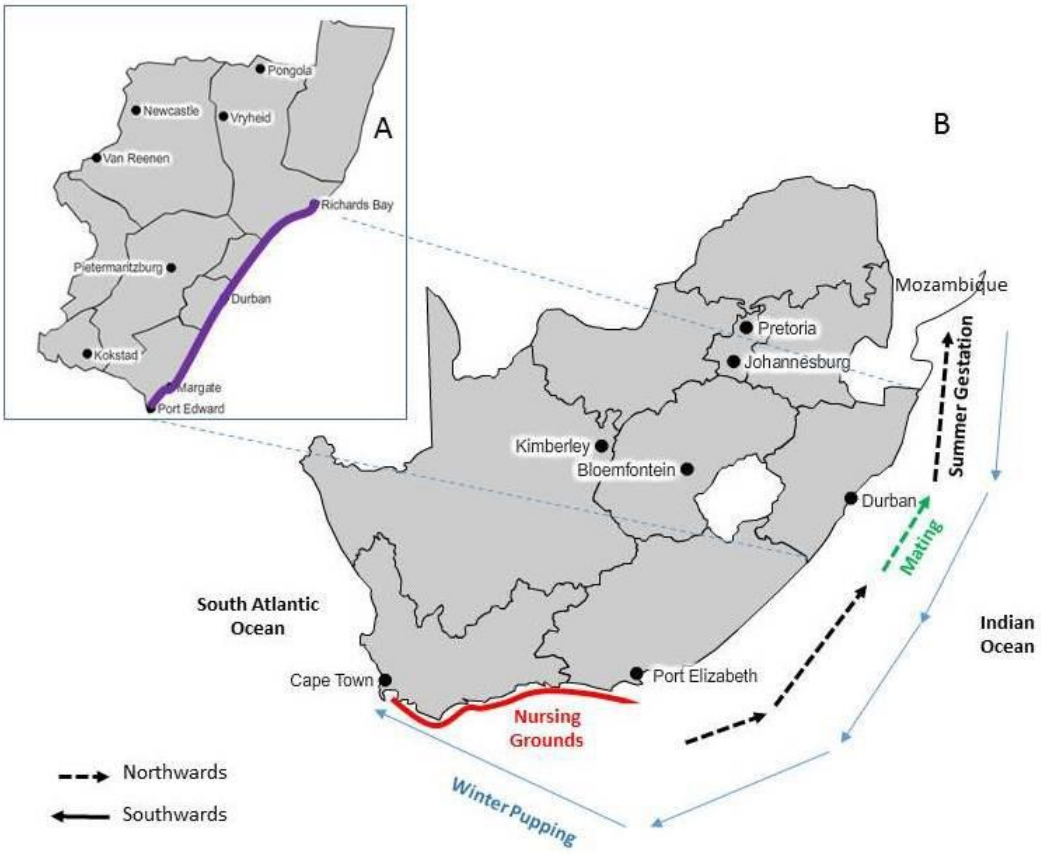


Figure 2.2: Illustration B shows the reproductive movements of *C. taurus* shark. Insert A (box) illustrates the KZNSB bather protection region along the coastline. Image adapted from (http://www.booktravel.travel/index.php).

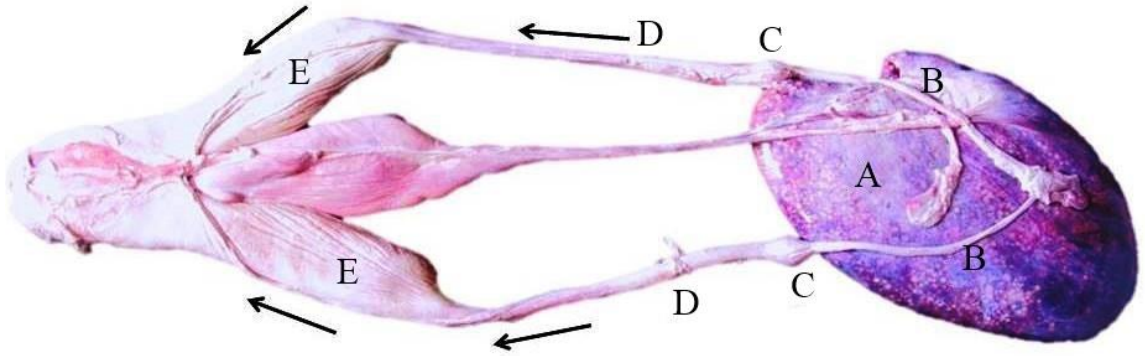


Figure 2.3: Reproductive tract of female *C. taurus*. The bi-tract consists of the A) ovary, B) anterior tube/oviduct, C) O.G, D) isthmus and E) uterus on the left and right sides of the female. Arrows (left and right sides) represent movement of ova.

2.2.3.1 Uterus

Gestation occurs in the uterus of all chondrichthyans. The uterus of *C. taurus* provides a matrotrophic environment, during gestation, where both uteri support the growth and development of the embryos until parturition.

2.2.3.2 Gestation

Gilmore (1983) has identified six stages of *C. taurus* embryo development [17,21,27]. They are:

1. Stage I-II: Pre-hatched embryos (13-60 mm). The embryos initial development occurs within capsules, derived from the O.G., filled with intracapsular fluid (ICF). Embryos will develop from fertilised ovum in the capsule (blastodisc) (**Figure 2.4A**). Literature indicates that three embryos have been recorded emerging from one capsule [17]. The encased embryos (EE) inside the capsule develop to approximately 57-60 mm TL (Total length i.e., length taken from tip of the snout to the end of the tail (at a swimming angle which is defined as measurement from snout to natural position of the tail) (**Figure 2.4B)** before it can escape the capsule. Each embryo emerges from its capsule retaining an external yolk sac (**Figure 2.4C-D**). Embryonic dentition (not resembling adult teeth) develops between 40-60 mm TL. This enables the embryo to

escape encapsulation and enter the next stage. However, Hamlett (1983) did note non-functional dentition in 30 mm and 35 mm TL *C. taurus* embryos; but only described functional teeth at 40 mm TL; with teeth appearing on both jaws around 45 mm TL and double rows of teeth appearing at 55 mm TL. Hamlett (1983) also reported a 49 mm TL FFE that appeared to have wide, erect teeth.

2. Stage III: Post-hatched embryos (60-100 mm), free-floating embryos (FFE), with yolk sac still evident. These embryos are released from the capsule (a collagenous sheath) into the uterus filled with fluid (i.e., uterine fluid or extra capsular fluid: UF/ECF). Rudimentary dentition is evident during this phase (**Figure 2.4D**).
3. Stage IV: Intrauterine cannibalistic phase (100-335 mm TL). The embryos have exhausted their yolk sac and there are several embryos in each uterus. The largest embryos begin to attack their siblings within the uterus (**Figure 2.4E-F**). External gill filaments were present.
4. Stage V: Oophagous phase (335-1000 mm TL), only a single embryo in each uterus. The fittest embryo (after consuming all other embryos), will consume capsules containing unfertilised eggs that the female produces. This yolk is stored in the stomach creating the typical distended embryo belly **Figure 2.4G**).
5. Stage VI: embryonic development occurs through gestation in the uterus until parturition where only one embryo per uterus is born. Oophagous/pre-parturition phase (900-1000 mm TL): Embryo also starts to take on a slender appearance, a morphology that aids with the birthing process. A larger liver and decreased yolk consumption, closer to near term embryos is noticed in this stage [27]. The embryo reaches a length/weight plateau which indicates a morphological and metabolic stasis period reached during gestation close to parturition (**Figure 2.4H**).

2.2.3.3 Uterine structure

The chondrichthyan uterus comprises four main tissue layers [29-31]. The layers are: 1) luminal epithelium with a basement membrane, 2) connective tissue that is vascularised, 3) smooth muscle (circular and longitudinal) and 4) simple squamous serosa [32,67-69].

The uterine structure which supports the *in utero* growth and development of embryos

has been investigated a number of viviparous species [27,32,70-72] . To support their developing young, many female elasmobranchs have developed different reproductive modes to satisfy their embryos' nutritional requirements, once the embryos' own nutrients are depleted (i.e., females are matrotrophic).

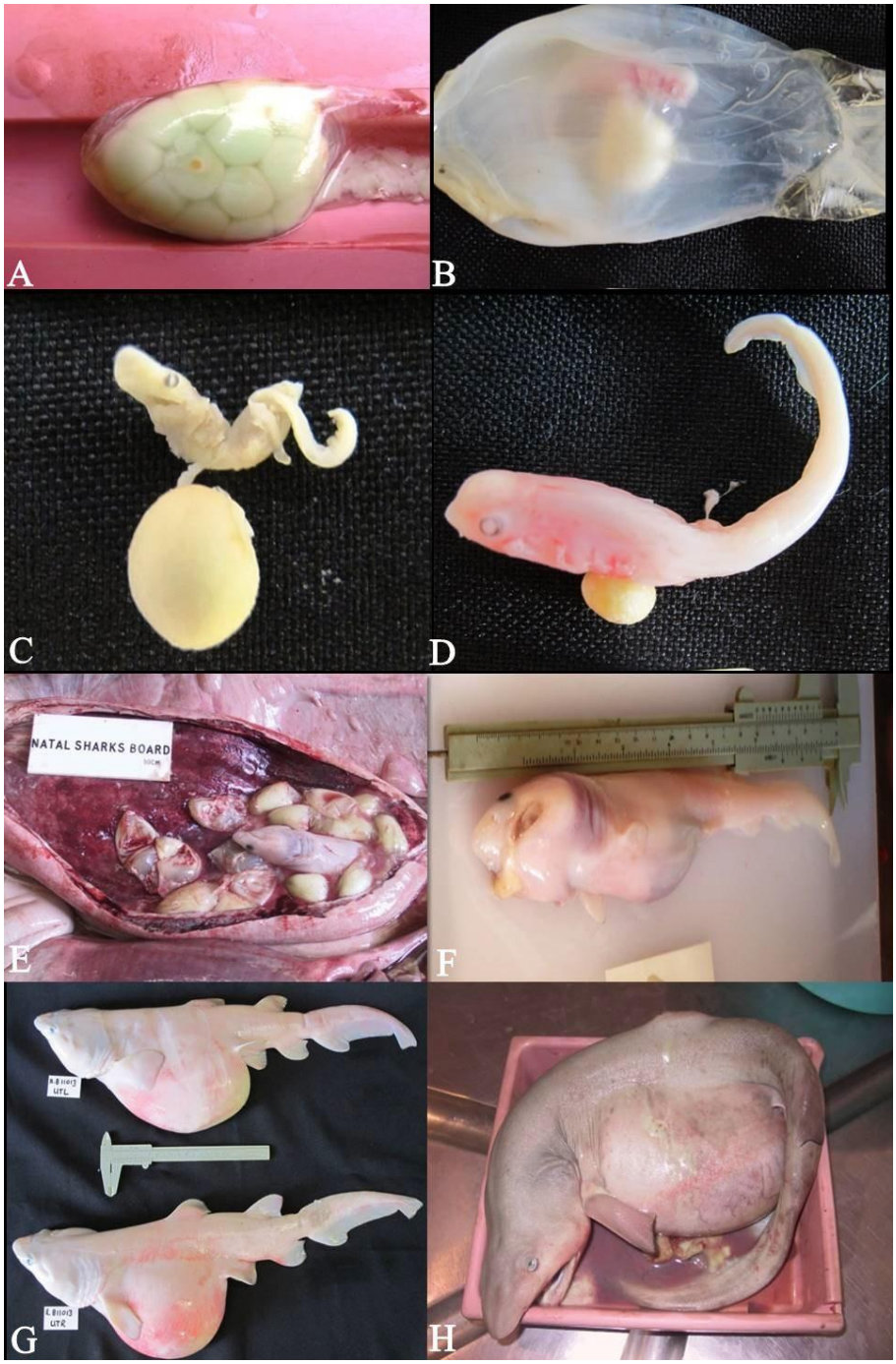


Figure 2.4: *C. taurus* embryology. Stage I-II: A) ova encapsulated with blastodisc, B) embryo encapsulated (EE) filled with ICF, C) a free-floating embryo (FFE) out of its capsule (15 mm TL) and Stage III: D) a longer FFE (65 mm TL) with yolk sac attached. Stage IV: E) embryo and capsules in uterus showing evidence of intrauterine cannibalism and F) embryo/pup (166 mm TL) with yolk in its mouth. Stage V: G) oophagous embryos from the left and right uterus (535 mm and 550 mm TL). Stage VI: H) embryo (900 mm TL) with slender features and protruding belly filled with yolk.

2.2.3.4 Reproductive mode: viviparity, oophagy and adelphophagy

Both main reproductive modes oviparity ("egg-bearing") and viviparity ('live bearing") entail that embryos are initially yolk reliant. Oophagous is a term used to denote embryos feeding on unfertilised eggs filled with yolk. Both these modes can be Lecithotrophic (i.e., embryo receive no other nutrition except yolk) or Matrotrophic i.e., additional maternal care, apart from yolk provision, during embryo development) (**Table 2.1)**. Viviparous species display an array of practices to extend nutrient provision through four viviparous modes [28] (**Table 2.1**) which depend on maternal tissues to satisfy their nutritional, respiratory and excretory needs[17,21,27,29,30,69].

Swenander (1907) was the first to describe embryos and oophagy (supply of unfertilised eggs) in a lamnoid, Porbeagle shark (*Lamna. nasus*). The yolk is deposited into the oocyte through a process called vitellogenesis. The yolk is synthesised in the liver of the female and transported to the follicles in the ovary. This is seen in all lamnoid species, except for *C. taurus* that extends this oophagous phase by consuming *in utero* siblings (intrauterine cannibalism/ adelphophagy) until there is only one embryo per uterus. The last mode (mode 4) is based on presence of placenta in the female.

Table 2.1: Chondrichthyan reproductive modes

Mode	Type	Definition	Mode No	Leicotrophy	Matrotrophy
Oviparity				x	
Viviparity	Aplacental	Yolk-Sac reliant through gestation	1	x	
		Depletion of embryonic sac. Uterine mucosa develops uterine villi or trophonemata, that secretes nutrient called histotroph	2		x
		Depletion of embryonic sac. Relies on oophagy (with/without adelphophagy)	3		x
	Placental	Placental (with or without appendicular)	4		x

Springer (1948) was the first to document the sacrifice of *C. taurus* embryos to produce few large, well developed sharks. This is what would later be referred to as intrauterine cannibalism [18]. Bass *et al*. (1975) provided the first description of *C. taurus* embryonic cannibalism in SA. Although adelphophagy has only been consistently recorded in *C. taurus* species [17,21], this trait has been reported to occasionally occur in another lamnoid, the shortfin mako (*I. oxyrinchus*) in the north-western (NW) Pacific [23]. However, it was only recorded in two of the six captured *I. oxyrinchus* females [23].

2.2.3.5 Uterine modifications

During pregnancy, the uterus becomes modified to support embryo development once the embryonic yolk supply is exhausted. The uterus needs to become modified to supply the embryos with accommodation, oxygen, nutrients, remove waste and regulate the intrauterine environment [29,32-34]. Studies show that these modifications are achieved through development of folds/ villi that may not be secretory, development of uterine compartments, reduction of epithelial layers between maternal and fetal tissue and dilatation of intercellular spaces [27,29,68,73,74]. The term uterine lamellae is also used for the description of folds found along the epithelium [73,74].

Histological investigations of *C. taurus* uterine specialisations have been documented to some extent. Hamlett and Hysell (1998) reported the lack of uterine lamellae (i.e., folds) present through the epithelium of a mature female, to the presence of lamellae and blood vessels in a mature G *C. taurus* [32]. They also noted that the uterus had no structural provision for uterine secretions [32]. Gilmore (1993) reported on increased uterine lamellae and micro-fold ridges on another lamnoid, *I. oxyrinchus*. He stated that the increased surface area and location of blood vessels could oxygenate the fluid found in the uterus (i.e. uterine fluid: UF) and enhance fluid secretion [17]. Aplacental studies have suggested that the vascularised uterine lamellae (UL)/(folds) function as a respiratory membrane [27,32].

2.2.3.6 Uterine Fluid (UF)

The fluid in the lumen of the uterus during gestation is referred to as the uterine fluid (UF) [27]. Some literature refers to it as the extra capsular fluid (ECF) [69]. It surrounds the capsules and embryos within the uterus. Studies have shown that UF is present during gestation [67,75]with an increase in volume during the gestation of free floating embryos (FFE) (Stage III). An investigation into the composition of the UF has occurred in a variation of shark species [76-84].

Many aplacental studies have suggested that the vascularised UL/folds function as a respiratory membrane [17,32,68,73,74,85]. Tomita *et al.* (2015) was the first to provide evidence that aplacental embryos can attain oxygen through the UF. It was shown that the uterine epithelium, in an aplacental species, can provide adequate oxygen supply to early-mid gestating *S. canicula* embryos [84]. This amount of oxygen, however, is inadequate for late stage *S. canicula* embryos and they are believed to acquire their primary source of oxygen from uterine seawater during the intermittent uterine flushing [76]. This uterine flushing, which creates intermittent exchange of UF with external seawater, could possibly play a similar role in similar aplacental reproductive modes where the UF facilitates respiration and waste removal [67,75]. This study together with other elasmobranch studies [74,84,86] showed that these aplacental embryos use the method of buccal pumping to ventilate their gills. Buccal pumping is the active process of drawing and controlling the intake of oxygen [87]. It is believed that buccal pumping allows the embryo to control the pumping action and keep the oxygen environment stable, when changes such as the introduction of seawater occurs [75,86].

The UF, in some species, is speculated to provide nutrients to developing embryos [67,75]. A recent publication termed the UF fluid, “embryotroph”, due to the increase in weight of aplacental Tiger shark embryos (*G. cuvier*) enclosed in this nutritional fluid [86]. It has also been speculated that UF could be laden with nutritive supplies that post-hatched *C. taurus* embryos utilise initially prior to external yolk indicated by the presence of gill filaments in this post hatch period [27].

Monitoring of changes in the chemical composition of the UF during gestation could provide evidence for uterine flushing, a process whereby a female shark can periodically flush her uteri with seawater, allowing the UF to resemble sea water [80,81,87]. It causes the UF, which appears more like the maternal plasma during early gestation, to change to a more seawater appearance during late gestation [75-77,79,80]. This process is believed to occur once embryos have hatched from their capsules [76,77,80,82]. Evidently osmoregulation would be required by embryos entertaining a seawater environment [80,81]. This fluid is believed to serve as an *in utero* oxygen, nutrient reservoir, provide lubrication for the uterus epithelium and assist with osmoregulation [17,27,80,81].

2.2.3.7 Intracapsular Fluid (ICF)

The fluid found within the capsules is referred to as the intracapsular fluid (ICF) [60]. The ICF has been examined fewer studies [75,88]. The ICF is greatly reduced or absent in later stages of gestation and its composition will be influenced by the enclosed embryos [75].

2.2.3.8 Plasma

Studies of shark plasma analytes have been undertaken, which focused on the general composition, osmoregulation, urea metabolism, immunological studies and their changes in response to some form of stress [52,89-95]. Studies investigating biochemical changes in elasmobranchs in response to stress has been recorded [96-98] including *C. taurus* [99]. Very few studies have established clinical reference values in sharks [34,96,100]. The most recent biochemical plasma profile was undertaken for male and female *C. taurus* [34]. The presence of blood vessels in the uterus indicates the important role its development plays in embryo development to allow a medium for respiratory exchange for the developing embryos.

Recent attention to capture-induced parturition in live bearing elasmobranchs [101] shows evidence of premature or aborted foetus that have an indirect effect on mortality rates of the species currently not recorded [101]. Recent study has shown that acute

induced capture stress (i.e., few minutes/hours) can compromise the pregnancy and life of the female and indicated the possible analytes most affected [102]. Post-mortem reference values can also assess when a female is reaching dangerous levels of stress [103]

2.2.4 Reproductive endocrinology

Little is known about the endocrine mechanisms [104] that regulate viviparity, oophagy and adelphophagy modes. Endocrine control of the reproductive tract and associated reproductive events and modes are achieved by regulating the interplay of morphological and physiological processes [26,105]. They play an important role in regulating major events in reproduction [106]. Biochemical steroidal reporting, with regards to reproduction, has been studied in some female shark species across the different reproductive modes [105,107-115]as well as skates and stingrays [108,116-118]. Serum hormone levels were assessed serially for mature NGF *C. taurus* sharks [114,119] which indicated the role progesterone and oestradiol played to control the reproductive cycles (ovulation and follicular phases) that allowed females of all modes to provide the necessary nutrients as well as the ability to rest and build the reserves needed for the next pregnancy. Literature shows that most of the endocrinology studies in elasmobranchs have focused on progesterone and oestradiol [26,105] with very little known about luteinising (LH) and follicle stimulating hormones (FSH) [120,121].

2.2.5 Maternal offloading

Oophagy has also been documented as a reproductive mode with a high level of maternal contaminant offloading [24,25]. Maternal offloading has been documented in elasmobranch matrotrophic sharks [122-124]. The bio-accumulated contaminants, from the females' diet and environment, can be passively transferred from the female to her offspring through maternal lipid mobilisation during lactation or oophagy. These lipids are derived from the liver, which accumulate the contaminants. Female *C. taurus* utilises both oophagy and adelphophagy nutrient provision pathways suggesting a high risk for developmental problems in embryos, in an already limited number of progeny.

2.3 Reasons for the study

The commercially targeted Dwarf ornate wobbegong shark (*O. ornatus*) has declined and are listed as "Vulnerable" in New South Wales (NSW) on the World Conservation Union (IUCN) Red list [125]. Pregnant wobbegong are known to spontaneously abort their developing embryos after capture. Placement of these late-termed embryos in small aquarium environments resulted in 100% mortality. The UF of the *O. ornatus* was found to change from complex (in early gestation) to simple conditions' which saw mid-to late termed embryos immersed into a seawater-like UF [8,76]. An artificial uterus was constructed for these embryos, with the placement of late-termed embryos in an artificial UF, comprising of filtered seawater [8]. Investigation into the uterus of the *O. ornatus* [76], as well a previous research on *S. acanthias* [71] and *M. antarcticus* revealed that the uterus modifies itself to facilitate respiration for their aplacental reliant embryos. The AU was prepared following the knowledge gained in all the above studies [76,126] and showed survival and growth of the embryos in the AU [8].

The "Near Threatened" status of *C. taurus* in SA caused by overexploitation, late maturity and low fecundity [11,12] suggests that this species could benefit from an AU breeding intervention. The techniques acquired in the *O. ornatus* breeding programme, can be applied to the information acquired and analysed on the uterus and fluid chemistry of *C. taurus*. Previous studies on *C. taurus* created some ambiguity or lacked all necessary information required. Clarification was needed on Gilmore (1993) conclusions that inferred the description of the uterine epithelium of the species *I. oxyrinchus* onto *C. taurus*. Hamlett and Hysell (1998) uterine epithelium changes and Otway [34] serum findings on *C. taurus* needed to be extended for the different reproductive stages of this species.

The aim of this study was to clarify the above queries through investigating the uterine tissue and biochemical analytes of the maternal fluids of the local *C. taurus* population in SA to better understand the aplacental maternal-embryonic relationship in this species. These results will feed into a breeding intervention program discussed by the

NSW DPI to increase the numbers of *C. taurus* population in Australia [9]. In addition, these results will serve as the first detailed description of the epithelium and wall of the uterine tissue as well as the composition and concentration of the chemistry analytes in the maternal plasma, ICF and UF in all reproductive stages of *C. taurus*. These results may also assist in other conversation practices such as appropriate management strategies for wild species or provision of more suitable husbandry for aquarium held species

2.4 References

1. Dudley, S. (2013) In Mann, B. (ed.), *Southern African Marine Linefish Species Profiles Special Publication No 9.* Durban Oceanic Research Institute, 134-136.
2. Pollard, D., Gordon, I., Williams, S., Flaherty, A. and McAuley, R. (2003) *Carcharias taurus (east coast of Australia). 2006 IUCN Red List of Threatened Species.* IUCN, Gland, Switzerland e.T44070A10854830. http://www.iucnredlist.org. Accessed 15 July 2013.
3. Pollard, D. and Smith, A. (2009) *Carcharias taurus. Version 2012.2: IUCN Red List of Threatened Species.* e.T3854A10132481. http://www.iucnredlist.org. Accessed 23 March 2010.
4. Chiaramonte, G., Domingo, A. and Soto, J. (2007) *Carcharias taurus (Southwest Atlantic Subpopulation).* The IUCN Red List of Threatened Species 2007.e.T63163A12625032. http://www.iucnredlist.org. Accessed 21 July 2015.
5. Olbers, J. and Cliff, G. (2017) *A brief survey of ragged-tooth shark (Carcharias taurus) abundance and behaviour during their annual gestation period on Quarter-mile reef, Sodwana Bay, iSimangaliso Wetland Park, South Africa.* Season Report for 2016/2017. Ezemvelo KZN Wildlife Internal Report, Pietermaritzburg, South Africa, pp 15.
6. Da Silva, C., Booth, A., Dudley, S., Kerwath, S., Lamberth, S., Leslie, R., McCord, M., Sauer, W. and Zweig, T. (2015) *The current status and management of South Africa's chondrichthyan fisheries.* African Journal of Marine Science, **37**, 233-248.
7. Lynch, T.P., Harcourt, R., Edgar, G. and Barrett, N. (2013) *Conservation of the critically endangered Eastern Australian population of the grey nurse shark (Carcharias taurus) through cross-jurisdictional management of a network of marine-protected areas.* Environmental Management, **52**, 1341-1354.
8. Otway, N. and Ellis, M. (2012) *Construction and test of an artificial uterus for ex situ development of shark embryos.* Zoo Biology, **31**, 197-205.
9. Venton, D. (2011) *Baby Sharks Birthed in Artificial Uterus.* Wired. http://www.wired.com/2011/09/artificial-shark-uterus. Accessed 23 June 2014.
10. Nadeem, H. (2015) *Dubai launches world's first breeding programme for endangered sand tiger sharks.* The National. http://www.thenational.ae.

Accessed 23 August 2016.

11. Dicken, M.L. (2006) P*opulation dynamics of the raggedtooth shark (Carcharias taurus) along the east coast of South Africa*. PhD Thesis, Rhodes University, South Africa.

12. Otway, N.M., Bradshaw, C.J. and Harcourt, R.G. (2004) *Estimating the rate of quasi-extinction of the Australian grey nurse shark (Carcharias taurus) population using deterministic age-and stage-classified models.* Biological Conservation, **119**, 341-350.

13. Dulvy, N.K., Baum, J.K., Clarke, S., Compagno, L.J., Cortes, E., Domingo, A., Fordham, S., Fowler, S., Francis, M.P. and Gibson, C. (2008) *You can swim but you can't hide: the global status and conservation of oceanic pelagic sharks and rays.* Aquatic Conservation: Marine and Freshwater Ecosystems, **18**, 459-482.

14. Dulvy, N.K., Sadovy, Y. and Reynolds, J.D. (2003) *Extinction vulnerability in marine populations.* Fish and Fisheries, **4**, 25-64.

15. Otway, N.M. and Parker, P.C. (2000) *The biology, ecology, distribution, abundance and identification of marine protected areas for the conservation of threatened Grey Nurse Sharks in south east Australian waters.* NSW Fisheries final report series No. 19. NSW Fisheries, Sydney, NSW, Australia., pp 132.

16. Goldman, K.J., Branstetter, S. and Musick, J.A. (2006) *A re-examination of the age and growth of sand tiger sharks, Carcharias taurus, in the western North Atlantic: the importance of ageing protocols and use of multiple back-calculation techniques.* Environmental Biology of Fishes, **77**, 241-252.

17. Gilmore, R.G. (1993) *Reproductive biology of lamnoid sharks.* Environmental Biology of Fishes, **38**, 95-114.

18. Springer, S. (1948) *Oviphagous embryos of the sand shark, Carcharias taurus.* Copeia, 153-157.

19. Cliff, G. (1989) *Breeding migration of the sand tiger shark Carcharias taurus in southern African waters.* In: 5th annual meeting of the American Elasmobranch Society, San Francisco, 17-23.

20. Dicken, M., Booth, A., Smale, M. and Cliff, G. (2007) *Spatial and seasonal distribution patterns of juvenile and adult raggedtooth sharks (Carcharias taurus) tagged off the east coast of South Africa.* Marine and Freshwater

Research, **58**, 127-134.

21. Gilmore, R.G., Dodrill, J.W. and Linley, P.A. (1983) *Reproduction and embryonic development of the sand tiger shark, Odontaspis taurus (Rafinesque).* Fishery Bulletin, **81**, 201-225.

22. Lucifora, L.O., Menni, R.C. and Escalante, A.H. (2002) *Reproductive ecology and abundance of the sand tiger shark, Carcharias taurus, from the southwestern Atlantic.* ICES Journal of Marine Science: Journal du Conseil, **59**, 553-561.

23. Joung, S.J. and Hsu, H.H. (2005) *Reproduction and embryonic development of the shortfin mako, Isurus oxyrinchus Rafinesque, 1810, in the northwestern Pacific.* Zoological Studies Taipei, **44**, 487.

24. Lyons, K., Carlisle, A., Preti, A., Mull, C., Blasius, M., O'Sullivan, J., Winkler, C. and Lowe, C.G. (2013) *Effects of trophic ecology and habitat use on maternal transfer of contaminants in four species of young of the year lamniform sharks.* Marine Environmental Research, **90**, 27-38.

25. Mull, C.G., Lyons, K., Blasius, M.E., Winkler, C., O'Sullivan, J.B. and Lowe, C.G. (2013) *Evidence of maternal offloading of organic contaminants in white sharks (Carcharodon carcharias).* PLoS ONE, **8**, e62886.

26. Koob, T.J. and Callard, I.P. (1999) *Reproductive endocrinology of female elasmobranchs: lessons from the little skate (Raja erinacea) and spiny dogfish (Squalus acanthias).* Journal of Experimental Zoology, **284**, 557-574.

27. Gilmore, R.G., Putz, O. and Dodrill, J.W. (2005) In Hamlett WC (ed.), *Reproductive biology and phylogeny of Chondrichthyes: Sharks, Batoids and Chimaeras.* Science Publishers, Enfield, New Hampshire, USA, 435-462.

28. Musick, J. and Ellis, J. (2005) In Jamieson BGM (ed.), *Reproductive biology and phylogeny of Chondrichthyes: sharks, batoids and chimeaeras*, 3, 45-79.

29. Wourms, J.P. (1977) *Reproduction and development in chondrichthyan fishes.* American Zoologist, **17**, 379-410.

30. Wourms, J.P. (1981) *Viviparity: the maternal-fetal relationship in fishes.* American Zoologist, **21**, 473-515.

31. Hamlett, W.C. (1983) *Maternal-fetal relations in elasmobranc fishes*. PhD Thesis, University of Clemson, USA.

32. Hamlett, W.C. and Hysell, M.K. (1998) *Uterine specializations in elasmobranchs.* Journal of Experimental Zoology, **282**, 438-459.

33. Hamlett, W.C., Kormanik, G., Storrie, M., Stevens, B. and Walker, T.I. (2005) *Chondrichthyan parity, lecithotrophy and matrotrophy.* Reproductive Biology and Phylogeny of Chondrichthyes: sharks, batoids and chimaeras, **3**, 394-434.

34. Otway, N.M. (2015) *Serum biochemical reference intervals for free-living Sand Tiger sharks (Carcharias taurus) from east Australian waters.* Veterinary Clinical Pathology, **44**, 262-274.

35. Cliff, G., Dudley, S. and Davis, B. (1990) *Sharks caught in the protective gill nets off Natal, South Africa. 3. The shortfin mako shark Isurus oxyrinchus (Rafinesque).* South African Journal of Marine Science, **9**, 115-126.

36. Cliff, G. and Dudley, S.F.J. (1992) *Protection against shark attack in South Africa, 1952-90.* Marine and Freshwater Research, **43**, 263-272.

37. Bass, A.J., Aubrey, J.D. and Kistnasamy, N. (1975). Oceanographic Research Institute, Durban, South Africa, 1-100.

38. Compagno, L. (1984) *FAO species catalogue. Vol. 4. Sharks of the World. An annoted and illustrated catalogue of sharks species known to date. Part. 1. Hexanchiformes to Lamniformes.* FAO Fisheries Synopsis, **4**, pp 249.

39. Dicken, M.L., Booth, A.J. and Smale, M.J. (2008) *Estimates of juvenile and adult raggedtooth shark (Carcharias taurus) abundance along the east coast of South Africa.* Canadian Journal of Fisheries and Aquatic Sciences, **65**, 621-632.

40. Klein, J.D., Bester-van der Merwe, A.E., Dicken, M.L., Mmonwa, K.L. and Teske, P.R. (2019) *Reproductive philopatry in a coastal shark drives age-related population structure.* Marine Biology, **166**, 26.

41. Ngcukana, L. (2010) *Shark swims free for 7436.* News24archives, https://www.news24.com. Accessed 12 December 2011.

42. Smale, M.J., Booth, A.J., Farquhar, M.R., Meÿer, M.R. and Rochat, L. (2012) *Migration and habitat use of formerly captive and wild raggedtooth sharks (Carcharias taurus) on the southeast coast of South Africa.* Marine Biology Research, **8**, 115-128.

43. Dulvy, N.K., Fowler, S.L., Musick, J.A., Cavanagh, R.D., Kyne, P.M., Harrison, L.R., Carlson, J.K., Davidson, L.N., Fordham, S.V. and Francis, M.P. (2014)

Extinction risk and conservation of the world's sharks and rays. eLife, **3**, e00590.

44. Davidson, L.N., Krawchuk, M.A. and Dulvy, N.K. (2016) *Why have global shark and ray landings declined: improved management or overfishing?* Fish and Fisheries, **17**, 438-458.

45. Goldman, K. (2002). *Aspects of age, growth, demographics and thermal biology of two Lamniform shark species.* PhD Thesis, College of William and Mary, School of Marine Science, Virginia Instituition of Marine Science, Williamsburg, VA.

46. Gelsleichter, J., Musick, J.A. and Nichols, S. (1999) *Food habits of the smooth dogfish, Mustelus canis, dusky shark, Carcharhinus obscurus, Atlantic sharpnose shark, Rhizoprionodon terraenovae, and the sand tiger, Carcharias taurus, from the northwest Atlantic Ocean.* Environmental Biology of Fishes, **54**, 205-217.

47. Bakker, J., Wangensteen, O.S., Chapman, D.D., Boussarie, G., Buddo, D., Guttridge, T.L., Hertler, H., Mouillot, D., Vigliola, L. and Mariani, S. (2017) *Environmental DNA reveals tropical shark diversity in contrasting levels of anthropogenic impact.* Scientific Reports, **7**, 16886.

48. Vermeulen, L. and Wepener, V. (1999) *Spatial and temporal variations of metals in Richards Bay Harbour (RBH), South Africa.* Marine Pollution Bulletin, **39**, 304-307.

49. Fatoki, O. and Mathabatha, S. (2001) *An assessment of heavy metal pollution in the East London and Port Elizabeth harbours.* Water SA, **27**, 233-240.

50. Kunito, T., Kubota, R., Fujihara, J., Agusa, T. and Tanabe, S. (2008) *Arsenic in marine mammals, seabirds, and sea turtles.* Reviews of Environmental Contamination and Toxicology, **195**, 31-69.

51. Skomal, G.B. and Mandelman, J.W. (2012) *The physiological response to anthropogenic stressors in marine elasmobranch fishes: a review with a focus on the secondary response.* Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, **162**, 146-155.

52. Hoffmayer, E.R. and Parsons, G.R. (2001) *The physiological response to capture and handling stress in the Atlantic sharpnose shark, Rhizoprionodon*

terraenovae. Fish Physiology and Biochemistry, **25**, 277-285.

53. Lyons, K. and Lowe, C.G. (2013) *Mechanisms of maternal transfer of organochlorine contaminants and mercury in the common thresher shark (Alopias vulpinus).* Canadian Journal of Fisheries and Aquatic Sciences, **70**, 1667-1672.

54. Eisler, R. (1984) *Trace metal changes associated with age of marine vertebrates.* Biological Trace Element Research, **6**, 165-180.

55. Carmo, W.P.D.d. and Fávaro, L.F. (2015) *Teratogenic processes in an embryo without gills and low yolk absorption of Zapteryx brevirostris (Elasmobranchii: Rhinobatidae).* Brazilian Journal of Oceanography, **63**, 497-500.

56. Turoczy, N., Laurenson, L., Allinson, G., Nishikawa, M., Lambert, D., Smith, C., Cottier, J., Irvine, S. and Stagnitti, F. (2000) *Observations on metal concentrations in three species of shark (Deania calcea, Centroscymnus crepidater, and Centroscymnus owstoni) from southeastern Australian waters.* Journal of Agricultural and Food Chemistry, **48**, 4357-4364.

57. Watling, R., McClurg, T. and Stanton, R. (1981) *Relation between mercury concentration and size in the mako shark.* Bulletin of Environmental Contamination and Toxicology, **26**, 352-358.

58. Watling, R., Watling, H., Stanton, R., McClurg, T. and Engelbrecht, E. (1982) *The distribution and significance of toxic metals in sharks from the Natal Coast, South Africa.* Water Science and Technology, **14**, 21-30.

59. Naidoo, K., Chuturgoon, A., Cliff, G., Singh, S., Ellis, M., Otway, N., Vosloo, A. and Gregory, M. (2017) *Possible maternal offloading of metals in the plasma, uterine and capsule fluid of pregnant ragged-tooth sharks (Carcharias taurus) on the east coast of South Africa.* Environmental Science and Pollution Research, **24**, 16798-16805.

60. Gajic, A. (2013) *Spiny dogfish shark secretion openings for fluorapatite, fluoride content in the enameloid of their teeth.* Editor-Chefe, 136.

61. Branstetter, S. and Musick, J.A. (1994) *Age and growth estimates for the sand tiger in the northwestern Atlantic Ocean.* Transactions of the American Fisheries Society, **123**, 242-254.

62. Pollard, D., Lincoln Smith, M. and Smith, A. (1996) *The biology and*

conservation status of the grey nurse shark (Carcharias taurus Rafinesque 1810) in New South Wales, Australia. . Aquatic Conservation: Marine and Freshwater Ecosystems, **6**, 1-20.

63. Dudley, S.F. and Simpfendorfer, C.A. (2006) *Population status of 14 shark species caught in the protective gillnets off KwaZulu–Natal beaches, South Africa, 1978–2003.* Marine and Freshwater Research, **57**, 225-240.

64. Dicken, M., Smale, M. and Booth, A. (2006) *Shark fishing effort and catch of the ragged-tooth shark Carcharias taurus in the South African competitive shore-angling fishery.* African Journal of Marine Science, **28**, 589-601.

65. Smale, M. (2002) *Occurrence of Carcharias taurus in nursery areas of the Eastern and Western Cape, South Africa.* Marine and Freshwater Research, **53**, 551-556.

66. Bansemer, C. and Bennett, M. (2009) *Reproductive periodicity, localised movements and behavioural segregation of pregnant Carcharias taurus at Wolf Rock, southeast Queensland, Australia.* Marine Ecology. Progress Series, **374**, 215-227.

67. Hamlett, W. and Koob, T. (1999) In Hamlett WC (ed.), *Sharks, Skates and Rays:The Biology of Elasmobranch Fish*. Baltimore, MD: Johns Hopkins University Press, 398-444.

68. Storrie, M.T., Walker, T.I., Laurenson, L.J. and Hamlett, W.C. (2009) *Gestational morphogenesis of the uterine epithelium of the gummy shark (Mustelus antarcticus).* Journal of Morphology, **270**, 319-336.

69. Swenander, G. (1907) *Über die Ernährung des Embryos der Lamna cornubica.* Zoologiske Studier Tollagnade Tullberg Uppsala, **1907**, 283-289.

70. Chen, M. (2001) *The form and structure of the uterus of spadenose shark, Scoliodon laticaudus.* Journal of Xiamen University (Natural Science), **40**, 92-96.

71. Jollie, W.P. and Jollie, L.G. (1967) *Electron microscopic observations on accommodations to pregnancy in the uterus of the spiny dogfish, Squalus acanthias.* Journal of Ultrastructure Research, **20**, 161-178.

72. Stevens, B. (2002) *Uterine and oviducal mechanisms for gestation in the common sawshark, Pristiophorus cirratus.* Bachelor of Science (Honors) Thesis,

University of Melbourne, Melbourne, Australia.

73. Sato, K., Nakamura, M., Tomita, T., Toda, M., Miyamoto, K. and Nozu, R. (2016) *How great white sharks nourish their embryos to a large size: evidence of lipid histotrophy in lamnoid shark reproduction.* Biology Open, **5**, 1211-1215.

74. Tomita, T., Nozu, R., Nakamura, M., Matsuzaki, S., Miyamoto, K. and Sato, K. (2017) *Live-bearing without placenta: Physical estimation indicates the high oxygen-supplying ability of white shark uterus to the embryo.* Scientific Reports, **7**, 11744.

75. Lombardi, J., Jones, K.B., Garrity, C.A. and Files, T. (1993) *Chemical composition of uterine fluid in four species of viviparous sharks (Squalus acanthias, Carcharhinus plumbeus, Mustelus canis and Rhizoprionodon terraenovae).* Comparative Biochemistry and Physiology Part A: Physiology, **105**, 91-102.

76. Ellis, M.T. and Otway, N.M. (2011) *Uterine fluid composition of the dwarf ornate wobbegong shark (Orectolobus ornatus) during gestation.* Marine and Freshwater Research, **62**, 576-582.

77. Evans, D., Oikari, A., Kormanik, G.A. and Mansberger, L. (1982) *Osmoregulation by the prenatal spiny dogfish, Squalus acanthias.* Journal of Experimental Biology, **101**, 295-305.

78. Files, T. and Lombardi, J. (1993) *Free amino acids in the uterine fluids of four species of viviparous sharks (Squalus acanthias, Carcharhinus plumbeus, Mustelus Canis and Rhizoprionodon terraenovae).* Comparative Biochemistry and Physiology Part B: Comparative Biochemistry, **104**, 583-588.

79. Kormanik, G.A. (1988) *Time course of the establishment of uterine seawater conditions in late-term pregnant spiny dogfish (Squalus acanthias).* Journal of Exploratory Biology, **137**, 443-456.

80. Kormanik, G.A. (1992) *Ion and osmoregulation in prenatal elasmobranchs: evolutionary implications.* American Zoologist, **32**, 294-302.

81. Kormanik, G.A. (1993) *Ionic and osmotic environment of developing elasmobranch embryos.* Environmental Biology of Fishes, **38**, 233-240.

82. Price, K.S. and Daiber, F.C. (1967) *Osmotic environments during fetal development of dogfish, Mustelus canis (Mitchill) and Squalus acanthias*

Linnaeus, and some comparisons with skates and rays. Physiological Zoology, **40**, 248-260.

83. Thorson, T.B. and Gerst, J.W. (1972) *Comparison of some parameters of serum and uterine fluid of pregnant, viviparous sharks (Carcharhinus leucas) and serum of their near-term young.* Comparative Biochemistry and Physiology Part A: Physiology, **42**, 33-40.

84. Tomita, T., Cotton, C.F. and Toda, M. (2015) *Ultrasound and physical models shed light on the respiratory system of embryonic dogfishes.* Zoology, **119**, 36-41.

85. Hamlett, W.C., Musick, J.A., Eulitt, A.M., Jarrell, R.L. and Kelly, M.A. (1996) *Ultrastructure of uterine trophonemata, accommodation for uterolactation, and gas exchange in the southern stingray, Dasyatis americana.* Canadian Journal of Zoology, **74**, 1417-1430.

86. Tomita, T., Nakamura, M., Sato, K., Takaoka, H., Toda, M., Kawauchi, J. and Nakaya, K. (2014) *Onset of buccal pumping in catshark embryos: how breathing develops in the egg capsule.* PLoS ONE, **9**, e109504.

87. Burger, J.W. and Loo, T.L. (1959) *Bromination of phenol red by the dogfish, Squalus acanthias.* Science, **129**, 778-779.

88. Castro, J.I., Sato, K. and Bodine, A.B. (2016) *A novel mode of embryonic nutrition in the tiger shark, Galeocerdo cuvier.* Marine Biology Research, **12**, 200-205.

89. Cooper, A.R. and Morris, S. (1998) *The blood respiratory, haematological, acid-base and ionic status of the Port Jackson shark, Heterodontusportusjacksoni, during recovery from anaesthesia and surgery: a comparison with sampling by direct caudal puncture.* Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, **119**, 895-903.

90. Hassanein, L. (2011) *The physiological and physical response to capture stress in sharks.* The Plymouth Student Scientist, **4**, 413-422.

91. Heberer, C., Aalbers, S., Bernal, D., Kohin, S., DiFiore, B. and Sepulveda, C. (2010) *Insights into catch-and-release survivorship and stress-induced blood biochemistry of common thresher sharks (Alopias vulpinus) captured in the*

southern California recreational fishery. Fisheries Research, **106**, 495-500.

92. Mandelman, J.W. and Skomal, G.B. (2009) *Differential sensitivity to capture stress assessed by blood acid–base status in five carcharhinid sharks.* Journal of Comparative Physiology B, **179**, 267-277.

93. Manire, C., Hueter, R., Hull, E. and Spieler, R. (2001) *Serological changes associated with gill-net capture and restraint in three species of sharks.* Transactions of the American Fisheries Society, **130**, 1038-1048.

94. Persky, M.E., Williams, J.J., Burks, R.E., Bowman, M.R., Ramer, J.C. and Proudfoot, J.S. (2012) *Hematologic, plasma biochemistry, and select nutrient values in captive smooth dogfish (Mustelus canis).* Journal of Zoo and Wildlife Medicine, **43**, 842-851.

95. Smith, H.W. (1929) *The composition of the body fluids of elasmobranchs.* Journal of Biological Chemistry, **81**, 407-419.

96. Cain, D.K., Harms, C.A. and Segars, A. (2004) *Plasma biochemistry reference values of wild-caught southern stingrays (Dasyatis americana).* Journal of Zoo and Wildlife Medicine, **35**, 471-476.

97. Sulikowski, J.A. and Howell, W.H. (2003) *Changes in plasma cortisol, glucose, and selected blood properties in the summer flounder Paralichthys dentatus associated with sequential movement to three experimental conditions.* Journal of the World Aquaculture Society, **34**, 387-397.

98. Wells, R., McIntyre, R., Morgan, A. and Davie, P. (1986) *Physiological stress responses in big gamefish after capture: observations on plasma chemistry and blood factors.* Comparative Biochemistry and Physiology Part A: Physiology, **84**, 565-571.

99. Haulena, M. (1999), *Plasma biochemical changes in response to transport, handling, and variation of water quality in Sand Tiger Sharks.* PhD Thesis. The University of Guelph, Canada.

100. Arnold, J.E. (2005) *Hematology of the sandbar shark, Carcharhinus plumbeus: standardization of complete blood count techniques for elasmobranchs.* Veterinary Clinical Pathology, **34**, 115-123.

101. Adams, K.R., Fetterplace, L.C., Davis, A.R., Taylor, M.D. and Knott, N.A. (2018) *Sharks, rays and abortion: the prevalence of capture-induced parturition*

in elasmobranchs. Biological Conservation, **217**, 11-27.

102. Wosnick, N., Awruch, C., Adams, K., Gutierre, S., Bornatowski, H., Prado, A. and Freire, C. (2018) *Impacts of fisheries on elasmobranch reproduction: high rates of abortion and subsequent maternal mortality in the shortnose guitarfish.* Animal Conservation, 10.1111/acv.12458.

103. Wosnick, N., Bornatowski, H., Ferraz, C., Afonso, A., Rangel, B.S., Hazin, F.H.V. and Freire, C.A. (2017) *Talking to the dead: using Post-mortem data in the assessment of stress in tiger sharks (Galeocerdo cuvier)(Péron and Lesueur, 1822).* Fish Physiology and Biochemistry, **43**, 165-178.

104. Gelsleichter, J. and Evans, A.N. (2012) In Carrier JC, Musick JA and Heithaus MR (eds.), *Biology of Sharks and their Relatives Second Edition*, 2, 313-348.

105. Callard, I., George, J.S., Koob, T. and Hamlett, W. (2005) In BGM, J. (ed.), *Reproductive biology and phylogeny of chondrichthyes: sharks, batoids and chimeras*, 3, 283-300.

106. Henningsen, A.D. (1999) *Review. Levels of recirculating reproductively-related steroid hormones in female elasmobranchs. Implications for reproduction in a captive environment.* Aquarium Sciences and Conservation, **2**, 97-116.

107. Callard, I.P., Fileti, L.A. and Koob, T.J. (1993) *Ovarian steroid synthesis and the hormonal control of the elasmobranch reproductive tract.* Environmental Biology of Fishes, **38**, 175-185.

108. Fletcher, G., Hardy, G. and Idler, D. (1969) *Production and metabolic clearance rates in sexually mature male and female skates (Raja radiata).* Endocrinology, **100**, 1483-1495.

109. Graham, J.D. and Clarke, C.L. (1997) *Physiological action of progesterone in target tissues 1.* Endocrine Reviews, **18**, 502-519.

110. Koob, T.J. and Callard, I. (1991) *Reproduction in female elasmobranchs.* Comparative Physiology, **4**, 155-200.

111. Manire, C.A., Rasmussen, L., Hess, D.L. and Hueter, R.E. (1995) *Serum steroid hormones and the reproductive cycle of the female bonnethead shark, Sphyrna tiburo.* General and Comparative Endocrinology, **97**, 366-376.

112. Rasmussen, L., Hess, D.L. and Gruber, S.H. (1992) In Hamlett WC (ed.), *Reproductive Biology of South American Vertebrates.* Springer, New York, 19-

42.

113. Rasmussen, L. and Murru, F. (1992) *Long-term studies of serum concentrations of reproductively related steriod hormones in individual captive carcharhinids.* Marine and Freshwater Research, **43**, 273-281.

114. Rasmussen, L.E. and Gruber, S.H. (1993) *Serum concentrations of reproductively-related circulating steroid hormones in the free-ranging lemon shark, Negaprion brevirostris.* Environmental Biology of Fishes, **38**, 167-174.

115. Sumpter, J. and Dodd, J. (1979) *The annual reproductive cycle of the female lesser spotted dogfish, Scyliorhinus canicula L., and its endocrine control.* Journal of Fish Biology, **15**, 687-695.

116. Koob, T., Tsang, P. and Callard, I. (1986) *Plasma estradiol, testosterone, and progesterone levels during the ovulatory cycle of the skate (Raja erinacea).* Biology of Reproduction, **35**, 267-275.

117. Perez, L.E. and Callard, I.P. (1992) *Identification of vitellogenin in the little skate (Rajah erinacea).* Comparative Biochemistry and Physiology Part B: Comparative Biochemistry, **103**, 699-705.

118. Snelson, F.F., Rasmussen, L., Johnson, M.R. and Hess, D.L. (1997) *Serum concentrations of steroid hormones during reproduction in the Atlantic stingray, Dasyatis sabina.* General and Comparative Endocrinology, **108**, 67-79.

119. Henningsen, A., Murru, F., Rasmussen, L., Whitaker, B. and Violetta, G. (2008) *Serum levels of reproductive steroid hormones in captive sand tiger sharks, Carcharias taurus (Rafinesque), and comments on their relation to sexual conflicts.* Fish Physiology and Biochemistry, **34**, 437-446.

120. Awruch, C. (2013) *Reproductive endocrinology in chondrichthyans: The present and the future.* General and Comparative Endocrinology, **192**, 60-70.

121. Buechi, H.B. and Bridgham, J.T. (2017) *Evolution of specificity in cartilaginous fish glycoprotein hormones and receptors.* General and Comparative Endocrinology, **246**, 309-320.

122. Gelsleichter, J. and Walker, C.J. (2010) In Carrier JC, Musick JA and Heithaus MR (eds.), *Sharks and their relatives II: biodiversity, adaptive physiology and conservation*. CRC Press, Boca Raton, 491-537.

123. Mull, C.G., Blasius, M.E., O'Sullivan, J.B. and Lowe, C.G. (2012) In ML, D.

(ed.), *Global perspectives on the biology and life history of the White Shark*. CRC Press, Boca Raton, Florida, 59-75.

124. Vas, P. (1987) *Observations on trace metal concentrations in a carcharhinid shark, Galeorhinus galeus, from Liverpool Bay.* Marine Pollution Bulletin, **18**, 193-194.

125. Cavanagh, R.e., Kyne, P., Fowler, S., Musick, J. and Bennett, M. (2003) In Cavanagh, R. D., Kyne, P. M., Fowler, S. L., Musick, J. A. and Bennett, M. B. (eds.). The University of Queensland, School of Biomedical Sciences, Brisbane, Australia, pp170.

126. Huveneers, C., Otway, N.M., Harcourt, R. and Ellis, M. (2011) *Quantification of the maternal–embryonal nutritional relationship of elasmobranchs: case study of wobbegong sharks (genus Orectolobus).* Journal of Fish Biology, **78**, 1375-1389.

BRIDGE
CHAPTER1/2 TO CHAPTER 3

The Introduction **(CHAPTER 1)** and Literature review **(CHAPTER 2)** introduced and expanded the knowledge of *C. taurus*. The natural low fecundity, due to intrauterine cannibalism, exacerbated by over-exploitation making it a vulnerable species. The aim of this study was to understand the natural aplacental maternal-embryonic environment to establish the proper *in vitro* conditions for breeding programmes aimed at increasing this species numbers. This was achieved by documenting and describing the changes in *C. taurus* female's reproductive physiology in the non-gravid reproductive staged (RS) females (immature-mature and sexually active; RS1-3) and gravid reproductive staged females (pregnant with capsules (RS4) and embryos phases (RS5A -RS5D).

The first step in describing the physiology was to understand the main morphometric indices of this species and how they related to a successful reproductive strategy. **CHAPTER 3** assessed the length (i.e., the precaudal length: PCL; the total length: TL), total weight of the female, hepatosomatic (i.e., the liver index), the gonadosomatic index (i.e., the ovary index), the width of the uterus and capsule count.

CHAPTER 3

Morphometrics of non-gravid and gravid female Ragged-tooth sharks (*Cacharias taurus*) on the east coast of South Africa

3.1 Abstract

Biological characteristics serve as indices to assist with evaluating elasmobranchs into their respective reproductive stages. The indices commonly used for assessment is length and weight due the non-invasive approach in obtaining these measurements. The capture of different reproductive stages of *C. taurus* females, provided an opportunity to assess and compare both the external (i.e. length and weight) and internal (hepatosomatic index, gonadosomatic index uterine width and capsules) morphometric indices of the female including their migration trends. The migration distribution and morphometric relationship equations were tabulated in relation to the reproductive stages of the females. The length and weights of the female's embryos were also tabulated in relation to the various gravid reproductive stages. Interestingly the trends of the hepatosomatic and gonadosomatic indices indicated the vital relationship between the liver and ovary of the female to sustain yolk production, that is eventually packaged into capsules for embryos to feed upon in the uterus. Additionally, assessment of the capsule count, through each gravid reproductive stage, suggests that rate at which yolk is supplied to these embryos decreases. The results highlight the importance of including these indices in the reproductive staging process during physiological assessments of a species.

Keywords: Morphometric, Hepatosomatic, Gonadosomatic, Uterus, Capsule, Embryo, Indices, length, weight

3.2 Introduction

Investigation into the reproductive biology of female *C. taurus* requires the examination and measurement of the internal and external body anatomy (i.e., morphometric) of the females at different reproductive stages. Biological characteristics serve as indices to assist in evaluating the reproductive stages of *C. taurus* females. Investigations have looked at different reproductive stages and divided them into juvenile, adolescent and mature stages [1-3]. The classification is often based on weight and lengths of the females which are non-invasive approaches [1,3-5]. However, a few investigations into the mature, GF *C. taurus* sharks have also been linked to the size of the *in utero* embryos and capsules [6-8].

The aplacental viviparous *C. taurus* supports her young by producing yolk filled ova, encased in a capsule (protective membranous sheath created by the O.G), that serves as the main nutrition for the embryos during gestation [9]. Gilmore *et al.* (1983) and Gilmore (1993) published detailed descriptions of the six stages (I-VI) of *C. taurus* embryology and its associated nutritional sources.

Currently no data exists on other morphometric indices such as hepatosomatic index (i.e., energy reserved indicator based on the liver weight/total body weight), gonadosomatic index (indicative of reproductive condition based on the ovary weight/total weight) and uterus width (UW) in relation to different reproductive stages of *C. taurus* females. In addition, the migration pattern of C. *taurus* females along the KZN coastline, especially during mating and gestation, were also recorded by observing the condition of these females in the months and seasons they were captured which helped to determine their migratory pattern [G Cliff, Natal Sharks Board, unpublished data].

The bather-protection nets deployed along the KZN coastline and, maintained by KZNSB (KNZSB Act 8, of 2008) caught *C. taurus* sharks of both sexes and reproductive stages [10-12]. One of the main objectives of this study was to document and compare the morphometrics measurements between NGF and GF. The capture of different staged *C.*

taurus female sharks provided an opportunity to investigate the common indices (i.e., length and weights), as well as morphometric indices: HSI, GSI and UW in all females as well as embryo and capsule information in GF, has never been reported.

3.3 Materials and Methods

3.3.1 Sampling and criteria

All *C. taurus* caught in bather protection nets along the KZN coastline were examined within 24 hours of capture. Ethical approval for this study was granted by the University of KwaZulu-Natal (076/10/Animal). All females in this study were captured on bather protective gill nets placed along our coastline. For specific details regarding the net installations, net services and operations please refer to [11,13,14]. Each female was assessed using a freshness index (i.e., evaluating colour of the redness of the gills (**APPENDIX A**) and the depth of the recession of eyes (**APPENDIX B)** prior to any collection of fluids and tissue. Sharks that appeared with pale gills and recessed eyes were omitted from the study as they were considered dead for over 24 hours. All information relating to the females were recorded on KZNSB dissection forms (**APPENDIX B**) and capsule sheet **(APPENDIX D)** using a key chart **(APPENDIX D).**

3.3.2. External measurements

External morphological measurements included body length (PCL: precaudal length, TL: total length and UC: Upper caudal length) (mm) and weight (kg). The PCL is the measurement from the tip of the snout to the precaudal notch while the UC is the measurement from the notch to the end of the tail (**APPENDIX E**)**.** The TL used in this study was determined by the conversion equation: $TL = PCL + 0.8UC$ [14]. This was to ensure consistency with Gilmore *et al.* (2005) who used the same relationship.

3.3.3 Internal measurements

Examination of female reproductive tracts included weighing of the single functional right ovary (g), liver (kg) and measurement of the UW (mm). The GSI and HSI percentages were calculated as follows:

(1) GSI (%) = ovary weight/total body weight (kg) *100

(2) HSI (%) = liver weight (kg)/ total body weight (kg) *100

The uterus (both left and right) of the GF *C. taurus* sharks contained capsules and/or embryos. All contents of the uterus were extracted by making a small incision into the uterine wall. All capsules present in the uterine fluid (UF) were extracted carefully. The lengths and weight of each capsule was documented (**APPENDIX D).** Capsules containing embryos (EE) and fluid (intracapsular fluid: ICF), all encapsulated (EE) and free-floating (FFE) embryos were examined, weighed and measured (**APPENDIX C).** The ICF, like the UF and the plasma, was removed with a syringe and stored at -20^{o}C for further testing. **CHAPTER 5** describes the processing of the plasma, ICF and UF for biochemical analysis. **CHAPTER 6** describes the processing of the plasma, ICF and UF from this study, for heavy metal analysis, which has also been documented [12]. **CHAPTER 7** includes further information of the processing of EE's < 100 mm TL and a few FFE' from this study which has been documented [15].

3.3.4 Reproductive staging

Reproductive stages were assigned to each *C. taurus* female based on maturity indicators applied from a classification system adapted from [16] and employed at KZNSB for over 50 years (**APPENDIX D**). These maturity indicators were measurements and weights of the female and her associated reproductive organs. A summary of the definitions of each stage are provided in **Table 3.1** while a detailed description of each can be found in **APPENDIX E** which had to be initially analysed from a larger KZNSB database of *C. taurus* females captured between 1980-2016. Some of these stages had to be further subdivided, the NGF mature inactive stage (RS2) consisting of females that were virgins (i.e., RS2A) and non-virgins (i.e., RS2B), and the GF stages (RS5) consisting of embryos at different developmental stages i.e. (RS5A-5D). The KZNSB database **APPENDIX E** was used to determine morphometric relationship equations (due to the sample size in the study being inefficient) and was referenced occasionally in transcript to confirm trends observed in our study under the limited morphometric data size. of this study.

3.3.5 Migration

The months and seasons in which these females were captured were also investigated to determine the reproduction-linked migratory movements of the ragged-tooth shark females linked to this study

.

3.3.6 Data Analysis

GRAPHPAD PRISM (Graph Pad Software Inc.; Version 7) was thereafter used for all analysis. All morphometric indices were analysed for normality which was determined through the Descriptive statistics of the female at all reproductive stages, the embryos and capsules found were tabulated. Relationship equations were derived using Microsoft Excel (2010) for all morphometric indices (lengths, weights, HSI and GSI) for all the reproductive stags of the females and indices (lengths and weight) as well as the embryos <100 mm TL and >100 mm TL. Shapiro-Wilk test was used to determine if the indices passed normality; where the level of significance was set at $p < 0.05$. Data that was not normally distributed was analysed using the unpaired, nonparametric Mann-Whitney test and One-way ANOVA (and nonparametric) with Tukeys multiple comparison tests (based on 2 tail with CI of 95%, $p < 0.05$) to determine any significant differences when comparing the medians of length, weight, UW, HSI and GSI in the NGF, GF and sub-group (RS1-RS5D) sharks. Parametric data was analysed using the Unpaired t test with Weltch correction and significance was displayed with p and t(df) values. All correlation was achieved using Spearman correlation (r_s, p) tests was used to determine any association between the various indices in the females (i.e., length vs. length; length vs. weight; length/mass vs. HSI/GSI; UW vs. length/weight/HSI/GSI of the females). The capsule production along the GF was also compared. In addition, correlation between the embryo's lengths and weights against the quantity of capsules and capsule weight was used to determine any associative relationships.

Table 3.1: A summary of the reproductive stage definitions used to divide *C. taurus* females into their respective maturity stages. A more detailed description can be found in APPENDIX E. Description of RS5A-RS5D is an extension of the embryological stages in Gilmore *et al*. (1983)

Reproductive Stage	Definition	Description
RS1	NGF (Immature (adolescent)	This female is inactive with ovary still developing. Not sexually active. Capture in January, March and October. Median UW is 33 mm.
RS2A	NGF (Mature, virgin)	Females has a developed ovary but has not ever mated (no ruptured hymen). No mating scars on the skin. Captured in March, May-July, October-November. Median UW is 58 mm.
RS2B	NGF (Mature, virgin/not a virgin but inactive)	Females have a developed ovary. They are either virgins or have mated previously (ruptured hymen). Old mating scars can be found on the skin in the latter. These females were not mating at the time of capture. Captured in April, July-August. Median UW is 68 mm
RS3	NGF (Mature, Active-Mating)	Females are found with a ruptured hymen and bear fresh mating lesions on the body. They are caught mainly in October-December, when mating mostly occurs. Median UW is 99 mm
RS4	GF (Pregnant with capsules)	Females are pregnant with capsules. Captured in December-January. Median UW is 101 mm
RS5A	GF (Pre-hatch stage)	Female are pregnant with embryos encased in the capsules. Capsules are present with either fertilised or unfertilised ova. Captured in January-March, September. Median UW is 166 mm .
RS5B	GF (Post-hatch stage)	Females are pregnant with embryos hatched from their capsules. Maximum of three embryos could hatch from one capsule. Embryos are between 60-100 mm TL. Captured in February and July. Median UW could not be calculated.
RS5C	GF (Intra-cannibalistic stage)	Females are pregnant with embryos in the intracannibalistic phase. Embryos are between 100-335 mm TL. Capsules (with unfertilised ova) are present to provide nutrition. Captured in February-April. Median UW is 145 mm
RS5D	GF (Oophagous stage)	Females are pregnant with one embryo in each uterus. Embryos are between 335-900 mm TL. Capsules are present providing food during this oophagous phase. Captured in January-September. Median UW is 168 mm
RS6	Post-partum	Females that have given birth. Captured in July-August. Median UW is 116 mm.

Abbreviations: GF: gravid females; mm: millimetre; NGF: non-gravid females, RS 1-5D: reproductive stage (1-5D); TL: total length, **UW: Uterine Width.**

3.4 Results

3.4.1 Captured Sharks

A total of 39 NGF and 35 GF *C. taurus* were captured over a period of four years. The NGF sharks comprised of the following females: (a) RS1 (*n* = 5), RS2B (*n* = 9), RS3 (*n* = 25) while the GF had: RS4 (*n* = 12) and RS5 (*n* = 23) [(RS5A (*n* = 5), RS5B (*n* = 1), RS5C (*n* = 5) and RS5D (*n* = 12)]. No RS2A and post-partum females were sampled in this study. There were times when some GF sharks fell into more than one gravid reproductive stage, due to the presence of embryos (in both uteri) at different developmental stages.

3.4.2 Morphometric relationships

The relationship equations were created for the intended purpose of being used in the field especially with estimating the HSI and GSI of the females which would require euthanizing the female or working with dead females. The relationship equations for different reproductive staged females (NGF, GF as well as sub groups RS1-5D) (**Table 3.2)**. The embryos from GF (**Table 3.3**) were derived using the data from this study as well as the KZNSB database **APPENDIX E** Relationship equations for females were derived for lengths: PCL vs. TL, lengths vs. weight; weight and lengths vs. GSI and HSI. The embryos equations only focused around the length and weights for embryos <100 mm TL and those >100 mm TL. This was due to no ovary being developed at these initial stages of development and the liver mass was not always reliable due to size and rate of decomposition. The PCL:TL equations in the females and the weight and lengths of the embryos (>100 mm TL) was shown to be the most reliable equations indicated by their trendline value, while more sampling is required to better the other relationship equations.

Table 3.2: Morphometric relationships of length, weight, hepatosomatic index (HSI) and gonadosomatic index (GSI) in *C. taurus* NGF (RS1-RS3) and GF (RS4-RS5) based on the larger KZNSB database (APPENDIX E).

Description	Relationship	Equation	*n*	r^2
NGF	PCL; TL	TL = 1.187 PCL + 222.66	1068	0.53
	PCL; Weight	Weight = $5.47e^{0.002PCL}$	1097	0.78
	PCL; HSI	HSI = $2.0706e^{0.0009PCL}$	983	0.19
	PCL; GSI	GSI = 8E-0.6e $^{0.01PCL}$	996	0.45
	Weight; HSI	HSI = $6.813e^{0.004WeightWeight}$	983	0.16
	Weight; GSI	GSI = $0.0228e^{0.025Weight}$	996	0.42
NGF: RS1	PCL; TL	TL = 1.218 PCL +160.56	160	0.97
	PCL; Weight	Weight = $3.907e^{0.002PCL}$	166	0.77
	PCL; HSI	HSI = 0.0111 PCL - 9.91	159	0.23
	PCL; GSI	GSI = 0.0004 PCL - 0.52	119	0.10
	Weight; HSI	HSI = 0.0544 Weight + 4.25	159	0.17
	Weight; GSI	GSI = 0.023Weight - 0.06	119	0.07
NGF: RS2B	PCL; TL	TL = 1.159 PCL + 272.31	458	0.97
	PCL; Weight	Weight = $6.94e^{0.002PCL}$	473	0.62
	PCL; HSI	HSI = $2.3225e^{0.0008PCL}$	432	0.11
	PCL; GSI	GSI =8E-06$e^{0.006PCL}$	449	0.32
	Weight; HSI	HSI =$6.968e^{0.034Weight}$	432	0.12
	Weight; GSI	GSI = $0.0248e^{0.022Weight}$	449	0.31
NGF: RS3	PCL; TL	TL = 1.087PCL + 428.91	443	0.15
	PCL; Weight	Weight = $19.19e^{0.001PCL}$	450	0.44
	PCL, HSI	HSI = 7.6938PCL	384	0.001
	PCL; GSI	GSI = $0.4307e^{0.001PCL}$	419	0.03
	Weight; HSI	HSI =$14.464e^{-0.001Weight}$	384	0.01
	Weight; GSI	GSI = -0.0016Weight + 2.1765	418	
GF	PCL; TL	TL = 1.134 PCL + 318.91	329	0.89
	PCL; Weight	Weight = $17.022e^{0.001PCL}$	345	0.42
	PCL; HSI	HSI = $9.635e^{1E-04PCL}$	277	0.001
	PCL; GSI	GSI = $3.656e^{-4E-04PCL}$	289	0.01
	Weight; HSI	HSI = $5.962e^{0.02PCL}$	277	0.012
	Weight; GSI	GSI = $1.031e^{0.004PCL}$	289	0.004
GF: RS4	PCL; TL	TL = 1.1003 PCL + 387.63	66	0.81
	PCL; Weight	Weight = $14.913e^{0.001PCL}$	66	0.49
	PCL; HSI	HSI = 11.454e-$^{5E-05PCL}$	121	0.001
	PCL; GSI	GSI = $0.6835e^{0.001PCL}$	123	0.013
	Weight; HSI	HSI = $8.1145e^{0.002PCL}$	121	0.04
	Weight; GSI	GSI = 2.0444e0.014PCL	123	0.003
GF: RS5	PCL; TL	TL = 1.144 PCL + 298.77	263	0.92
	PCL; Weight	Weight = $17.023e^{0.001PCL}$	278	0.42
	PCL; HSI	HSI = 13.002e-$^{3E-04PCL}$	205	0.007
	PCL; GSI	GSI = $9.131e^{-9E-04PCL}$	217	0.004
	Weight HSI	HSI = $6.124e^{0.001PCL}$	204	0.004
	Weight; GSI	GSI = $0.688e^{0.006PCL}$	217	0.009

Abbreviations: GSI: Gonadosomatic Index; HSI: Hepatosomatic Index; n: sample size; PCL: precaudal length; r^2: r value for trendline RS 1-5: Reproductive Stage (1-5); GF: gravid females; NGF: non-gravid females; TL: total length.

Table 3.3: Morphometric relationships of length, weight, HSI and GSI for *C. taurus* embryos <100 mm TL and > 100mm TL. Measurements based on this study and KZNSB database.

Description	Relationship	Equation	*n*	r^2
***C. taurus* embryos (<100 mm TL**	Weight on TL	Weight = $0.1231e^{0.043TL}$	39	0.54
***C. taurus* embryos (>100 mm TL)**	TL on PCL	TL = 1,3464PCL - 4.6124	521	0.99
	Weight on PCL	Weight = $71.27e^{0.007PCL}$	502	0.89
	Weight on TL	Weight = $126.3e^{0.004TL}$	515	0.99

Abbreviations: mm: millimetre; *n:* sample number; PCL: precaudal length; r^2: r value for the trendline TL: total length.

3.4.3 Seasonal movements

The seasonality of catches is summarised in **Table 3.4.** Very few NGF were caught in the early part of the year with larger numbers being captured towards the end of the year, peaking in the spring (September-November). Females in RS4, in this study sample were most commonly captured in December while RS5 female shows larger capture between February-July. Closer examination showed that RS5A was captured in February-March, RS5B was captured in February, RS5C was captured in February-March and RS5D was captured in March-July

Table 3.4: Seasonality and sample size of catches of *C. taurus* NGF and GF

Reproductive Stage	Month	Season (South Hemisphere)	Female *n*	Substages
RS1	January	Middle summer	1	
	March	Early Autumn	1	
October		Middle Spring	3	
RS2B	April	Middle Autumn	1	
July-August		Middle-late Winter	2	
September- November		Spring	6	
October- November		Middle and late Spring	20	
December		Early Summer	5	
RS4	November	Late Spring	3	
December		Early Summer	7	
January		Middle Summer	2	
RS5	February	Late Summer	9	RS5A (*n* = 4*); RS5B (*n* = 1*); RS5C (*n* = 4*)
	March	Early Autumn	7	RS5A (*n* = 1); RS5C (*n* = 1); RS5D (*n* = 5)
	April	Middle Autumn	2	RS5D (*n* = 2)
	May	Late Autumn	4	RS5D (*n* = 4)
	July	Early Winter	1	RS5D (n =1)

Abbreviations: *n*: sample size; NA: Not Applicable; RS: reproductive stage; *: has recurring females sharing more than one reproductive stage

3.4.4 Morphometrics of C. taurus females

The median (and mean) results for the PCL, TL, UW (mm), weight (kg), HSI (%) and GSI (%) for all females is summarised in **Table 3.5.** All the capsules and embryos found in the GF is summarised in **Table 3.6.** The median length at 50% maturity for *C. taurus* females was calculated at 1750 mm [14].

3.4.4.1 Comparison and the correlation of length and weight

The length (PCL and TL) and weight indices increased as females matured and fell pregnant (**Table 3.5**). The RS3 females had significantly longer PCL (p = 0.0002) and TL (p = 0.0003) compared to RS2B. The same NGF group of females showed a significantly higher weight than RS1 females (p = 0.0001) (**Table 3.5**). Length and weight showed positive correlations in all females. The NGF sharks (RS1 + RS2B +

RS3) showed a significant positive correlation between PCL and weight (r_s = 0.812; p < 0.0001) and TL and weight (r_s = 0.813; p <0.0001). The GF (RS4+RS5) showed a significant positive correlation between PCL and weight (r_s = 0.69; p = 0.02).

3.4.4.2 Comparison of the uterus width (UW) and its correlation to other morphometric indices

The UW width increased as females matured (33-99 mm for RS1-RS3; **Table 3.5**). This increase continued for gravid stages RS4 (101 mm) and RS5A (166 mm). Thereafter RS5C showed a decrease (145 mm) followed by an increase (168 mm) in RS5D females (**Table 3.5**). The GF had significantly larger UWs during their pregnancy compared to the NGF sharks (p < 0.0001) **(Table 3.5**). The UW of the NGF in RS1 group was significantly lower than RS2B (p = 0.005; t(10.69) = 3.54) and RS3 (p < 0.0001, t(14.12) = 8.96) (**Table 3.5)**. The females in RS3 had a significantly higher UW than RS2B (p < 0.001, t(19.49) = 4.99) sharks in the NGF stage (**Table 3.5).**

3.4.4.3 Comparison and correlation of hepatosomatic and gonadosomatic indices

The HSI was significantly higher in the NGF sharks compared to the GF (p < 0.0001, t(65.39) = 4.99) (**Table 3.5;Figure 3.1A)**. The HSI concentrations increase during the resting periods between pregnancies, reaching a peak in mating females (RS3). HSI was significantly lower in RS1 females compared to RS2B (p = 0.007, t(8.96) = 3.5^{A}) and RS3 (p = 0.008, t(3.61) = 5.32^{B}) (**Table 3.5; Figure 3.1A).** Pregnant females showed a decreasing liver capacity as pregnancy progressed (i.e., RS4-RS5D) **(Table 3.5; Figure 3.1A).** HSI which was significantly higher in RS4 than RS5 (p < 0.001) and subgroups RS5A (p < 0.001, t(8.35) = 5.37^{C}), RS5C (p = 0.002, t(8.77) = 4.23^{D} and RS5D (p < 0.001, t(14.75) = 8.21^{E}) (**Table 3.5)**. This trend, in this study, matched data from the larger KZNSB database. The study showed HSI and GSI to have a negative association (r_s = -0.1; p = 0.32) for pregnant females bearing embryo\pups ≤826 mm TL, which is a weak, nonsignificant correlation. However, increase in embryo size sampling would create a true positive association as indicated by KZNSB database that contained embryos of sizes 1035/1064 mm TL.

The GSI of the GF was significantly higher than that of NGF sharks ($p < 0.0001$) (**Table 3.5; Figure 3.1B)**. The GSI increased with the onset of maturity (RS1). The GSI was significantly higher in RS3 compared to RS1 ($p = 0.0008^{F}$) and RS2B ($p = 0.0007^{G}$) (**Table 3.5; Figure 3.1B**). This was further corroborated with a non- significant positive correlation of HSI and GSI for the TNP group ($r_s = 0.15$; $p = 0.36$). The highest GSI was observed in early pregnancy in the presence of small EEs (RS5A). Although a single female was captured for RS5B (as indicated in **Figure 3.1A**), the ovary weight was not taken and therefore was omitted from **Figure 3.1B**. Thereafter a decrease in GSI is noticeable from the intrauterine cannibalistic stage (RS5C) to the oophagous (RS5D) gravid stage. This decreasing trend coincided, but to a far less extent, than indicated by the larger sampled KZNSB database that contained embryos up to 1035/1064mm TL (in the oophagous staging). The extent of this decrease, however, was not evident in our study as indicated by a significantly higher GSI's in RS5 ($p < 0.012$) and RS5D ($p = 0.04$) compared to RS4 (**Table 3.5, Figure 3.1B**). The decrease was not evident in our study due to sampled embryos being smaller than 826 mm TL (**Table 3.6).** This discrepancy is further corroborated with the significant negative relationship between embryo TL and GSI/HSI ($r_s = -0.6$; $p < 0.0001$).

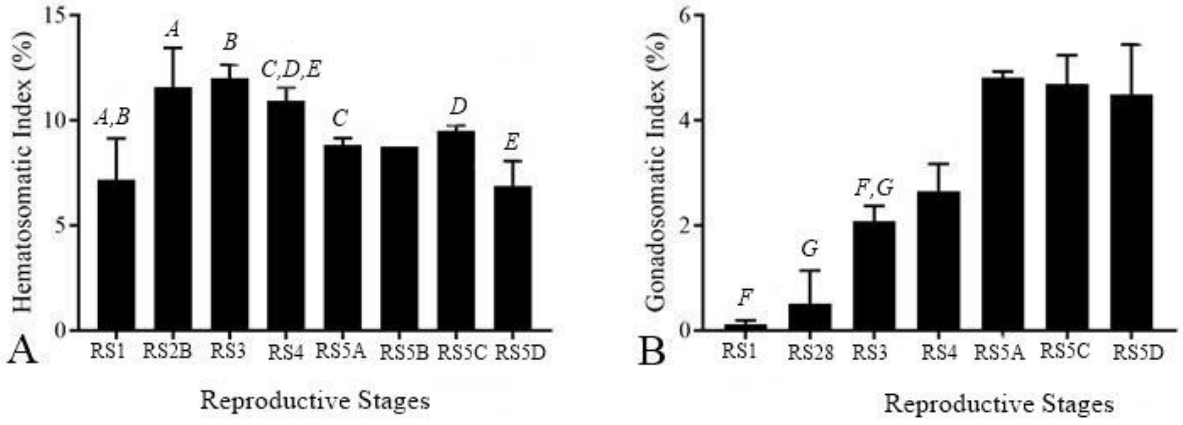


Figure 3.1: The median A) HSI and B) GSI values for each reproductive stage of *C. taurus* females. Medians attached with superscripted letters (*A-G*) indicated a significant difference between stages ($p \leq 0.05$). Abbreviations: HSI: Hepatosomatic Index; GSI: Gonadosomatic Index; RS: (Reproductive Stage 1-5D).

3.4.5 Capsules and embryos

A summary of the capsules and embryos found in the GF appear in **Table 3.6.** This table indicates a discrepancy in 1) * the ratio count of capsules to females and 2) # embryos to females in certain stages due to either to no capsules present in one of the uteri or incomplete measurements taken for an embryo. This data shows the expected increase in length and weight of the embryos through the GF stages of *C. taurus* sharks. It also shows an increase in capsule formation as pregnancy proceeds from (RS4) (103/106 capsules in the left and right uterus respectively) with a peak occurring at RS5C (197/177 capsules) in the left and right respective uterus **(Table 3.6**). Thereafter the capsule count is reduced in RS5D (50/48 capsules). Correlation of the capsule count against embryo length (**Figure 3.2A**) and embryo weight **(Figure 3.2B**) indicated an inverse relationship in both. This relationship is not maintained when considering the capsule weight against embryo length or weight, as capsule weight appears to decrease from RS4 (when they first appear) (318g/376g) to RS5C (30.6g/41.8g) in the left and right respective uterus. Thereafter the capsule weight increases in RS5D (1278g/122g), a stage which represents the largest embryos.

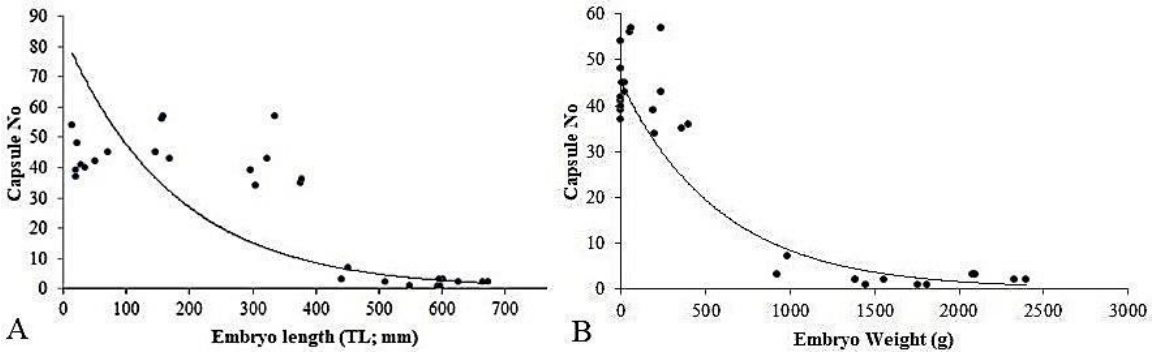


Figure 3.2: Capsule and embryo length (A) and weight (B) relationships in *C. taurus* GF (RS4-RS5D). Abbreviations: g: gram; mm: millimetre, No: number/size; TL: Total length.

Table 3.5: Median ± IQR; Mean ± SEM and Ranges for the PCL, TL (mm), Weight (kg), HSI (%), GSI (%), UW (mm) of all *C. taurus* females captured in this study.

RS	Indice	*n*	Median ± IQR	Mean ± SEM	Range	RS	Indice	*n*	Median ± IQR	Mean± SEM	Range
NGF	PCL	38	1961 ± 185	1940 ± 22.87	1524-2170	**RS5**	PCL	32	1990 ± 101	2014 ± 13.95	1858-2198
	TL	37	2566 ± 224	2522 ± 27.95	2017-2757		TL	30	2582 ± 138	2605 ± 17.2	2426-2838
	Weight	38	148 ± 36.2	139 ± 4.69	59-176		Mass	31	148 ± 25	148,9 ± 3.21	118-198
	HSI	37	11,56 ± 2.43	11,30 ± 0.34	6.11-15.09		HSI	31	9,25 ± 3.46	8,912 ± 0.34	5.32-12.12
	GSI	36	1,82 ± 1.60	1,44 ± 0.15	0.103-2.66		GSI	28	4,03 ± 2.27	3,745 ± 0.29	0.35-6.03
	UW	35	83±57	84.7±5.7	25-150		UW	9	145±95	180±25.72	104-350
RS1	PCL	5	1700±247	1676±56,9	1524-1826	**RS5A**	PCL	5	2036±106	2021±24.04	1960-2070
	TL	5	2241±262	2201±64,3	2017-2386		TL	5	2628±125	2624±28.3	2552-2070
	Weight	5	90±32,5	82,6±7,9	59-103		Weight	5	150±30	150±7.01	130-169
	HSI	4	7,2±2,9	7,5±0,8	6,1-9,7		HSI	5	8.85±10.4	8.68±0.32	7.47-9.43
	GSI	4	0,1±0,1	0,1±0,03	0,1-0,2		GSI	4	4.8±3.4	4.36±0.46	3.02-4.93
	UW	5	32±22	36.4±5.2	25-52		UW	4	155.5±57.7	160.3±15.17	130-200
RS2B	PCL	9	1904±137	1907±25,5	1810-2060	**RS5B**	PCL	1	2070±0	2070±0	ND
	TL	8	2465±129	2489±34,9	2370-2679		TL	1	2694±0	2694±0	ND
	Weight	9	129±27	129,4±5,2	108-154		Weight	1	161±0	161±0	ND
	HSI	9	11,6±3,7	11,4±0,8	7,4-15,0		HSI	1	8.75±0	8.75±0	ND
	GSI	9	0,5±0,9	0,7±0,2	0,1-1,8		GSI	1	NA	NA	NA
	UW	8	59±26.7	63.9±5.8	39-87		UW	1	130±0	130±0	NA
RS3	PCL	25	2020±112	2004±16.6	1840-2170	**RS5C**	PCL	5	1988±16	2009±38.76	1900-2120
	TL	25	2629±165	2596±19.6	2418-2757		TL	5	2582±170	2602±39.76	2492-2707
	Weight	25	160±29	153.7±3.1	125-176		Weight	5	150±32	142.8±7.86	120-164
	HSI	25	12±1.8	11.8±1.2	9.7-15.1		HSI	5	9.5±1.61	9.07±0.44	7.46-9.87
	GSI	24	2.1±0.5	1.9±0.5	0.6-2.6		GSI	5	4.69±1.62	4.48±0.44	2.93-5.55
	UW	22	109.5±36.3	103.3±5.4	60-150		UW	2	124.5±41	124.5±20.5	104-145
GF	PCL	32	1990 ± 101	2014 ± 13.95	1858-2198	**SS5D**	PCL	12	1980±59	1994±27.45	1858-2198
	TL	30	2582 ± 138	2605 ± 17.2	2426-2838		TL	12	2564±75	2581±32.39	2426-2838
	Weight	31	148 ± 25	148,9 ± 3.21	118-198		Weight	12	143±32.5	144.8±5.37	118-175
	HSI	31	9,25 ± 3.46	8,912 ± 0.34	5.32-12.12		HSI	12	6.89±2.0	7.06±0.341	5.32-9.25
	GSI	28	4,03 ± 2.27	3,745 ± 0.29	0.35-6.03		GSI	12	4.49±1.67	4.08±0.542	0.35-6.03
	UW	16	130±59.3	149.9±17.03	87-350		UW	2	300±100	300±50	250-350
RS4	PCL	12	2025±131	2034±1896	1920-2120						
	TL	10	2633±180	2626±27.06	2502-2736						
	Weight	11	151±18	154.1±5.41	135-198						
	HSI	11	10.93±1.27	10.83±0.23	9.56-12.12						
	GSI	9	2.66±1.05	2.84±0.35	1.85-5.36						
	UW	7	102±42	111.3±8.9	87-149						

Abbreviations: GSI: Gonadosomatic index; HSI: Hepatosomatic Index; IQR: Interquartile range; kg: kilogram; mm: millimetre; n: number of females; NA: Not applicable; PCL: precaudal length; RS: Reproductive stage's: Standard error of mean; GF: gravid females, TL: Total length; NGF: non-gravid, UW: Uterine width; %: percentage.

Table 3.6: The capsule and embryo data from gravid *C. taurus* females (RS4-RS5D)

		Capsules				Embryos				
RS		Cap_n /F_n	Median± IQR	Mean± SEM	Range	Weight/TL/ Sex Ratio	$Embryo_n$ /F_n	Median ± IQR	Mean± SEM	Range
RS4	L	103//11*	5±14	9,36±2,8	1-27	Weight (g)	NA	NA	NA	NA
						TL (mm)	NA	NA	NA	NA
						Sex Ratio	NA	NA	NA	NA
	R	106//9*	11±16	11,78±2,8	1-24	Weight (g)	NA	NA	NA	NA
						TL (mm)	NA	NA	NA	NA
						Sex Ratio	NA	NA	NA	NA
RS5	L	424//20*	38±44,5	30,29±6	1-57	Weight (g)	31//20	56±1394	783,7±222	0,1-4940
						TL (mm)	32//20	152±515	272,4±48	14-824,8
						Sex Ratio	32//20	NA	NA	9M:6F:17U
	R	394//19*	36±41	26,27±5	1-57	Weight (g)	28//19	62,5±1428	727,1±212	0,1-4880
						TL (mm)	28//19	186,4±524	277±50	9-762
						Sex Ratio	28//19	NA	NA	9M:7F:
	A	0/1	NA	NA	NA	Weight (g)	3//1	1±0,6	0,8±0,2	1-0,4
						TL (mm)	3//1	35±7	32,67±2,3	28-35
						Sex Ratio	3//1	NA	NA	U
RS5A	L	132*//5	41 ±17	44 ±5	37-54	Weight (g)	13#//5	0,4±0,8	0,5±0,1	0,1-1
						TL (mm)	14#//5	30,5±18	30±3,3	14-52
						Sex Ratio	14//5	NA	NA	U
	R	169//4	41±7,3	42,25±2	39-48	Weight (g)	12//4	0,2 ±0,9	0,52±0,2	0,1-2
						TL (mm)	12//4	21,5±23,5	25,5±3,9	9-50
						Sex Ratio	12//4	NA	NA	U
	A	0/1	NA	NA	NA	Weight (g)	3//1	1±0,6	0,8±0,2	1-0,4
						TL (mm)	3//1	35±7	32,67±2,33	28-35
						Sex Ratio	3//1	NA	NA	U
RS5B	L	45//1	NA	NA	NA	Weight (g)	1//1	3,6±0	3,6±0	NA
						TL (mm)	1//1	71±0	71±0	NA
						Sex Ratio	1//1	NA	NA	U
RS5C	L	197//5	50,5±16,25	49,25±4,4	39-57	Weight (g)	5//5	71±175	116,6 ±41,8	25-240
						TL (mm)	5//5	209,2±164,2	228,7±37,6	146-335
						Sex Ratio	5//5	NA	NA	3M:1F:1U
	R	177//5	43±17,25	44,3±4,8	34-57	Weight (g)	5//5	66±177	116±42,3	21-234
						TL (mm)	5//5	204,8±151,6	231,9±34,7	157,8-324
						Sex Ratio	5//5	NA	NA	3M:2F
RS5D	L	50//12	2,5±12,2	8,3±5,4	1-35	Weight (g)	12//12	1520±932	1975±368,9	363-4940
						TL (mm)	12//12	583,5±134,6	590,2±36,8	376,6-824,8
						Sex Ratio	12//12	NA	NA	6M:5F:1U
	R	48//11*	2±2	6,9±4,9	1-36	Weight (g)	11//11	1560±738	1797±346	400-4880
						TL (mm)	11//11	596±113	572,2±31,9	377,8-762
						Sex Ratio	11//11	NA	NA	6M:5F

Abbreviations: Cap_n/Embryo: capsules/embryo sample size; F: Female; F*n*: Female sample size; g: gram; IQR: interquartile range; L: left uterus; M: Male; mm: millimetre; NA: Not applicable; R: Right uterus; SEM: Standard error of measurement; RS: Reproductive Stage 4-5D; TL: Total length; U: Unidentified. *Indicates discrepancy in the female count (in the Cap_n/F_n column) and #Indicates discrepancy in the embryo count (in the $Embryo_n/F_n$ column)

3.5 Discussion

Female *C. taurus* sharks occur along the south and central KZN coast throughout the year [1,17,18]. This is based on catches in the bather-protection nets [11,14] and reports from divers and anglers. Catches of NGF peaked in the later part of the year, in readiness for mating in Spring (October-November) [17] **(Table 3.4).** Non-gravid *C. taurus* females appear along the KZN coastline throughout the year making a distinct breeding pattern difficult to identify. The GF, in this study, were captured moving north to warmer waters for their gestation period after recently mating (indicated by fresh lesion scars on the skin observed during sharks dissections at KZNSB and reported [1, G Cliff Kwa-Zulu Sharks Board, unpublished data]. The RS4 GF captured, in northern waters (November-January), indicated that females begin migrating before actual embryos have begun developing *in utero*. According to the KZNSB database (**APPENDIX E**), this staging can also extend to February-May. The RS5A-D females, not near term, were mostly captured in northern KZN waters (February-July). Some females were captured enroute to the colder waters of the Cape to give birth. When parturition is imminent, females move south to the Eastern Cape in July and August to give birth in cooler waters [10,17] in September-November [18]. The largest of the pups in this study were captured in Durban moving to the Eastern Cape while all other lengths (<825 mm TL) were mostly captured in Richards Bay (further north). This suggested that females still require warm waters well into the oophagous stage (i.e., RS5D) and supports the notion that they use the cooler waters as a pupping ground. The catches of these GF also peaked in the later part of the year as seen in the KZNSB database (**APPENDIX E**), but was not evident in our study due to the small sample size. The migration patterns of the pregnant females are associated with their stage of reproduction. The overlap in the months/seasons in the GF further suggest the females practice a biennial reproductive cycle as supported by previous studies in other papers [1,3]. Females are known to migrate back to KZN after parturition possibly due to philopatric behaviour [1].

This chapter describes the gross morphometrics of NGF (RS1-RS3; PCL: 1332-2290/TL: 1754-2906 mm) and GF (RS4-RS5D; PCL: (1858-2198 mm /TL 2426-2838 mm) females **(Table 3.5).** Females maturing (RS2B) between 2180-2906 mm TL comes

close to the minimum length for maturation documented for *C. taurus* off the east coast of the USA [19]; and the south west Atlantic [3] but does have a longer range. The maturity length of 2509 mm TL in this study is higher than the 2200 mm TL reported for the grey nurse [3,20] and previous SA reported lengths [21] but appears smaller than the 2660 mm TL reported for the Sand tiger off eastern Australia [21]. Literature suggests South Africa's *C. taurus* females are longer than recorded pregnant females of this species in other regions [22,23].

The uterus shows an increase in width as the females mature, as would be expected. The decrease in width in the RS5C females could be attributed to a decrease in total weight within the uterus due to intrauterine cannibalism which would see a decrease in embryo number. The increase in width hereafter in RS5D females would be attributed to the weight gain of the single embryo within the uterus.

The increasing HSI, as the females mature, peaks at the mating stage (RS3) (**Table 3.5; Figure 3.1A)**. The large liver of the female supports the energy expenditure required during vitellogenesis, oocyte maturation and gestation [24]. Vitellogenin, a hepatic high molecular mass protein, is incorporated into oocytes to form yolk proteins which are involved in teleost embryo development [25]. In addition, oocyte maturation, in the *S. acanthias*, correlated with an increase in plasma vitellogenin that accumulated in the ovary [7]. Once mature, the ova are released during ovulation to be fertilised in early pregnancy stages (during mating RS3) prior to the ova being encapsulated (RS4) or to be released and encapsulated as unfertilised eggs during late pregnancy (RS5D) for nutritional support to the embryos [7]. The aplacental viviparous *C. taurus* female supports her young by producing yolk filled ova, encased in a capsule (**Figure 2.4A**). One capsule can contain a maximum of 23 ova, of which a maximum of 2-3 will develop into an embryo [7]. The six stages (I-VI) of *C. taurus* embryology and its associated nutritional sources, has been extensively investigated [7,8,26] (see **CHAPTER 2.2.3.2** for elaboration). Naidoo *et al.* (2017) described the dentition of the EEs and some FFEs observed in this study. The small HSI increase in the intrauterine cannibalistic phase (RS5C) (**Figure 3.1A)** could indicate that the heaviness of the liver is due to more yolk precursors being required for the nutrition of unfertilised eggs to the two surviving embryos in RS5D. The overall decrease in HSI during the gravid stages is a result of

the energy expended by GF during vitellogenesis, oocyte maturation and the gestation process of 9-12 months [7] and is indicative of the critical role played by the liver in sequestering the lipid reserves required during pregnancy [24].

The GSI was highest in the pre-hatched phase (RS5A) (**Table 3.5; Figure 3.1B**) as the ovary contains developed follicles which provide nourishment for the developing embryos. The GSI thereafter decreases as the embryo grows and requires eggs to feed on. Capsule formation, used as an approximation to the rate of ovulation, would suggest that ova release is low in early pregnancy stage (RS4) and appeared highest in the RS5C (**Table 3.6**). The slow rate in capsule production during RS1- RS5A (**Table 3.6)** was supported by the growing (heavier) ovary (**Figure 3.1B)** during those stages. A peak in capsule production was observed in the RS5C females (**Table 3.6**) which corresponded with a drop in GSI (**Figure 3.1**). A decrease in capsule production continued in the RS5D females (**Table 3.6**; **Figure 3.1B**, however, these capsules were heavier than the capsules present in RS5C This suggests that the largest size embryos (in RS5D) are associated with lowest capsule count (in RS5D) as indicated by the non-linear inverse relationship between embryos and capsules (**Figure 3.2).** The capsule drop could result from larger embryos consuming more than can be produced as well as well as the female now producing fewer larger eggs than many smaller ones as in the previous stages. Studies have shown that the ovulation rate can increase to provide 11-13 capsules a day to ravenous 335-1000 mm TL FFEs [7,8].

It is worth noting that the extent to which GSI decreases in the late stage pregnant females (RS5D) was not made evident in this study (**Table 3.5; Figure 3.1B),** as when compared to the fuller KZNBS database. This was due to the sample length of the embryos in this study being l ≤826 mm TL in the selection of early-mid pregnant females in this study compared to the well-developed 1035/1064 mm TL embryos in the late stage pregnant females in the KZNSB database. The largest range of length recorded for *C. taurus* embryos is 950-1300 mm TL [27]. The low GSI is attributed to the increasing energy demands of the developed and larger embryos necessitating an increase in ovulation and capsule production. The GSI also continued to decrease in post-partum females presumably due to the absorption of any remaining follicles and follicle growth coming to a rest. In addition, the negative trend of HSI and GSI in our study for

embryos ≤826 mm TL would change into a positive association for a larger sample set where embryos sizes 1035/1064 mm TL, as indicated by the KZNSB database.

The results in terms of HSI and GSI indicates that the liver and ovary undergo marked changes in the transition from adolescence to maturity and pre- and post- pregnancy indicating the rate at which these organs function to supply yolk precursors and release/ovulate mature ova in the pursuit to accommodate the embryos reliance on yolk for embryonic growth. The embryos requirement for nutrition is indicated clearly by the heavy yolk laden stomach of near-termed embryos **(Figure 2.4G-H).** This study indicated that length and weight values alone cannot be used to assign females according to reproductive stage. The results in this chapter will provide a useful guide in the field especially during necropsy of *C. taurus* females.

3.6 Conclusion

The best morphological indices to classify NGF and GF (*C. taurus*) would be a combination of lengths, weight, HSI, GSI and UW indices. This chapter tabulated the range of these indices for all staged females. The results indicate the essential role HSI and GSI play in reproductive staging of the females and highlights the fact that staging cannot be based solely on length and/or weight. The essential function of HSI and GSI to provide nutrition to the developing aplacental embryos within the female's uterus, indicates further investigation into the uterine tissue is warranted to understand the how the uterine structure aids to support these developing embryos.

3.7 Acknowledgements

Immense gratitude to: the KZNSB research staff (Mr P Zungu, Mr E Makhatini, Mr B Zungu and Mrs AJ Ganesan) and the KZNSB operations crew for the tracking and proper handling of all sharks included in this study; Dr K Dhuness, Mrs R Sobaren Singh and Ms T Naidu for their assistance in the final manuscript verification process. Gratitude to the New South Wales Fisheries, Department of Primary Industries, for the scholarship, purchasing of necessary equipment and funding of analysis on this project. National

1633 Research Fund (Deutscher Akademischer Austauschdienst; DAAD) is also gratefully
1634 acknowledged.
1635

3.8 References

1. Dicken, M.L. (2006) Population dynamics of the raggedtoothshark (Carcharias taurus) along the east coast of South Africa. PhD Thesis, Rhodes University, South Africa.
2. Henningsen, A., Murru, F., Rasmussen, L., Whitaker, B. and Violetta, G. (2008) *Serum levels of reproductive steroid hormones in captive sand tiger sharks, Carcharias taurus (Rafinesque), and comments on their relation to sexual conflicts.* Fish Physiology and Biochemistry, **34**, 437-446.
3. Lucifora, L.O., Menni, R.C. and Escalante, A.H. (2002) *Reproductive ecology and abundance of the sand tiger shark, Carcharias taurus, from the southwestern Atlantic.* ICES Journal of Marine Science: Journal du Conseil, **59**, 553-561.
4. Dicken, M., Booth, A., Smale, M. and Cliff, G. (2007) *Spatial and seasonal distribution patterns of juvenile and adult raggedtooth sharks (Carcharias taurus) tagged off the east coast of South Africa.* Marine and Freshwater Research, **58**, 127-134.
5. Dicken, M., Smale, M. and Booth, A. (2006) *Shark fishing effort and catch of the ragged-tooth shark Carcharias taurus in the South African competitive shore-angling fishery.* African Journal of Marine Science, **28**, 589-601.
6. Gajic, A. (2013) *Spiny dogfish shark secretion openings for fluorapatite, fluoride content in the enameloid of their teeth.* Editor-Chefe, 136.
7. Gilmore, R.G. (1993) *Reproductive biology of lamnoid sharks.* Environmental Biology of Fishes, **38**, 95-114.
8. Gilmore, R.G., Dodrill, J.W. and Linley, P.A. (1983) *Reproduction and embryonic development of the sand tiger shark, Odontaspis taurus (Rafinesque).* Fishery Bulletin, **81**, 201-225.
9. Hamlett, W.C. and Hysell, M.K. (1998) *Uterine specializations in elasmobranchs.* Journal of Experimental Zoology, **282**, 438-459.
10. Cliff, G. (1989) *Breeding migration of the sand tiger shark Carcharias taurus in southern African waters.* In: 5th annual meeting of the American Elasmobranch Society, San Francisco, 17-23.
11. Cliff, G. and Dudley, S.F.J. (1992) *Protection against shark attack in South*

Africa, 1952-90. Marine and Freshwater Research, **43**, 263-272.

12. Dudley, S. (2013) In Mann, B. (ed.), *Southern African Marine Linefish Species Profiles Special Publication No 9.* Durban Oceanic Research Institute, 134-136.
13. Cliff, G., Dudley, S. and Davis, B. (1990) *Sharks caught in the protective gill nets off Natal, South Africa. 3. The shortfin mako shark Isurus oxyrinchus (Rafinesque).* South African Journal of Marine Science, **9**, 115-126.
14. Dudley, S.F. and Simpfendorfer, C.A. (2006) *Population status of 14 shark species caught in the protective gillnets off KwaZulu–Natal beaches, South Africa, 1978–2003.* Marine and Freshwater Research, **57**, 225-240.
15. Naidoo, K., Chuturgoon, A.A., Cliff, G., Ellis, M.T., Otway, N.M., Gregory, M.A., Singh, S.D. and Naidu, S.L. (2017) *Dentition facilitates the release of encapsulated Ragged-tooth Shark (Carcharias taurus) embryos.* Environmental Biology of Fishes, **100**, 1343-1354.
16. Stehmann, M.F. (2002) *Proposal of a maturity stages scale for oviparous and viviparous cartilaginous fishes (Pisces, Chondrichthyes).* Archive of Fishery and Marine Research, **50**, 23-48.
17. Bass, A.J., Aubrey, J.D. and Kistnasamy, N. (1975). Oceanographic Research Institute, Durban, South Africa, 1-100.
18. Smale, M. (2002) *Occurrence of Carcharias taurus in nursery areas of the Eastern and Western Cape, South Africa.* Marine and Freshwater Research, **53**, 551-556.
19. Branstetter, S. and Musick, J.A. (1994) *Age and growth estimates for the sand tiger in the northwestern Atlantic Ocean.* Transactions of the American Fisheries Society, **123**, 242-254.
20. Last, P. and Stevens, J. (1994) *Sharks and rays of Australia.* Commonwealth Scientific and Industrial Research Organization, Australia, pp 513
21. Otway, N.M. (2015) *Serum biochemical reference intervals for free-living Sand Tiger sharks (Carcharias taurus) from east Australian waters.* Veterinary Clinical Pathology, **44**, 262-274.
22. Gilmore, R.G. (1983) *Observations on the embryos of the longfin mako, Isurus paucus, and the bigeye thresher, Alopias superciliosus.* Copeia, 375-382.
23. Springer, S. (1948) *Oviphagous embryos of the sand shark, Carcharias taurus.*

Copeia, 153-157.

24. Koob, T.J. and Callard, I.P. (1999) *Reproductive endocrinology of female elasmobranchs: lessons from the little skate (Raja erinacea) and spiny dogfish (Squalus acanthias).* Journal of Experimental Zoology, **284**, 557-574.

25. Selman, K. and Wallace, R.A. (1989) *Cellular aspects of oocyte growth in teleosts.* Zoological Science, **6**, 211-231.

26. Gilmore, R.G., Putz, O. and Dodrill, J.W. (2005) In Hamlett WC (ed.), *Reproductive biology and phylogeny of Chondrichthyes: Sharks, Batoids and Chimaeras.* Science Publishers, Enfield, New Hampshire, USA, 435-462.

27. Chen, M. (2001) *The form and structure of the uterus of spadenose shark, Scoliodon laticaudus.* Journal of Xiamen University (Natural Science), **40**, 92-96.

BRIDGE

CHAPTER 3 TO CHAPTER 4

After the detailed assessment of morphometric indices in *C. taurus* female **(CHAPTER 3)**, understanding the histology of the uterus where embryo development occurs was needed. **CHAPTER 4** described the epithelium and wall of the NGF (immature and mature and sexually active; RS 1 and 3) and GF (pregnant with capsules; RS4 and embryos in different gestation phases; RS5A, RS5C-RS5D) *C. taurus* females. Females (RS2A and RS5B) could not be histologically assessed in this chapter. This chapter both confirmed and cleared ambiguity posed by previous studies on *C. taurus* histology and extended it with reproductive stages presented in this study.

CHAPTER 4

Histology of non-gravid and gravid female Ragged-tooth sharks (*Carcharias taurus*) on the east coast of South Africa

Naidoo K[1], Chuturgoon AA[1], Gregory MA[2], Ellis MT[4], Cliff G[5], Otway NM[6], Singh SD[3], SL Naidu[7], V Baruth[7]

[1]Discipline of Medical Biochemistry, [3]Biomedical Resource Unit and [2]School of Health Sciences, University of KwaZulu-Natal (UKZN), Durban, South Africa, [5]KwaZulu-Natal Sharks Board (KZNSB), Umhlanga, South Africa

[4]Gladstone Ports Corporation, Gladstone, Queensland, Australia

[6]New South Wales Department of Primary Industries, Port Stephens Fisheries Institute, Taylors Beach, New South Wales, Australia

[7]Microscopy and Microanalysis Unit (MMU), University of KwaZulu-Natal, Pietermaritzburg, South Africa

*Corresponding author email: chutur@ukzn.ac.za

To be submitted to Environmental Biology of Fishes

4.1 Abstract

The elasmobranch uterus is known to undergo modifications to promote embryonic development. This study describes the histological transformation of the epithelium and wall of the uterus, while also confirming if the uterus serves a secretory function in non-gravid and gravid females. Light Microscopy (viewed with Haematoxylin and Eosin and Periodic Acid Schiff/Alcian blue stains) and Scanning Electron Microscopy showed the presence of uterine lamellae on the epithelial surface, projecting into the lumen of the uterus, once the female matures. This structure increased in number and length, as gestation progressed. Micro-ridges (containing blood vessels), appearing only in gravid females, extensively increased on the surface of these lamellae as pregnancy increased. Observations on the uterine wall, from immaturity to gestation, showed a decrease in size with an increase in vascularity. No secretory structures, that could provide nutrition, were found in the uterus. The increase of surface area by the lamellae structures and increase in blood vessels in close proximity to the lumen of the uterus with a reduction in wall thickness to aid in diffusion, suggests the uterus functions to provide respiratory and osmoregulatory support to the developing embryos that does not include the provision of nutrition. This article represents the first detailed description of the uterine tissue through all reproductive stages of *C. taurus* female which also confirms no presence of secretory structures to account for the high volume of uterine fluid. However, further examination of cells lining the epithelium needs to be elucidated. Any contribution to the fluid that bathes the embryos *in utero* would serve as a critical component to include in any models aimed at increasing the numbers of this species.

Keywords: *Carcharias taurus*, epithelium, female, gravid, non-gravid, microscopy, Ragged- tooth shark, uterus, wall.

4.2 Introduction

Viviparous chondrichthyans are matrotrophic (i.e., they supplement the embryonic internal yolk stores once exhausted). Female chondrichthyan reproductive tracts have been studied in oviparous (egg laying) [1] and viviparous (live bearing) [1-3] species and have the same general structure with a pair of functional uteri [3,4]. Matrotrophic species can modify their uterus to support the development of their young by ensuring accommodation, oxygen supply as well as the synthesis and secretion of nourishing material [2,5,6]. They supplement embryo nutrition through four main pathways i.e., via uterine secretions (histotrophy), provision of unfertilised ova (oophagy), sibling cannibalism (intrauterine cannibalism) or placental transfer (placentatrophy) [2,6-9].

Oophagous viviparity (with or without embryophagy) occurs in sharks of the Order Lamniformes [5]. Our understanding of the reproductive biology of lamnoid sharks has been largely based on the development of embryos and not on the uterine structure or maternal contributions [10]. Embryo nourishment of the lamnoid aplacental *C. taurus* occurs through oophagy and intrauterine cannibalism. Oophagy is a trait found in all lamnoid sharks while the additional nutritional support of intrauterine cannibalism only occurs consistently in *C. taurus* [6,10] and occasionally in *I. oxyrinchus* [5]. The uterus plays a unique role in supporting these large embryos in the absence of a placenta [6].

Embryo development (Stage I-VI) in *C. taurus* has been well documented [10]. The EE and FFE embryos, from *C. taurus* females in this study, has already been documented [11] (see **CHAPTER 7)**. In early pregnancy (prior to embryology stage I-II), capsules (protective membranous sheath) are produced to enclose ova released from the ovary. There are six types of capsules, ranging from those with no ova to those with fertilised and unfertilised ova (up to 23 ova in one capsule) [5,6]. These capsules also enclose the developing embryo in an ICF [12]. Following growth, the embryos escape encapsulation and develop *in utero* within the UF. This fluid is believed to facilitate respiration and waste removal of the embryos [7,12]. In addition, this is the only species known to incorporate a unique nutritional strategy of embryophagy resulting in a single well-developed embryo being born from each uterus [6,12]. Descriptions of the lamnoid uterine epithelium has been documented [10,13-15]. Description of the uterus of *C. taurus* has been presented with some ambiguity [2,5]. Gilmore (1993) described the tissue of a

pregnant *I. Oxyrinchus* and made inferences on *C. taurus* reproduction. This study described the tissue as having a respiratory function, but also described the presence of secretory structures, which could suggest a supply of nutrition to developing embryos [5] while the study by Hamlett and Hysell (1998), based on *C. taurus*, suggested no uterine secretory function in the two females investigated [2]. This latter result, based on a small sample set, served as a point of ambiguity with the previous study [5] that required clarification. In addition, no uterine wall observations were documented in *C. taurus* females.

These *C. taurus* species migrate along the east coast of SA. The large numbers are inadvertently captured in the bather protection nets managed by KwaZulu-Natal Sharks Board [16-19]. These captures and the knowledge of the reproductive migration of these females along the east coast of SA [18,19] afforded us the opportunity to investigate uterine tissue transformation of the epithelium and the wall examined through the NGF and GF reproductive stages of this shark . This paper met one of the objectives of the study, which was to clarify the ambiguity surrounding the uterine function that has resulted from the two previous studies, while also extending the knowledge of *C. taurus* uterine tissue transformation and any secretions, through the reproductive stages (RS1-RS5D), to support the development of the aplacental embryos. The second objective of the study, was to investigate the biochemical analytes in the maternal fluids of *C. taurus*, which was investigated but reported elsewhere (**CHAPTER 5;** Naidoo *et al*., in prep).

4.3 Materials and Methods

4.3.1 Sampling and classification

C. taurus females caught in bather protection nets along the KZN coastline were examined after receiving ethical approval from the University of KwaZulu-Natal (076/10/Animal). The methodology involved an external examination of all captured females to inspect their freshness which was determined by gill colour, eye recession and overall odour of the animal. Morphological measurements were used to determine the female's reproductive classification (RS1 - RS5D) (**Table 3.1)**. These included external measurements (i.e., lengths and weight) and internal measurements (i.e., the

weight of the ovary and liver as well as the UW). A detailed study design can be found in **CHAPTER 3.2**. A summary of this classification and morphometric calculations, based on all *C. taurus* captures at KZNSB for over 36 years, is presented in a supporting table (**APPENDIX E**). However, a simple guideline to the definitions of each reproductive stage are presented in **Table 3.1**.

4.3.2 Uterine processing

Histological samples were taken from the anterior and posterior portions of both the left and right wall of each uterus of all NG and GF. The tissue was bisected, one sample being fixed for LM in 10% formalin in saline, dehydrated in alcohol (30% - 100%) prior to wax embedding. Eight repeat serial sections (of 3 µm) of the anterior (area where the isthmus enters the uterus) and posterior ends of the uterine tissue were cut, and heat affixed to glass slides and stained with H&E as well as PAS-AB for LM analysis. Purple indicated neutral mucopolysaccharides (PAS^+) and light blue indicated acid mucosubstance (AB^+). The sections were examined using a Nikon Eclipse 80i with NISD software (images were taken using objectives X10 - X100) (Software link: https://www.nikon.com/products/microscope-solutions/support/download/software/imgsfw/nis-d_v5110064.htm). The other sample was fixed in 3% glutaraldehyde in 0.1M phosphate buffer, washed in 0.1M phosphate buffer prior-to and after fixation in 1% osmium tetroxide. The tissue was sequentially dehydrated using acetone (25%- 100%) prior to being critically point dried. Samples were gold coated in a Polaron SC500 sputter coater and viewed using a field emission scanning electron microscope (FE-SEM-Zeiss Ultra Plus, Germany). All processing, examination/measuring and image capturing was undertaken at the Microscopy and Microanalysis Unit (MMU) (University of KwaZulu-Natal, Durban).

After analysis of the tissue and to understand its morphology, measurements of the epithelium involved measuring each uterine lamella (UL/folds) using the NISD software. The length of the UL was measured from its tip to its base and its width was measured from side to side. The number of blood vessels within the lumen and periphery of each UL was also quantified. Measurements of the uterine wall also undertaken with the middle wall area (submucosa, muscularis and the areolar tissue)

measured from the basal lamina to the end of the areolar tissue and serosa (S) measured from the beginning of the dense muscle area to the mesothelial area. Combined, the middle wall and the S gave the thickness (width) of the uterine wall (**APPENDIX E**)

4.4 Results

4.4.1 shark sampled

After the processing and analysing of uterine tissue from 40 females (which consisted of an uneven dispersal of NGF and GF reproductive stages) based on **APPENDIX F (**and **CHAPTER 3),** a final count of nine NGF and 20 GF was reported. The low count of NGF was due to the uneven distribution of the NGF sub stages as well as problems encountered in processing of the tissue. The NGF samples comprised of RS1 (n = 1); RS2B (n = 3); RS3 (n = 5) whereas the GF comprised of RS4 (n = 8); RS5 (n = 12); RS5A (n = 1) RS5C (n = 4) and RS5D (n = 7). Tissue samples from females in stages RS2A and RS5B were not assessed in this study.

4.4.2 Uterine transformation

The reproductive tract of *C. taurus* females comprised of one functional right ovary that extends to two left and right oviducts. Ovulated ova proceed to the O.G, isthmus and uterus (**Figure 2.3**). The epithelium and wall segments of the uterus for both the anterior and posterior sections of the uterus in NGF and GF were examined and found to be similar. The most striking feature of the uterus was the evident transformation, in its epithelium and wall structures, through the immature to gravid reproductive stages. The changes in the uterine morphology are described here with illustrations provided via H&E and PAS-AB histological staining.

4.4.2.1 Uterine wall

Investigation into the uterine wall of the NGF and GF sampled in this study confirmed the following sections of the tissue: 1) the mucosa (M) (comprising of consist of the luminal epithelium, the basement membrane and the lamina propria (LP), 2) the middle wall (comprising of the submucosa with vascular elements, muscularis mucosa (MM) and area of loose areolar tissue) and 3) the Serosa (S) (comprising of the smooth

muscle layers with single outer mesothelial layer) (**Figure 4.1** and **Figure 4.2**).

4.4.2.1.1 Description of the uterine wall in NGF

All layers of the uterus from the immature to mature, sexually active females (RS3) stained for PAS (i.e. purple: normally indicative of neutral mucopolysaccharides), except for RS1 that stained PAS and AB (neutral and acidic mucopolysaccharides) but no mucus cells were found. Hence the term PAS^{+} not used. Few blood vessels were found in the uterine wall of the NGF. An increase in the width of the wall (4165-7296 µm) was noted as females developed from immature to sexually mature active females (**Table 4.1).** The middle wall (680-5796 µm) and S (500-1500 µm) segments increased with maturity **(Table 4.1).**

4.4.2.1.2 Description of the wall in GF

All layers of the uterine tissue in GF sharks stained for PAS but no mucus cells were found. Blood vessels of varying sizes were dispersed throughout the wall. The width of the uterine wall decreased (from 11 374 µm to 4836 µm) (**Table 4.1; Figure 4.2**) as the pregnancy progressed. The middle wall (thickness: 9288 - 4065µm) and S (thickness 2086 - 722µm) segments decreased as the females progressed in pregnancy **(Table 4.1).**

4.4.2.2 Uterine epithelial projections

The changes in the uterine epithelial morphology from immaturity to pregnancy are described below using images from H&E and PAS-AB staining with measurements were taken from the uterine epithelium of NGF and GF (**Table 4.1)**

4.4.2.2.1 NGF: description of the immature females (RS1)

The epithelia of immature females (RS1) were shown to be relatively flat to smooth with an undulating surface. The surface was lined by a basal lamina (BL) (**Figure 4.3A**), populated by columnar basal cells (BC) (**Figure 4.3A**) stained for PAS and AB but no mucus secreting cells were found. Hence the terms PAS^{+} and AB^{+} not used. No blood vessels were found in the mucosa but small blood vessels did appear in the middle wall area (**Figure 4.3A**). The middle wall and S areas took up both stains (**Figure 4.3**).

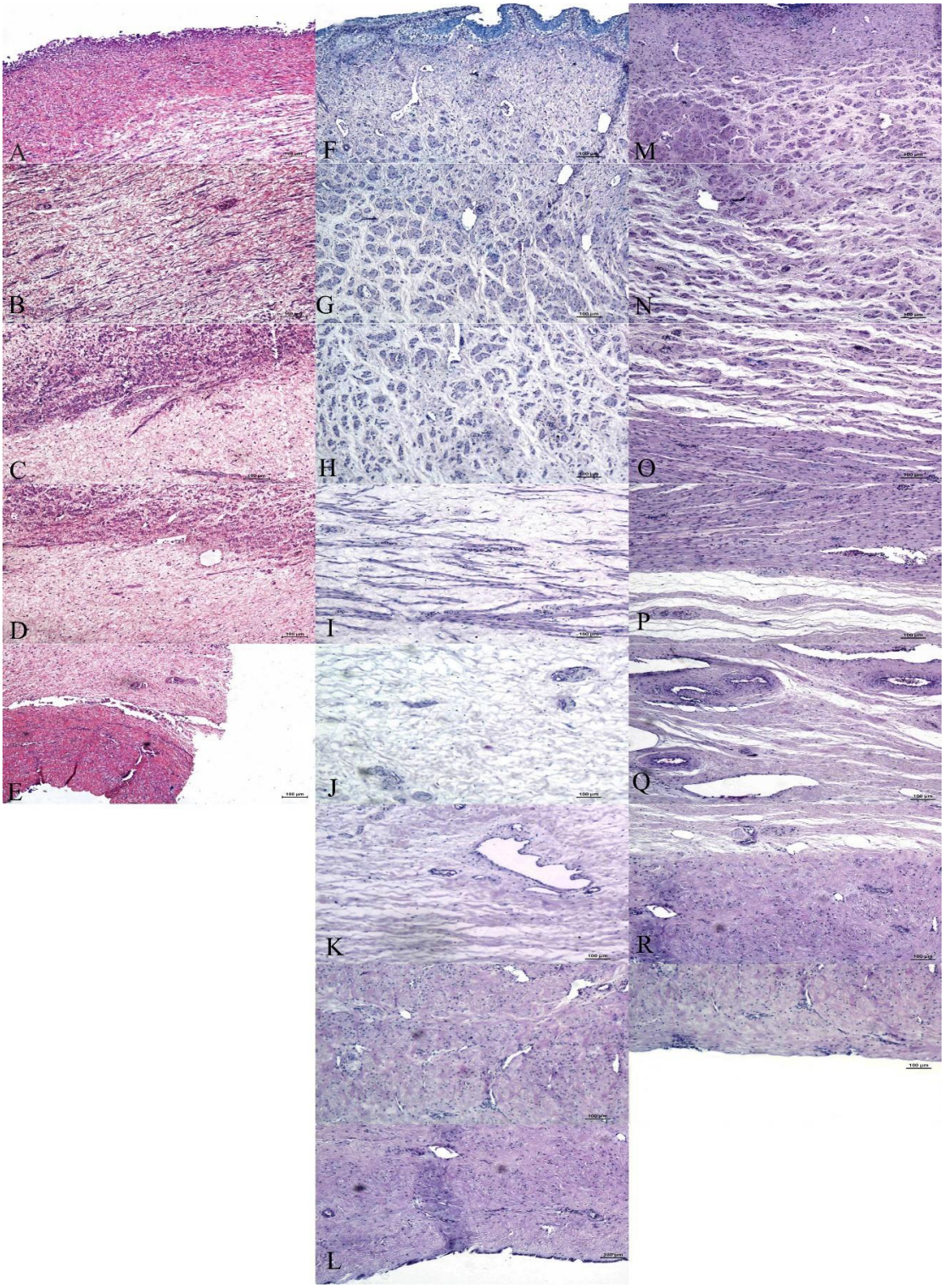


Figure 4.1: PAS-AB images demonstrating the difference in wall thickness between *C. taurus* NGF: Images A-E) are immature females (RS1), Images F-L) are mature but virgin females (RS2B) and Images M-R) are mature and mating females (RS3). Images A, F and M represents the mucosa section (i.e. epithelial and LP layer); Images B, C, G-I and M-P represents the middle wall section (i.e. MM layer); as well as Images C-D, J-K and P-Q that represents the areolar tissue layer). Images E, L and R-S represents the serosa section (Scale Bar = 100 µm for all images).

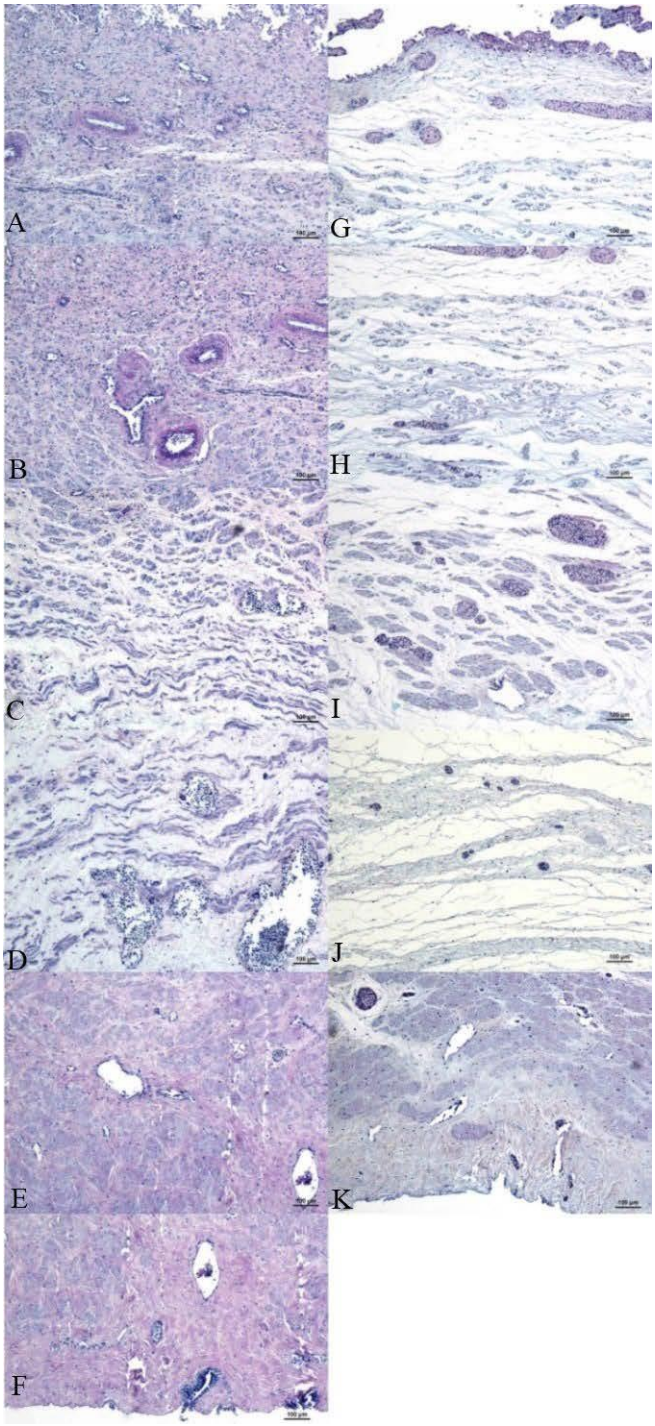


Figure 4.2: PAS-AB stained images of uterine tissue demonstrated the difference in the thickness of the wall between early *C. taurus* GF (RS4; Images A-F) and late G (RS5D; Images G-K). Images A and G represents the mucosa section (i.e. epithelial and LP layer); Images B-C and H-I represents the middle wall section (i.e. MM layer). Images D and J also represents the middle wall section (i.e. areolar tissue layer). Images F and K represents the Serosa section (Scale Bar = 100 µm for all images)

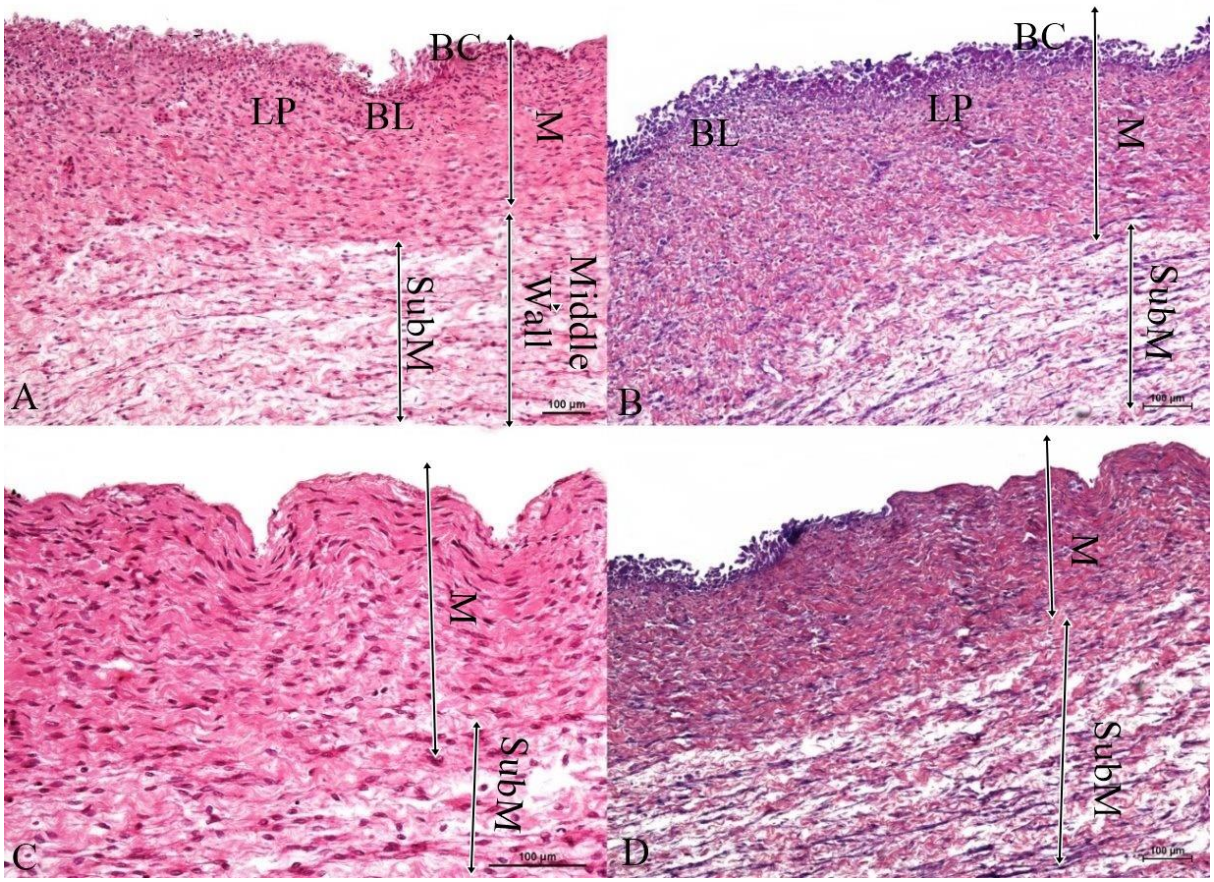


Figure 4.3: Images A and C (H&E staining) and Images B and D (PAS-AB staining) of the immature *C. taurus* NGF (RS1) epithelium and wall of the uterus. Images A and B indicate the basal lamina (BL), basal cells (BC), and Lamina propria (LP) in the Mucosa (M). The SubM (submucosa) which is part of the middle wall area, is shown below the M region, as shown in Images C and D. Scale Bar = 100 µm for all images.

4.4.2.2.2 NGF: description of the mature non-active females (RS2B)

The undulating M (in RS1) changed to mucosa with small, thick and short UL protruding into the lumen of the uterus of these mature females (RS2B) (**Figure 4.4A).** Each UL was derived from the penetrating SubM below and covered by the existing BL Although the UL stained for PAS, no distinct mucus cells were found. This lamina was populated with nucleated mature columnar basal cells (MBC) and possible replacement stem cells in a pseudo stratified epithelium. The connective tissue forming the body of the fold stained for PAS but no distinct mucus cells were found. In addition, there was a stratified layer (SL) of cuboidal/squamous cells that filled the apical surface, surrounding the columnar cells below which stained for AB, especially in the trough (T) areas of the UL, no mucus cells were found. This layer does not appear in the immature

female. There were slight indications of BV development in the middle wall area of the tissue **(Figure 4.4**). The UL and the wall appeared larger than the immature female (RS1) (**Table 4.1).**

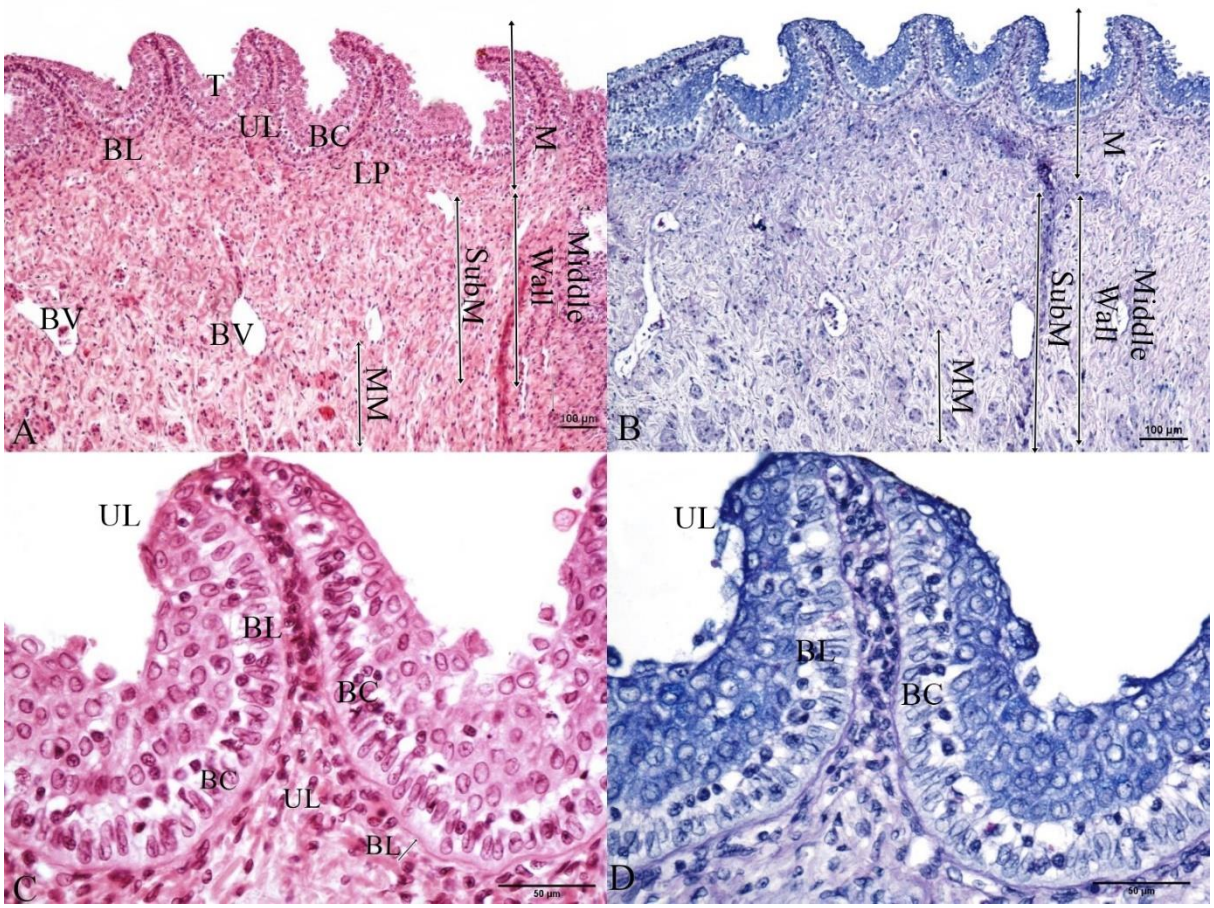


Figure 4.4: Images A and C (H&E staining) (Scale Bar = 100 µm) shows the epithelium and the wall of the uterus while Images B and D (PAS-AB staining) (Scale Bar = 50 µm) shows the wall of the uterus in the mature, inactive *C. taurus* NGF (RS2B). Image A and B shows the uterine lamellae (UL) lined by basal cells (BC) on the basal lamina (BL) and lamina propria (LP) pushing into the lumen of the uterus that forms the Mucosa (M). This formation creates troughs (T) between the UL. The submucosa (SubM) and muscularis mucosa (MM), layers that form the middle wall area also shown below the M region with small BV. Images C and D show a closer view of the UL, BC and BL areas.

4.4.2.2.3 NGF: description of the mature sexually active females (RS3)

The same UL, seen in RS2B, appeared more irregular, thinner and longer in the M area in the sexually active females **(Table 4.1)**. The connective tissue forming the body of the fold stained for PAS, no distinct mucus cells were found (**Figure 4.5B and D)** and was lined with MBC (**Figure 4.5A**) and stem cell replacement. The length and width of the

UL and the uterine wall thickness were greater than those of RS1 and RS2) NGF (**Table 4.1).** However, RS3 NGF had variation between the epithelium. Some UL showed BV formation (**Figure 4.5B)** while others did not **(Figure 4.6).** Blood vessel formation also occurred in varying degrees, within the wall of all RS3 females. There also appeared to be a SL of cells lining the UL with the greatest density in the trough (T) regions of the epithelium (**Figure 4.5A-B**).

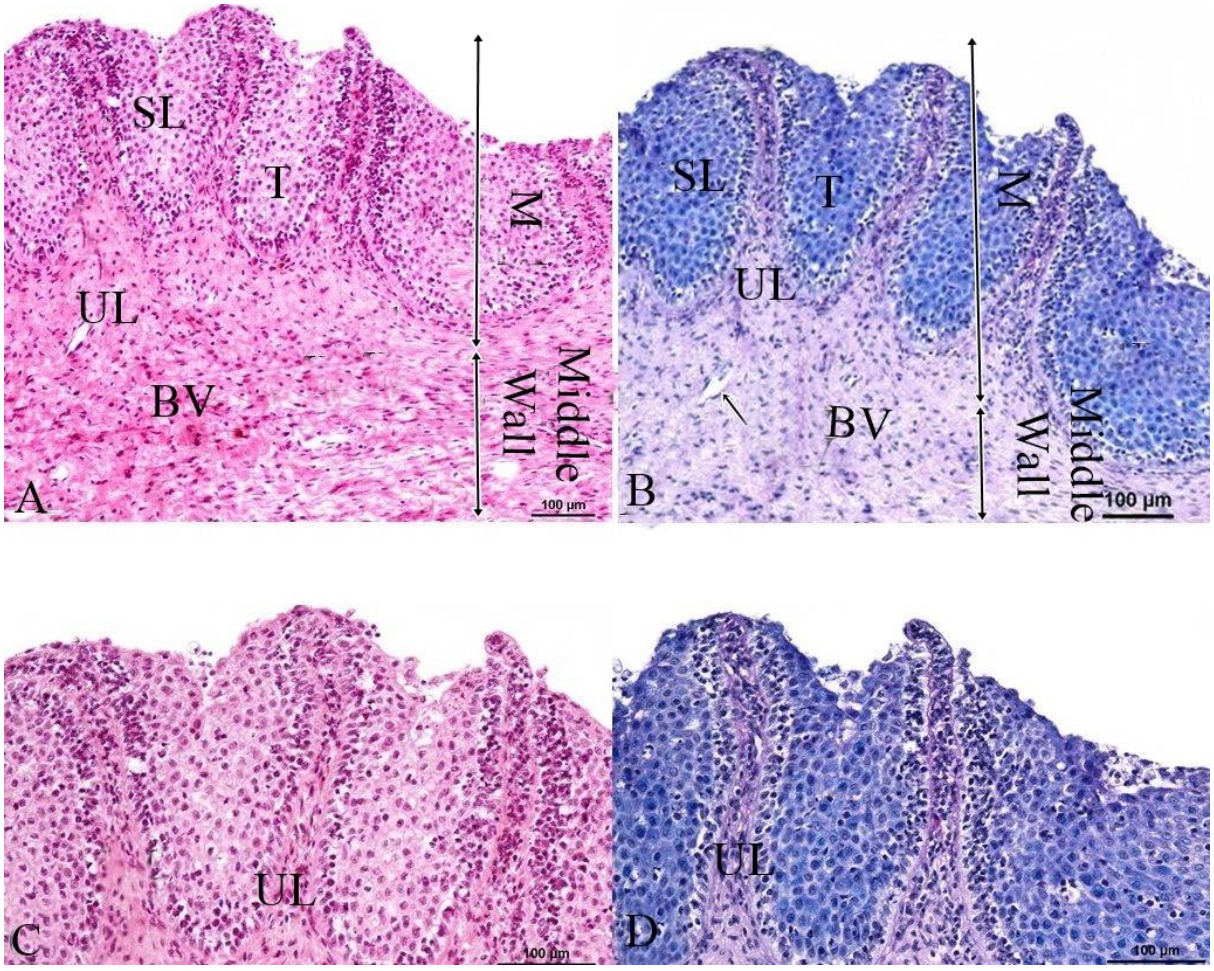


Figure 4.5: Images A and C (H&E staining) shows the epithelium and wall of the uterus while Images B and D (PAS-AB staining) show the epithelium of the uterine tissue in in a sexually active *C. taurus* GF (RS3). Image A and B shows the uterine lamellae (UL) lined by basal lamina (BL) and mature basal cells (MBC) in the mucosa region (M). The mucosa (M) region filled with stratified layer of cells (SL) in the trough (T) areas of the epithelium. Blood vessels (BV) are shown in the submucosa (SubM) area under the M region shown in closer view in Images C and D. Scale Bar = 100 µm for all images.

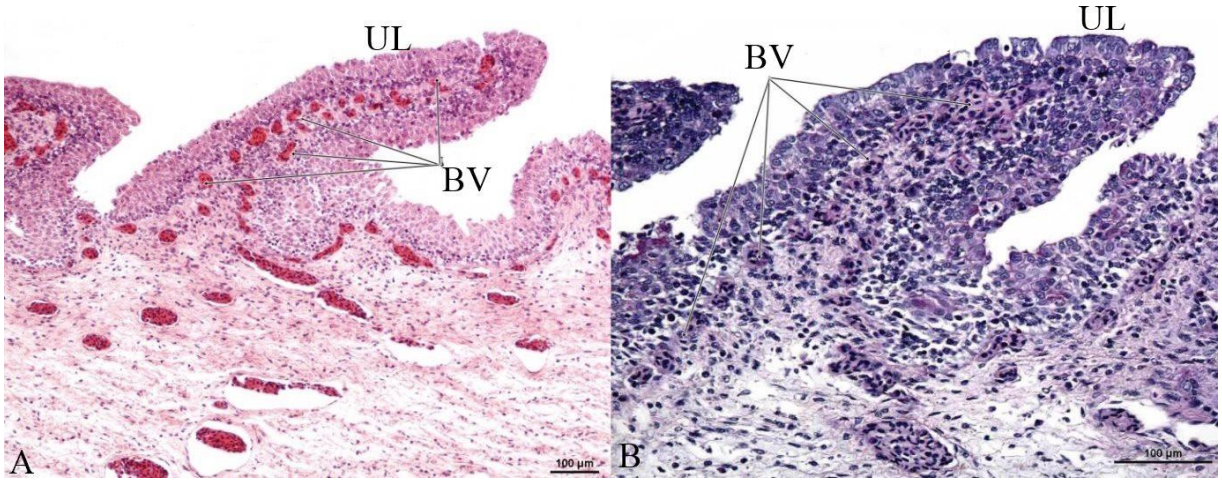


Figure 4.6: Image A (H&E staining) and Image B (PAS-AB staining) of the epithelium of the uterus with the presence of blood vessels (BV) within the uterine lamellae (UL) in a sexually active *C. taurus* GF (RS3) (Scale Bar = 100 µm for all images).

4.4.2.2.4 Measurements of the epithelium of NGF sharks

The ranges in length (153-318 µm), width (62-143 µm) as well as the distance between the UL (127-326) increased as the females matured (**Table 4.1**). Blood vessel (BV) formation also became visible in RS3 females with a median count of 11 within each UL in this stage.

4.4.2.2.5 Epithelium description of GF that only contain capsules (RS4)

This stage involves the presence of an embryo. The different lengths of UL (thin and long) protruding into the lumen of the uterus dominated this stage **(Figure 4.7A-B)**. These tubular structures were derived from the penetrating SubM below, covered by the existing BL, attached to nucleated columnar basal cells (BC) (**Figure 4.7E-F**). The connective tissue, forming the body of the UL, stained for PAS, no distinct mucus cells were found (**Figure 4.7D** and **F).** The lamellae appear to be in transition with many different lengths and types; with some extending from other UL (**Figure 4.7E-F)**. Small irregular spaces (arterial loops; AL) were found emerging along the periphery and within each UL itself (**Figure 4.7C-D**), which were to become sinusoidal blood vessels (BV) (indicated by arrows). These structures stained for AB and became lined by squamous epithelium, with no mucus cells. The BC also stained AB especially at the top end of the cell (indicated by asterisk in **Figure 4.7E)** and may prevent abrasion to the underlying surface when capsules enter the uterus. However no mucus cells were found.

4.4.2.2.6 GF: Description of epithelium in females with pre-hatched/encapsulated embryos (RS5A)

Stage RS5A is representative of embryos that are still encased in their respective capsules within the lumen of the uterus with protruding UL (**Figure 4.8A-D).** The UL reduce in thickness (distance between the BL and tip) and show an increase in the number of bud-like/arteriole loop (AL) projections (**Figure 4.8C-D**) along the periphery/length of each UL. These buds appeared to be encased by simple squamous epithelium. There were fewer UL and an absence of columnar cells present in the earlier stages. However, the decrease in cells could be as consequence of the technical processing of the tissue as there was an increase in the appearance of these cells in subsequent stages.

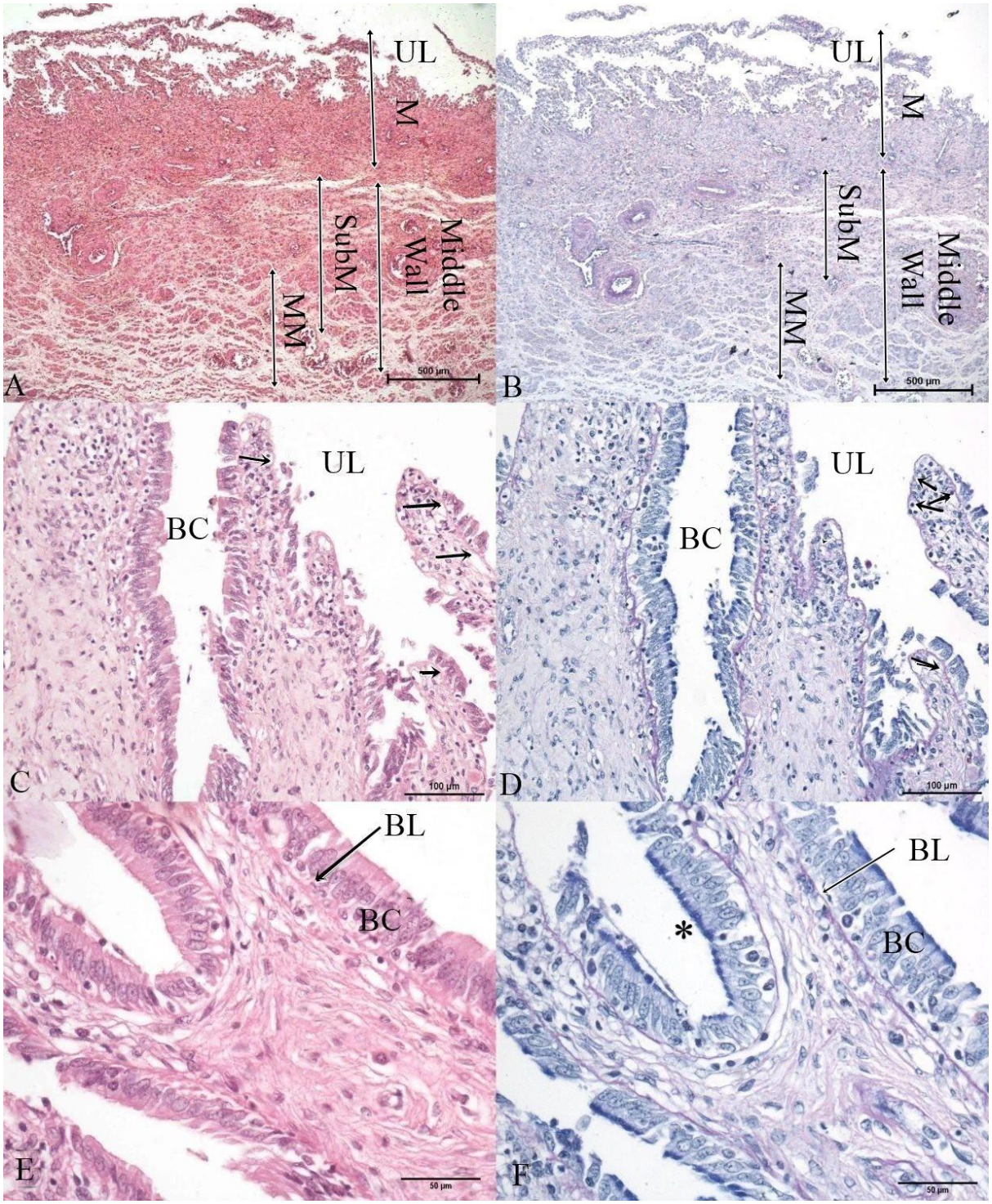


Figure 4.7: Images A, C and E (H&E stains), Images B, D and F (PAS-AB) stains of the wall and uterine lamella (UL) the epithelium of the uterus in *C. taurus* GF (RS4). Image A and Image B (Scale Bar = 500 µm) shows the UL in the mucosa (M) and submucosa (SubM) and muscularis (MM) layers of the middle wall. Image C and Image D (Scale Bar = 100 µm) depicts the UL lined with basal cells (BC) and shows signs of arterial loops (AL) (indicated by arrows). Images E and F (Scale Bar = 50 µm) shows a closer view of the UL shows the BC stained, especially at the top end of the cell (indicated by asterisk).

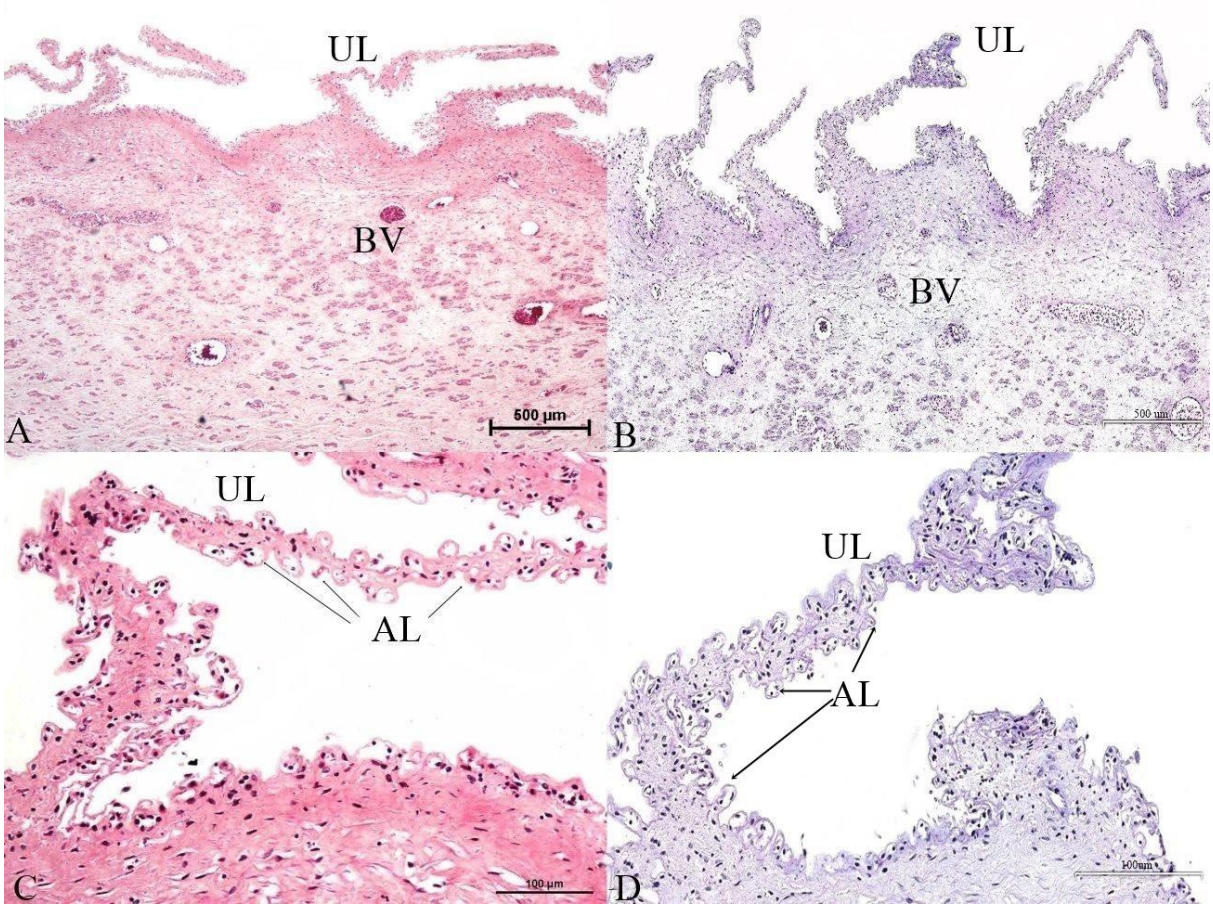


Figure 4.8: Uterine epithelium and wall of *C. taurus* GF (RS5A). Image A and C (H&E stain, Scale = 500 µm) shows the epithelium and the wall of the uterus while Image B and D (PAS-AB stain, Scale = 100µm) shows the epithelium. Images A and B shows the thin, long UL extending into the lumen. Images C and D shows a closer view of the increased bud like/arterial loops (AL), along the periphery of the UL.

4.4.2.2.7 GF: description of epithelium of females with hatched embryos during the intrauterine cannibalistic phase (RS5C)

In RS5C females, embryos undergo intrauterine cannibalism. The UL still protrude into the lumen (**Figure 4.9A-D**) where they now came into contact with FEEs. A distinction in this phase was the increase in bud-like/AL structures along the periphery of the UL connected to the existing BL covering the lamellae that are filled with red blood cells (RBC) (**Figure 4.9C-D**). The BC cells are evident, at certain areas, along the UL and additionally there was an increase in BV in the LP of the uterine wall. The length of the UL and BV's appeared to increase when compared to RS5A and RS5D females.

4.4.2.2.8 GF: description of epithelium in females with single oophagous embryo in each uterus (RS5D)

The uterine epithelium in RS5D females supports one embryo in each uterus. The lamellae were dominated by capillaries located along their length resulting in a protrusion of the capillary and the BL covering the capillary (**Figure 4.10A** and **D)**. These projecting capillaries were filled with RBC (**Figure 4.10C-D**) that appeared spaced out along the length of the UL, extending from the base. Significant difference in UL length was found between RS5D and RS4 (means: 1488 vs. 507, $p = 0.03$; $t(6.99) = 2.5$) and RS5D and RS3 (means: 1488 vs. 374, $p = 0.02$, $t(6.82) = 2.9$).

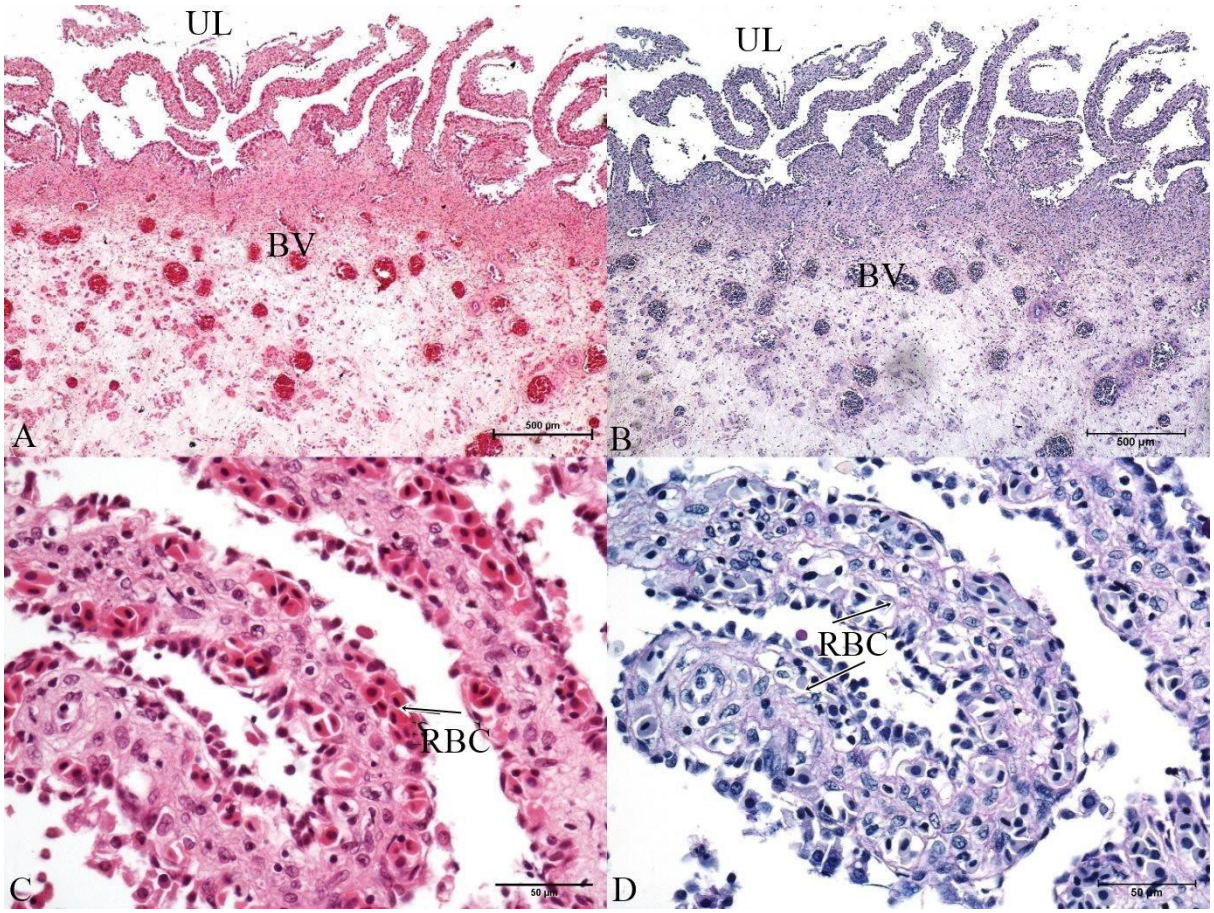


Figure 4.9: Image A and Image C (H&E stain, Scale Bar = 500 µm) shows the uterine epithelium and wall and Image B and D (PAS-AB stain, Scale Bar = 50 µm) shows the epithelium of the uterus in the GF *C. taurus* shark (RS5C). Images C and D shows the increased bud like blood projections along the periphery of UL filled with red blood cells (RBC).

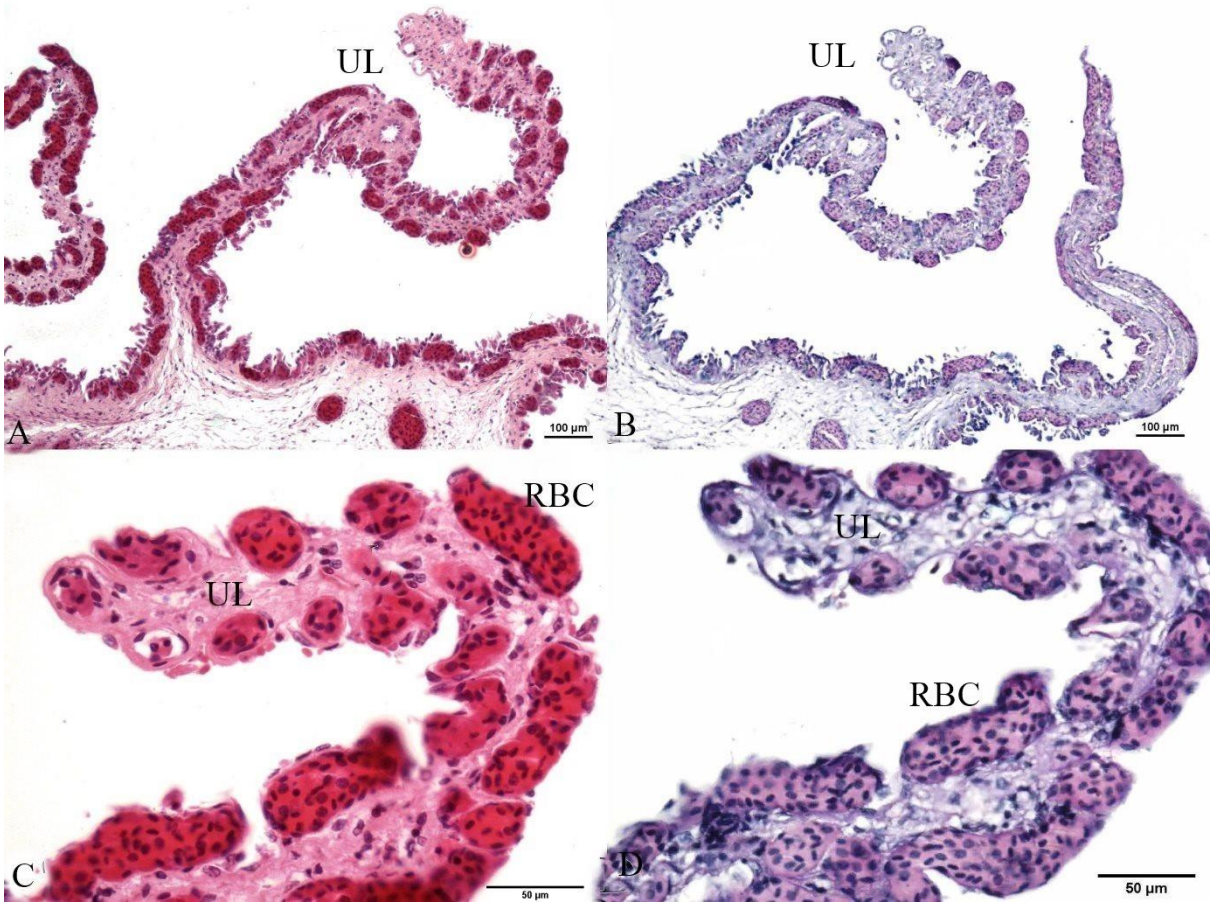


Figure 4.10: Uterine epithelium of *C. taurus* GF (RS5D) is represented in Images A and C (H&E stain, Scale Bar = 100 µm) and Image B and D (PAS-AB, Scale Bar = 50 µm). All images show increase in length of bud like blood projections of blood vessels (BV) filled with red blood cells (RBC) along the periphery of each uterine lamella (UL).

4.4.2.2.9 Measurements of the epithelium of GF

The ranges in length (418-1377 µm) **(Table 4.1**) of the UL continued to increase in the GF sharks (RS4-RS5D) while the width varied continuing to increase in early gravid stages RS4-RS5A (170-179 µm) and then decreasing to 163 µm in RS5D (**Table 4.1**). Distance between the UL showed increased variation (**Table 4.1**). The mean number of BV in the UL also increased (13-19) as pregnancy progressed.

Table 4.1: Median measurements (µm) of the uterine epithelium and wall of *C. taurus* NGF (RS1-RS3) and GF (RS4-RS5D) stages

	NGF			GF			
	RS1 (*n* = 1)	**RS2B** (*n* = 3)	**RS3** (*n* = 5)	**RS4** (*n* = 8)	**RS5A** (*n* = 1)	**RS5C** (*n* = 4)	**RS5D** (*n* = 7)
UL length (µM)	153	309	318	418	765	928	1377
UL width (µM)	95.5	129	143	170	179	134	163
Distance between UL (µM)	127	299	326	231	627	853	669
Average BV in UL	none	none	11	13	13	14	19
Middle wall (µM)	680	5242	5796	9288	5119	4154	4065
Serosa (S) (µM)	500	1409	1500	2086	1125	1131	772
Total wall (µM) (Middle wall + Serosa)	4165	6652	7297	11374	6244	5285	4837

Abbreviations: *n*: number of females; RS1-RS5D: Reproductive Stages (1-5D); NGF: non-gravid females; GF: gravid females, UL: Uterine lamellae; µM: micrometre

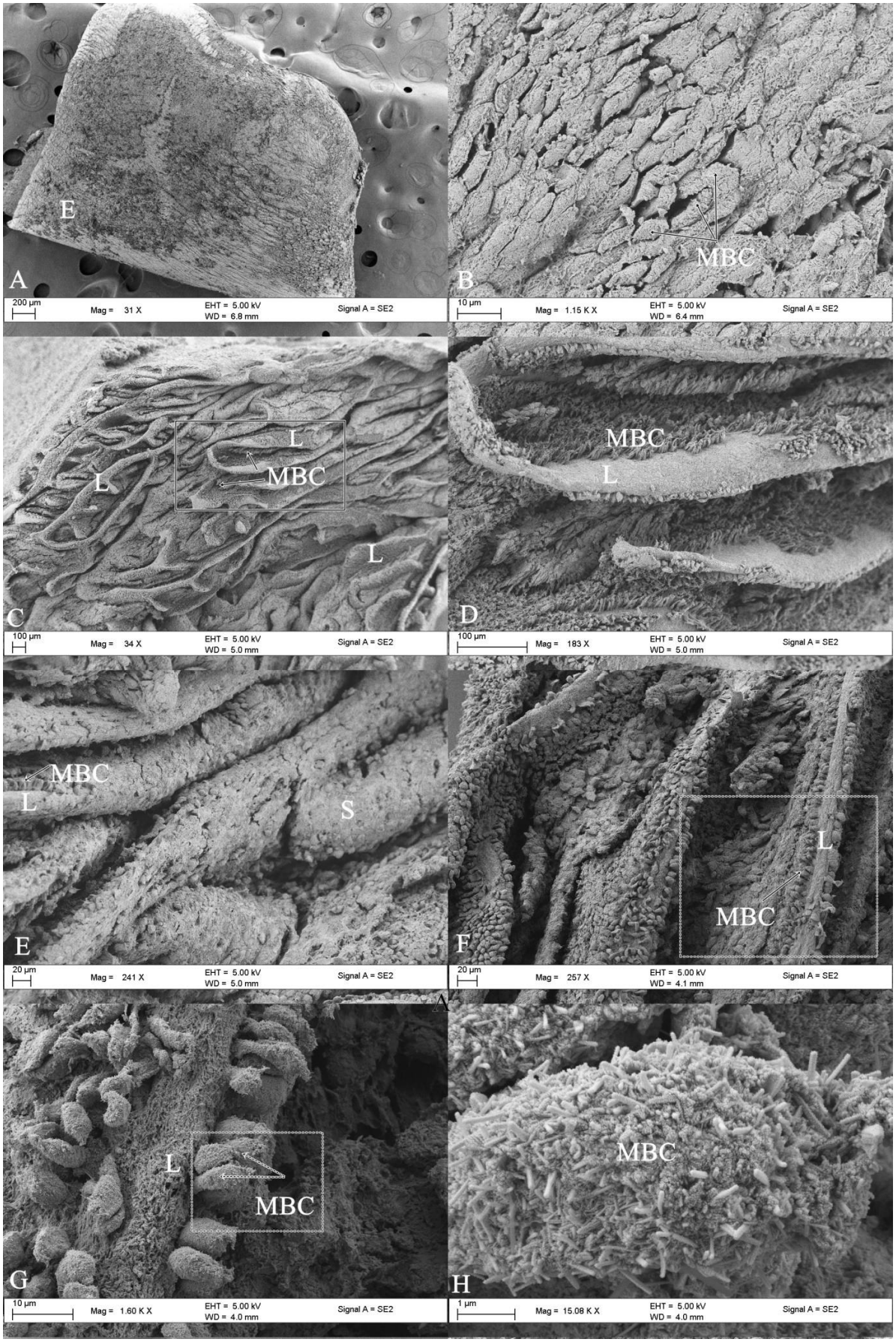
4.4.2.2.10 NGF: scanning electron microscopy of the uterine epithelium

Scanning electron microscopy revealed the outer structure of the epithelium of the NGF female in more detail. The fold/finger-like projections, as observed using light microscopy, were actually "plate like" in structure **(Figure 4.11C)**. The projections, viewed under light microscopy, were cross/oblique sections through tightly packed plates/UL, attached perpendicularly to the uterine wall (**Figure 4.11C**). The UL changed from smooth tissue in immature females (RS1) **(Figure 4.11A)** and mature virgins females (RS2B) (**Figure 4.11C)** to tissue that showed the presence of "cable-like" micro-ridges (MR's) containing BV present on the surface of the UL in RS3 females **(Figure 4.11J).**

In addition, the MBC, columnar in appearance, appeared in immature and mature, sexually active females **(Figure 4.11B** and **D**). However, on some areas of the UL (more exposed) these cells appeared columnar **(Figure 4.11D)** but were dehydrated/damaged. The UL, not exposed, contained rounder and fuller columnar cells attached to the wall **(Figure 4.11F-G**). There was also a form of some secretion (SC) around the cells **(Figure 4.11E**) and evidence of the SL between the UL, as documented by light microscopy (**Figure 4.11I**).

4.4.2.2.11 GF: scanning electron microscopy of the uterine epithelium

The “plate-like” lamellae observed in the NGF were also seen in the GF sharks (RS4-RS5D) (**Figure 4.12A-H).** The UL were arranged in an accordion-like fashion, in all the gravid stages and presented with MR’s on its surface, that became more prominent, as the pregnancy progressed. The increase MR thereby indicated the increase the presence of BV which complimented the light microscopy images depicting their formations along the periphery of the UL (**Figure 4.8 – Figure 4.10**).


E
A
200 µm Mag = 31 X EHT = 5.00 kV WD = 6.8 mm Signal A = SE2
MBC
B
10 µm Mag = 1.15 K X EHT = 5.00 kV WD = 6.4 mm Signal A = SE2
L
MBC
L
L
C
100 µm Mag = 34 X EHT = 5.00 kV WD = 5.0 mm Signal A = SE2
MBC
L
D
100 µm Mag = 183 X EHT = 5.00 kV WD = 5.0 mm Signal A = SE2
MBC
L
S
E
20 µm Mag = 241 X EHT = 5.00 kV WD = 5.0 mm Signal A = SE2
L
MBC
F
20 µm Mag = 257 X EHT = 5.00 kV WD = 4.1 mm Signal A = SE2
L
MBC
G
10 µm Mag = 1.60 K X EHT = 5.00 kV WD = 4.0 mm Signal A = SE2
MBC
H
1 µm Mag = 15.08 K X EHT = 5.00 kV WD = 4.0 mm Signal A = SE2

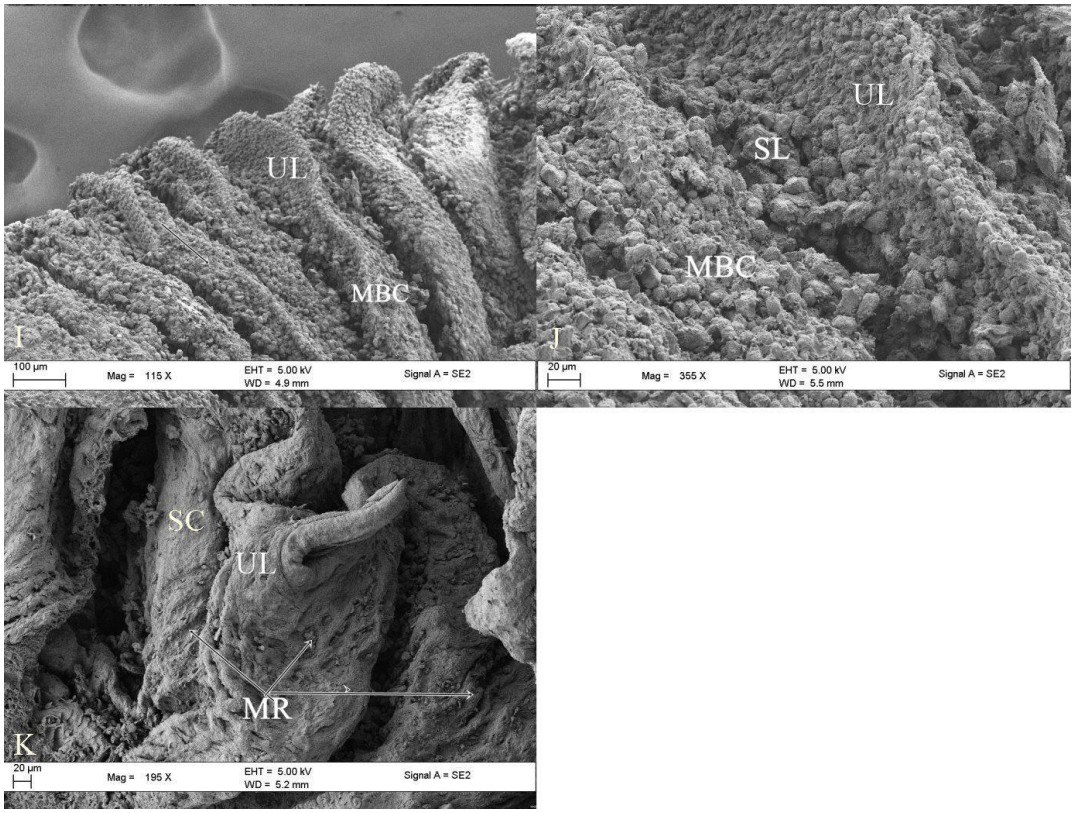


Figure 4.11: SEM images of *C. taurus* NGF (RS1-RS3). Shows the presence of "plate-like" structure of the uterine lamellae, mature basal cells (MBC), stratified layer of cells (SL) and secretory substance (SC) on some of the epithelium of the immature (RS1: Image A, Scale Bar = 20 µm) and Image B, Scale Bar = 10 µm), mature and inactive (RS2B: Images C and D, Scale Bar = 100 µm, Images E-F, Scale Bar = 20 µm) and Image G, Scale Bar = 1 µm) and the mature, sexually active (RS3:Images I, (Scale Bar = 100 µm and Images J-K, Scale Bar = 20 µm).

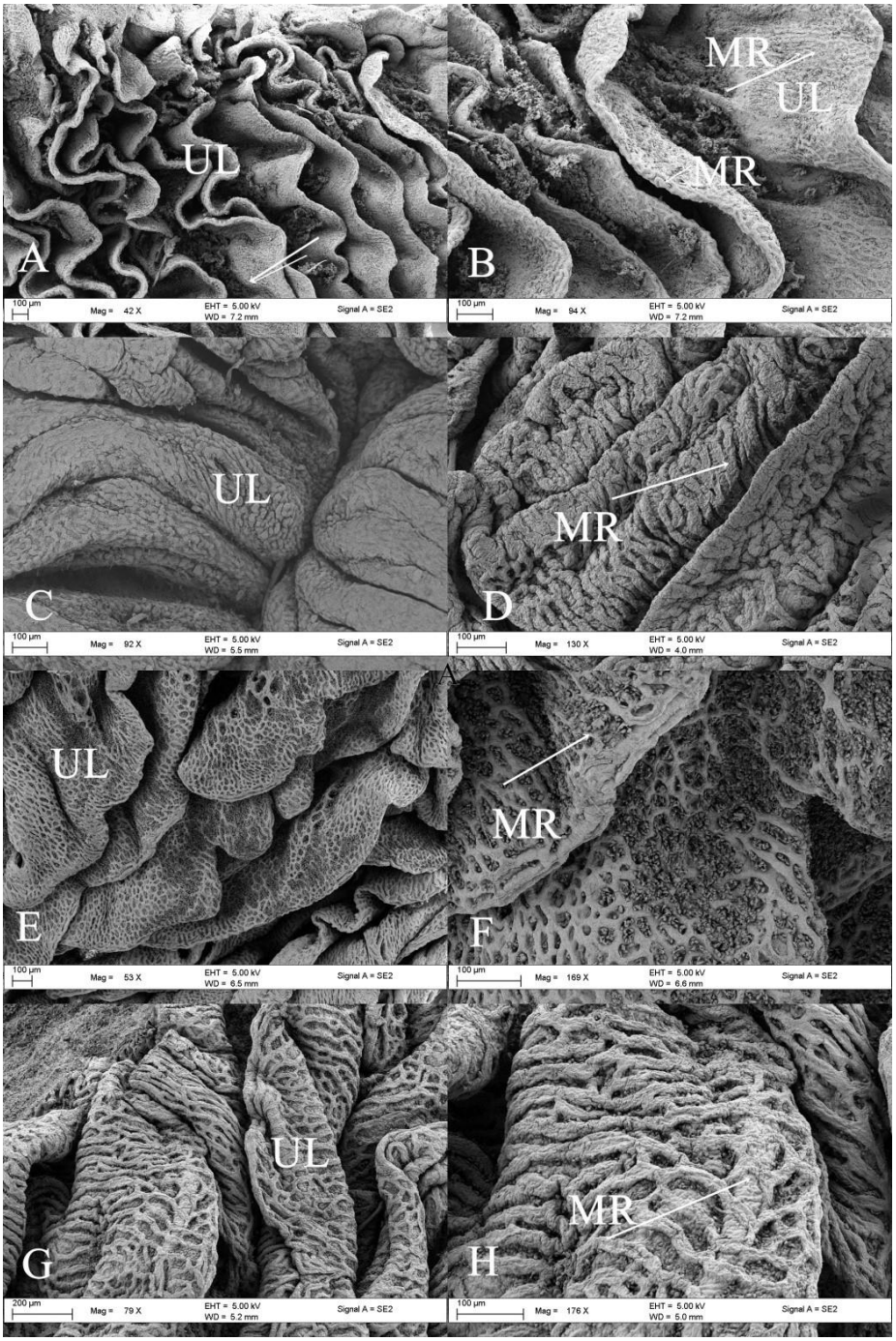


Figure 4.12: SEM images of uterus of *C. taurus* GF (RS4-RS5). Shows the uterine lamellae (UL) surrounded by micro-ridge (MR) structures that become prominent as pregnancy progresses in RS4: Images A and B, RS5A: Images C and D, RS5C: Images E and F and RS5: Images G and H. Scale Bar = 100 µm for all images except Image G (Scale Bar = 200 µm).

4.5 Discussion

In lamniform sharks, such as *C. taurus*, oophagy is one of the primary modes of embryonic nutrition [8,20]. However, *C. taurus* embryos are normally found surrounded by fluid within the uterus. Hence clarification was needed to confirm if there are any contribution to this fluid by uterine secretions (histotrophy) which could suggest maternal nourishment as indicated in other sharks [21-23]. Descriptions of the uterus of *C. taurus* have previously been presented [2,5] with ambiguity. Both studies described the increased vascularisation of the tissue concluding that the tissue served a respiratory function. Gilmore (1993) based his findings on the tissue/s of a pregnant *I. oxyrinchus* while Hamlett and Hysell (1998) based their findings on a single NGF and GF. Gilmore (1993) also described the tissue as possibly having a secretory function. This study aimed to seek clarification on the literature and extend it.

The UL (i.e., epithelial folds) increased in length, width and frequency from the onset of maturity (RS2B) (**Figure 4.4; Table 4.1**). The UL are created through the SubM pushing upward through the top layer of tissue (**Figure 4.4**). We suggest that the act of mating (and fertilisation that follows thereafter) is the trigger for the immense change in the epithelium to accommodate the ensuing pregnancy. Hamlett and Hysell (1998) recorded no UL appearance in the NGF *C. taurus* female and suggested that vascularised UL's become visible when embryos appear. Our study, however, showed the presence of UL in *C. taurus* NGF. A stratified layer of cells (SL) were found on the apical uterine surface of NGF sharks (RS2B and RS3) (**Figure 4.5** and **Figure 4.7I**). The thickness of these cells would provide function to the underlying epithelium of the uterus.

Blood vessel development, possibly originating from the SubM **(Figure 4.6)**, first appeared in the UL's of some mature, sexually active females (RS3) females **(Figure 4.5; Table 4.1)** while not appearing in other RS3 females **(Figure 4.6).** The variation in blood vessels indicates females at different stages of UL development. The different rates of tissue transformation, which could also be affected by virgins mating for the first time versus those reoccurring due to uterine regeneration dependant on time after post-partum [24]. Blood vessel development in UL's dominated in GF (**Figure 4.7-Figure 4.10; Table 4.1**). Arterial BV

loops distributed themselves from the inner connective tissue of the UL (in early staged females RS5A) (**Figure 4.7-Figure 4.8**) to form "bud-like" structures along the periphery that became covered by a simple layer of squamous epithelium filled with red blood cells B (**Figure 4.9-Figure 4.10**). The peripheral location of the BV's increased its proximity to the lumen of the uterus, which aids the respiration of the embryos found in the UF [6]. This study confirmed and extended Hamlett and Hysell (1998) results by postulating that the G *C. taurus* described was in fact an early to mid-pregnant RS5A or RS5B) indicated by the central location of BV in the UL. Closer inspection revealed that UL projection were tightly packed "plates", folded in an accordion fashion, attached perpendicularly to the wall and lined with a thick cable network of MR containing the BVs. These MR structures, became visible in the NGF (RS3) **(Figure 4.12**), suggest the ability to supply oxygen to the uterine surface is 56 times greater compared to a uterus with a smooth surface [24]. Gilmore (1993) described similar "microfold ridges" in another aplacental lamnoid shark, the shortfin mako and postulated that the large surface area of the uterus and the location of high vascularisation allowed oxygen to enter the UF. This study showed for the first time that the MR structure does exist in *C. taurus* females. The prominent appearance of vascularisation, as pregnancy progressed, suggests that plasma plays a vital role in the development of *C. taurus* embryos found in the UF [2,13,25].

The thickness of the UW increased as the females matured (NGF: RS1-RS3) (**Figure 4.1; Table 4.1).** The largest width of the uterine wall was identified in females that were pregnant with capsules (GF: RS4) (**Figure 4.7; Table 4.1**). The thickness of the wall layers thereafter reduced as the female progressed during pregnancy (G: RS5A-RS5D) (**Figure 4.8-4.10)**. The thinning of the wall aids in the reduction of diffusional distances [2,26]. Neutral mucopolysaccharides were the predominant characteristic for the uterine epithelium and wall in all stages.

Histological examination of the uterine tissue, through all NG and GF, did not reveal any secretory structures/apparatus of the uterine lining such as villi usually responsible for uterine histotrophy observed in other studies [2,5,6,20] compared to the reported weight gain of 1, 200.000% in *C. taurus* embryos [3]. This suggests that the additional

nutrient strategy employed by *C. taurus* females (i.e. intrauterine cannibalism and oophagy) could outweigh any UF nutritional benefit which could explain the lack of secretory structures. However, the composition of the UF, close examination of cellular structure at the epithelium and other avenues of transfer such hemotrophic transfer from maternal blood to the UF, occurring across the uterine lining [2,12,20,27] and possible minimal histotrophy [28] still needs to be investigated to determine if minimal secretion (organic/inorganic) could exist.

This paper serves as the first detailed description of the uterus of *C. taurus* female which showed the following matrotrophic characteristics with: 1) increased vascularisation and peripheral location of the BV's; 2) reduction in uterine wall thickness of the wall, during gestation, l reduced the distance for diffusion of necessary compounds and 3) increased development of longitudinal UL increased the surface area [2,26,28]. These characteristics support the developing embryos through accommodation and the assistance with respiration and osmoregulation [20]. The role of the uterine lining in promoting UF needs further study as UF plays a pivotal component to model in future breeding programmes intent to increase this species size.

4.6 Conclusion

This study is the first to describe the transformation of *C. taurus* NGF and GF uterus wall and epithelium. The increased presence of long UL lined with MR structures containing capillaries that increase as pregnancy progresses together with the reduction in wall thickness indicates that this tissue is created for respiration and not nutrient secretion. Embryonic nutritional provision via secretory structures was not shown. The study also showed the first evidence for the presence of MR's in this species which further supports this tissue's function for respiration. Future studies should microscopically investigate the cells of the UL and the wall of the uterine tissue using TEM techniques to elucidate an understanding into the cellular structures that encompass this tissue. The increased vascularisation and close proximity to the UF indicating the importance of the maternal fluids was explored elsewhere (**CHAPTER 5).**

4.7 Acknowledgements

We owe immense gratitude to: the KZNSB research staff (Mr P Zungu, Mr E Makhatini, Mr B Zungu and Mrs AJ Ganesan) and the KZNSB operations crew for the tracking and proper handling of all sharks included in this study; Dr J Wesley-Smith, Mrs S Singh, Mrs DG Pepperall and Mrs J Maxwell for their histological advice and service; Dr K Dhuness for his assistance in the editing process. The senior author is grateful to the New South Wales, Department of Primary Industries, for the scholarship, purchase of necessary equipment and funding of analysis on this project. The National Research Fund (Deutscher Akademischer Austauschdienst) is also gratefully acknowledged.

4.8 References

1. Chen, M. (2001) *The form and structure of the uterus of spadenose shark, Scoliodon laticaudus.* Journal of Xiamen University (Natural Science), **40**, 92-96.
2. Hamlett, W.C. and Hysell, M.K. (1998) *Uterine specializations in elasmobranchs.* Journal of Experimental Zoology, **282**, 438-459.
3. Wourms, J.P. (1981) *Viviparity: the maternal-fetal relationship in fishes.* American Zoologist, **21**, 473-515.
4. Musick, J. and Ellis, J. (2005) In Jamieson BGM (ed.), *Reproductive biology and phylogeny of Chondrichthyes: sharks, batoids and chimeaeras*, 3, 45-79.
5. Gilmore, R.G. (1993) *Reproductive biology of lamnoid sharks.* Environmental Biology of Fishes, **38**, 95-114.
6. Gilmore, R.G., Putz, O. and Dodrill, J.W. (2005) In Hamlett WC (ed.), *Reproductive biology and phylogeny of Chondrichthyes: Sharks, Batoids and Chimaeras.* Science Publishers, Enfield, New Hampshire, USA, 435-462.
7. Hamlett, W. and Koob, T. (1999) In Hamlett WC (ed.), *Sharks, Skates and Rays:The Biology of Elasmobranch Fish*. Baltimore, MD: Johns Hopkins University Press, 398-444.
8. Wourms, J.P. (1977) *Reproduction and development in chondrichthyan fishes.* American Zoologist, **17**, 379-410.
9. Wourms, J.P., Grove, B.D. and Lombardi, J. (1988) *The maternal-embryonic relationship in viviparous fishes.* Fish Physiology, **11**, 1-134.
10. Gilmore, R.G., Dodrill, J.W. and Linley, P.A. (1983) *Reproduction and embryonic development of the sand tiger shark, Odontaspis taurus (Rafinesque).* Fishery Bulletin, **81**, 201-225.
11. Naidoo, K., Chuturgoon, A.A., Cliff, G., Ellis, M.T., Otway, N.M., Gregory, M.A., Singh, S.D. and Naidu, S.L. (2017) *Dentition facilitates the release of encapsulated Ragged-tooth Shark (Carcharias taurus) embryos.* Environmental Biology of Fishes, **100**, 1343-1354.
12. Lombardi, J., Jones, K.B., Garrity, C.A. and Files, T. (1993) *Chemical composition of uterine fluid in four species of viviparous sharks (Squalus acanthias, Carcharhinus plumbeus, Mustelus canis and Rhizoprionodon*

terraenovae). Comparative Biochemistry and Physiology Part A: Physiology, **105**, 91-102.

13. Chen, C.T., Liu, K.M. and Chang, Y.C. (1997) *Reproductive biology of the bigeye thresher shark, Alopias superciliosus (Lowe, 1839)(Chondrichthyes: Alopiidae), in the northwestern Pacific.* Ichthyological Research, **44**, 227-235.

14. Otake, T. and Mizue, K. (1981) *Direct evidence for oophagy in thresher shark, Alopias pelagicus.* Japanese Journal of Ichthyology, **28**, 171-172.

15. Shann, E. (1923), *Proceedings of the Zoological Society of London*. Wiley Online Library, **93**, 161-171.

16. Dicken, M.L. (2006) *Population dynamics f the raggedtooth shark (Carcharias taurus) along the east coast of South Africa*. PhD Thesis, Rhodes University, South Africa.

17. Dudley, S. (2013) In Mann, B. (ed.), *Southern African Marine Linefish Species Profiles Special Publication No 9.* Durban Oceanic Research Institute, 134-136.

18. Olbers, J. and Cliff, G. (2017) *A brief survey of ragged-tooth shark (Carcharias taurus) abundance and behaviour during their annual gestation period on Quarter-mile reef, Sodwana Bay, iSimangaliso Wetland Park, South Africa.* Season Report for 2016/2017. Ezemvelo KZN Wildlife Internal Report, Pietermaritzburg, South Africa, pp 15.

19. Smale, M. (2002) *Occurrence of Carcharias taurus in nursery areas of the Eastern and Western Cape, South Africa.* Marine and Freshwater Research, **53**, 551-556.

20. Hamlett, W.C., Kormanik, G., Storrie, M., Stevens, B. and Walker, T.I. (2005) *Chondrichthyan parity, lecithotrophy and matrotrophy.* Reproductive Biology and Phylogeny of Chondrichthyes: sharks, batoids and chimaeras, **3**, 394-434.

21. Castro, J.I., Sato, K. and Bodine, A.B. (2016) *A novel mode of embryonic nutrition in the tiger shark, Galeocerdo cuvier.* Marine Biology Research, **12**, 200-205.

22. Conrath, C.L. and Musick, J.A. (2012) *Reproductive biology of elasmobranchs.* Biology of Sharks and their Relatives, 291-312.

23. Sato, K., Nakamura, M., Tomita, T., Toda, M., Miyamoto, K. and Nozu, R. (2016) *How great white sharks nourish their embryos to a large size: evidence*

of lipid histotrophy in lamnoid shark reproduction. Biology Open, **5**, 1211-1215.

24. Tomita, T., Nozu, R., Nakamura, M., Matsuzaki, S., Miyamoto, K. and Sato, K. (2017) *Live-bearing without placenta: Physical estimation indicates the high oxygen-supplying ability of white shark uterus to the embryo.* Scientific Reports, **7**, 11744.

25. Files, T. and Lombardi, J. (1993) *Free amino acids in the uterine fluids of four species of viviparous sharks (Squalus acanthias, Carcharhinus plumbeus, Mustelus Canis and Rhizoprionodon terraenovae).* Comparative Biochemistry and Physiology Part B: Comparative Biochemistry, **104**, 583-588.

26. Jollie, W.P. and Jollie, L.G. (1967) *Electron microscopic observations on accommodations to pregnancy in the uterus of the spiny dogfish, Squalus acanthias.* Journal of Ultrastructure Research, **20**, 161-178.

27. Hamlett, W.C. (1983) *Maternal-fetal relations in elasmobranch fishes.* PhD Thesis, University of Clemson, USA.

28. Storrie, M.T., Walker, T.I., Laurenson, L.J. and Hamlett, W.C. (2009) *Gestational morphogenesis of the uterine epithelium of the gummy shark (Mustelus antarcticus).* Journal of Morphology, **270**, 319-336.

BRIDGE

CHAPTER 4 TO CHAPTER 5

After documenting the morphology of the uterine epithelium and wall in (**CHAPTER 4**) the important role of the maternal plasma and uterine fluid it became important to explore the maternal body fluid. **CHAPTER 5** investigated and tabulated the composition and concentration of biochemical analytes in the three main maternal fluid compartments within the respective *C. taurus* NGF (immature and mature and sexually active; RS 1 and 3) and GF (pregnant with capsules; RS4 and embryos in different gestation phases; RS5A, RS5C-RS5D). Females (RS5B) could not be assessed in this chapter.

CHAPTER 5

Biochemistry of the maternal fluid (plasma, intracapsular and uterine fluid) of non-gravid and gravid female Ragged-tooth sharks (*Carcharias taurus*) on the east coast of South Africa

Naidoo K[1], Chuturgoon AA[1], Cliff G[2], Ellis MT[3], Otway NM[4], Gregory MA[5], Singh SD[6]

[1]Discipline of Medical Biochemistry, [5]School of Health Sciences and [6]Biomedical Resource Unit, University of KwaZulu-Natal (UKZN), Durban, South Africa,

[2]KwaZulu-Natal Sharks Board (KZNSB), Umhlanga, South Africa

[3]Gladstone Ports Corporation, Gladstone, Queensland, Australia

[4]New South Wales Department of Primary Industries, Port Stephens Fisheries Institute, Taylors Beach, New South Wales, Australia

*Corresponding author email: chutur@ukzn.ac.za

Prepared for submission to Environmental Biology of Fishes

5.1 Abstract

Cacharias taurus females exhibits the reproductive mode of aplacental viviparity inclusive of a unique cannibalistic trait which when added to its late maturity and low fecundity of two pups born every two years, creates concern as to whether this species can rebound fast enough from their current "Vulnerable" status. Understanding the biochemical composition and concentration of the main maternal fluids that surround the embryos during their gestation is important when attempting to mimic *in utero* conditions during breeding programmes aimed at increasing the number of this species. The maternal plasma, uterine- and intracapsular- fluids of *C. taurus* females in all reproductive stages, were analysed for biochemical analytes (i.e., ions, enzymes and electrolytes) with a clinical biochemistry analyser while the reproductive hormones were determined using Chemi-illumenescence Assay. Descriptive statistics on all fluid components was tabulated. The remaining fluids were shown to have a similar composition to the maternal plasma, with all fluids having some organic content present. Follicle stimulating hormones were indicative of possible ovulation periods while progesterone was shown to have a controlling effect on oestradiol function. The data generated in this study can also serves as post-mortem references to identify when females are reaching dangerous levels of stress. This article represents the first comprehensive biochemical report on the maternal fluid of *C. taurus* females that surround the embryos during gestation and will serve as critical *in utero* components to model *in vitro* when attempting to propagate this species.

Keywords: biochemistry, *Carcharias taurus*, gravid, hormones, intracapsular fluid, non- gravid, plasma**,** ragged-tooth, uterine fluid

5.2 Introduction

Limited information on blood biochemistry [1] and minimal studies on the UF and ICF in *C. taurus* creates an incomplete understanding of the role these fluids play in the reproductive strategy of this species. The extensive documentation of *C. taurus* embryology indicates that the ICF and UF plays a significant role during the viviparous aplacental *in utero* development [2,3]. In addition, the presence of highly vascularised uterine tissue in GF sharks also suggests maternal blood plays an important role during pregnancy [3,4].

Some compositional breakdown in plasma has been achieved in a few elasmobranchs [5-8]. Knowledge of blood biochemistry of *C. taurus* is limited to these studies [1,9,10]. Rasmussen and Murru (1992) and Henningsen *et al.* (2008) studies each analysed progesterone and oestradiol serially in a single captive mature female [9,10]. Otway (2015) study established the first baseline values for serum analytes in juvenile and mature females of this species [1], however such studies do not exist for this species found in SA water. Gravid females develops fluid (UF) in the uterus, during early stages of pregnancy [2,3] which has been quantified in many elasmobranch species [5,7,8,11-13]. It has been postulated that UF facilitates respiration and waste removal in gravid elasmobranchs [13,14]. The UF increase in volume after embryos are released which also suggests a nutritive role [4,13,15-18]. The ICF, bound by capsule barrier has similar properties to the UF [14]. Studies have also shown that serum steroid levels are important in regulating events in elasmobranch reproduction [9,13,19-21].

We conducted this study in KZN-SA due to capture of sharks in the local bather protection nets maintained by the KZNSB coupled with the knowledge of the coastal migration of this species along the east coast of SA [22-24]. The capture of NGF and GF sharks, in these nets provided an opportunity to investigate the biochemical analytes present in their plasma, ICF and UF in respective females. These analytes included reproductive hormones, are affected by various stress factors such as anthropogenic threats (e.g., capture) and natural events (e.g., natural earth disaster, predator avoidance) [25-29] which influence the maturation and reproductive process. Uterine investigations reported elsewhere (**CHAPTER 4)**, on the same staged females, showed increased blood vessel formation in close proximity to the UF indicated the importance of further

investigation into the maternal fluids. This paper is the first detailed record of the biochemical analytes in the maternal plasma, ICF and UF in *C. taurus* NGF and GF that would serve as critical components to consider for breeding models aimed at increasing this species numbers.

5.3 Material and Methods

5.3.1 Shark sampling and staging

This species, *C. taurus* NGF and GF, were captured in KZNSB bather nets and evaluated into their respective reproductive stages as detailed in **Table 3.1.**

5.3.2 ICF, UF and blood sampling and processing

Maternal blood was initially drawn from the female's lateral vein, using a heparinized 10 mL syringe with an 18G needle, and transferred into vacutainers coated with anticoagulant (BD Diagnostic). Vacutainers were centrifuged (4000×g, 10 min, RT) and the serum supernatant removed and placed on ice.

During the dissection, the content of each uterus was extracted by making a small incision into the uterus wall. Uterine fluid was initially scooped into collection vials (from both uteri). All capsules found were also examined. Some capsules that contained EEs also contained capsule fluid (ICF) which was removed very carefully with a 10 mL syringe (Terumo). All EEs and FFE's were collected, examined, weighed and measured. The description of the EE's < 100 mm TL and a few FFE's, from this study, has been documented [30].

An aliquot (2ml) of serum, ICF and UF was transported on ice to the clinical laboratory at the National Health Laboratory Services (NHLS) (Durban, SA) where all samples were maintained at -20^{O}C until analysis. Remaining samples were kept at -20^{O}C for future analysis. The analytical methods used to determine the composition of the all fluids were summarised in **Table 5.1**. These methods used the Synchron CX7 or DXC800 clinical biochemistry analyser, at 37^{0}C, following standard NHLS operating procedures.

All endocrinology was determined on the Siemens Centaur (XP) analyser, using Chemi-illumenescence Assay, in the Chemical Pathology department at Inkosi Albert Luthuli Central Hospital (Durban, SA).

5.3.3 Analysis

Statistical analysis, including outlier identification, were determined using GRAPHPAD PRISM (Graph Pad Software Inc.; Version 7) and Microsoft Office Excel (2010).

Descriptive statistics of all the analytes were tabulated and represented as median and mean for the purpose of comparison with other studies. Some of the major stress analytes that were not tabulated were referenced in text as (Median ± IQR; *p* value).

The Shapiro- Wilk test and P-Anderson Darling tests were used for different procedures to determine if data passed normality. Comparative *p* values were deduced using either Mann-Whitney test for data that not pass normality and the Unpaired t test with Weltch correction, One-way ANOVA (and non-parametric) with Tukeys multiple comparison test for data that was normal. Only significant values (*p*) and *t*(*df*) values are indicated in text while descriptive stats can be found in the respective tables. Non-significant values were also reported in the same manner when important analytes needed to be reported. Levels of significance were set at $p < 0.05$, based on two-tailed tests. Correlations occurred through Spearman correlation (r_s, p) tests were used for non-parametric analysis while Pearson correlation (r_p, p) used for parametric analysis.

In the case where results from this study had to be compared to a separate study, the one unpaired t test with Holm Sidak method for multiple comparisons was used resulting in tabulated *p* and *t* values. Albumin and globulin were not analysed in the fluids as recent literature reported that *C. taurus* do not seem to have albumin despite it being reported in previous studies [1].

Table 5.1: Methods used for measuring analytes in the plasma of all females *C. taurus* females

Analytes	Method
ALP (at 410nm)	kinetic rate
ALT (at 340 nm)	kinetic rate
Creatinine	Jaffe Rate
Urea	enzymatic conductivity
Bicarbonate, Na, K, Cl	ion-selective electrode
AST, gamma-glutamyltransferase, LDH, CK	enzymatic rate
Mg, P, cholesterol and triglycerides	timed-endpoint
TP	time-endpoint biuret
TB	timed-endpoint diazo
LH, FSH, Progesterone and Oestradiol	chemiluminescent immunoassay

Abbreviations: ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: aspartate; CK: creatinine kinase; FSH: Follicle stimulating hormone; LDH: lactate dehydrogenase; LH: Luteinising hormone; Mg: magnesium; P: Phosphate; TB: Total Bilirubin; TP: Total protein.

5.4 Results

5.4.1 shark Sampling

A total of 34 (from 39) NGF and 22 (from 35) GF sharks were reported for plasma in this study. The NGF comprised of immature (adolescent) (RS1; n = 3), the mature inactive (RS2B; n = 9) and mature and sexually active (RS3; n = 22) while the GF included pregnant females with capsules (RS4; n = 8) and GF with capsules and embryos (RS5; n = 14) (i.e., females with the pre-hatch embryos (RS5A; n = 1), females with intrauterine cannibalistic embryos (RS5C; n = 4) and females with oophagous embryos (RS5D; n = 9). The UF was recorded for a total of 21 GF which comprised of RS4 (n = 4) and RS5 (n = 17) females. The latter consisted of RS5A: n = 2; RS5B: n = 2; RS5C: n = 2 and RS5D: n = 11. In addition, ICF was evaluated in a total of eight capsules found in a stage RS5A female. Each of these stages was defined in **Table 3.1** with further detail represented in **APPENDIX E** Descriptive statistics for each analyte was presented for the (1) plasma in NGF and GF, (2) UF in GF sharks and 3) ICF in the pre-hatched GF. The discrepancy in the sampled numbers for the plasma and UF against the total sample size, was due to some fluid material could not be analysed as well as outliers being identified and removed from analysis. The ICF could only be obtained from females that contained encapsulated embryos.

5.4.2 Fluid composition

This study described 17 biochemical analytes (Na, K, Cl, Urea, Creatinine, Calcium, Phosphorus, Total Protein (TP), Total Bilirubin (TB), Cholesterol, Magnesium, Triglycerides, Alkaline Phosphatase (ALP), Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Lactate dehydrogenase (LDH), Creatinine Kinase (CK) as well as four hormones (Luteinising hormone (LH), Follicle stimulating hormone (FSH), Progesterone and Oestradiol) found in the plasma (**Table 5.2-Table 5.3** and **APPENDIX G-APPENDIX J)**, UF **(Table 5.3-Table 5.4; APPENDIX K-APPENDIX M)** and ICF **(Table 5.5**) of the females regardless of the reproductive stage. All these biochemical analytes also serve as stress analytes/markers due to the allostatic overload leading to disruption in all these analytes [31]. These results are consistent with loss of allostatic balance in elasmobranchs, condition closely related to stressful/lethal events [32,33]. However, some analytes are more commonly associated with stress than others in elasmobranchs i.e. K, CK, LDH and AST. It is for this latter reason that these four analytes were tabulated separately as major stress analytes for all three maternal fluids, while the remainder of the biochemical analytes are termed minor stress analytes which was only analysed for plasma together with some major analytes.

Table 5.2: Descriptive statistics (Median ± IQR; Mean ± SEM and ranges) for the NGF and GF *C. taurus* maternal plasma

		NGF						GF						
	Units	*n*	Mean	SEM	Median	IQR	Min	Max	*n*	Mean	SEM	Median	IQR	Min	Max	*p*
Sodium	mmol/l	17	247,9	2.8	245,0	14.5	226.0	276.0	16	236.1	5.6	238.5	28.5	187.0	266.0	NS
Potassium	mmol/l	16	12,7	1,0	12,5	8,2	6,5	18,9	17	19,8	2,5	17,2	15,1	8,2	41,9	0.04
Chloride	mmol/l	16	223,6	3.4	222,0	13.3	200,0	255.0	21	213.4	3,7	209.0	28.0	182,0	241.0	NS
Na:K ratio		16	22,2	2,1	20,4	16,2	12,6	38,9	13	14,7	2,2	13,7	13,1	4,5	28,7	0.02
Anion Gap		34	20,1	8,5	5,0	35,7	-8,0	279,9	15	38,2	3,1	34,3	22,3	24,2	57,6	0.0002
Urea	mmol/l	34	382,4	5,2	382,6	39,2	292,5	436,2	21	390,4	6,1	387,6	40,8	327,1	443,7	NS
Creatinine	mmol/l	17	36,8	4,2	36,0	22,0	11,0	75,0	20	81,5	12,6	74,5	69,5	24,0	229,0	0.004
Calcium	mmol/l	34	4,1	0,1	4,1	0,7	2,8	5,4	16	4,3	0,1	4,3	0,4	3,6	4,7	NS
Phosphate	mmol/l	18	5,2	0,5	4,7	3,0	2,0	10,1	21	7,9	0,9	7,8	6,7	2,3	17,7	NS
Ca:P ratio		18	1,0	0,1	1,0	0,7	0,4	1,9	18	0,8	0,1	0,8	0,6	0,0	1,9	NS
Total Protein	g/L	34	27,2	0,9	27,0	8,0	18,0	38,0	21	26,2	0,7	27,0	4,0	21,0	32,0	NS
Total Bilirubin	umol/L	33	3,5	0,4	3,0	3,0	0,0	7,0	20	4,1	0,6	3,0	4,1	1,0	9,0	NS
Cholesterol	mmol/L	33	0,5	0,0	0,4	0,5	0,1	1,2	22	0,5	0,1	0,5	0,5	0,0	1,0	NS
Magnesium	mmol/L	33	2,9	0,2	2,4	1,5	0,9	5,7	22	2,8	0,2	2,6	2,0	1,5	4,6	NS
Triglyceride	mmol/L	30	0,4	0,0	0,3	0,2	0,1	0,9	22	0,6	0,1	0,5	0,5	0,1	1,1	0.02
ALP	U/L	32	18,6	1,7	18,5	15,0	3,0	46,0	20	7,0	0,7	7,0	4,5	1,0	14,0	<0.0001
AST	U/L	32	1465,0	229,2	1293,0	1454,2	95,0	4380,0	21	1763,0	325,5	1295,0	2463,0	108,0	5039,0	NS
ALT	U/L	30	35,6	7,7	10,0	61,8	2,0	122,0	21	82,5	22,3	30,0	196,5	0,0	291,0	NS
LH	IU/ml	28	0,1	0,0	0,1	0,0	0,1	0,1	18	0,1	0,0	0,1	0,0	0,1	0,1	NS
FSH	IU/ml	30	0,7	0,1	0,7	0,6	0,2	1,8	17	0,7	0,1	0,8	0,8	0,3	1,2	NS
Progesterone	nmol/L	27	3,9	0,5	2,9	4,3	0,3	10,6	21	3,4	0,5	3,3	3,1	0,8	8,1	NS
Oestradiol	nmol/L	28	1,5	0,2	1,5	2,3	0,0	3,9	22	1,2	0,2	1,4	1,8	0,0	2,6	NS

Abbreviations: ALP: alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; Ca:P: Calcium: Phosphate ratio; Dist.: Distribution; FSH: Follicle stimulating Hormone; GF: gravid females; g/L: grams/litre; LH: Luteinising Hormone; IQR: interquartile range; mmol/L: millimole/litre; mIU/ml: milli-international units/millilitre; Min: minimum; Max: maximum; *n*: sample size; NGF: Non-gravid females; Na: K: Sodium: Potassium ratio; nmol/L: nanomole/litre; SEM: Standard error

5.4.2.1 Biochemical analytes (minor and major stress analytes) in the plasma of NGF and GF

Descriptive statistics was summarised for the plasma in NGF and GF (**Table 5.2**) as well as the respective sub-groups (RS1-RS3) (**APPENDIX G-APPENDIX H)** and (RS4 - RS5D) **(APPENDIX I-APPENDIX J**). Stage RS5B was not represented as the material haemolysed. The comparison of the plasma between NGF and GF showed the following: The Na: K ratio (p = 0.02, t(26.5) = 2.46), anion gap (p = 0.0002) and ALP (p = <0.0001, t(41.5) = 6.33) were significantly higher in NGF sharks (**Table 5.2**). The NGF sub-groups **(APPENDIX -APPENDIX H)** showed significant differences in levels of Na (RS2B vs. RS3: p = 0.006) and Cl (RS2B vs. RS3: p = 0.004), Urea (RS1 vs. RS2B: p = 0.04, t(10) = 2.37) and Mg (RS2B vs. RS3: p = 0.02, t(9.8) = 2.95). The creatinine (p = 0.004) and triglycerides (p = 0.02) were significantly higher in GF (**Table 5.2**). The major stress analytes CK (Median ± IQR: 58 ± 905 vs. 211 ± 41928; p = 0.07), LDH (Median ± IQR: 1504 ± 11409 vs. 848.5 ± 37409; p = 0.786) and AST (Median ± IQR: 1293 ±1454 vs. 1295 ± 2463; p = 0.56) showed no significance, except for K (Median ± IQR: 12,45 ± 8.2 vs. 17.2 ± 15.1; p = 0.02, t(21.10) = 2.62; **(Table 5.2**), when compared between the plasma of NGF vs. GF. Major stress analytes K, CK, LDH and AST were compared between the plasma and UF in GF in section 5.4.2.1 above. TP showed no significant difference between the females (p = 0.40, t(52.71) = 0.84; (**Table 5.2**).

Table 5.3: Descriptive statistics (Median ± IQR, Mean ± SEM and ranges) for the stress analytes in the plasma, UF and ICF in GF (*C. taurus*)

	Units	*n*	Median	IQR	Mean	SEM	Min	Max
Plasma: GF								
K	mmol/L	17	17,2	15,05	19,78	2,517	8,2	41,9
CK	IU/L	19	211	41928	20410	8579	5	129533
LDH	U/L	12	848,5	37409,75	20556	10321	9,9	106366
UF: GF								
K	mmol/L	12	9,75	6,075	10,32	0,8623	6,3	14,9
CK	IU/L	18	1995	3905	2341	581,2	0,9	7505
LDH	U/L	9	8	369	184,9	108,1	0,9	871
ICF: GF								
K	mmol/L	8	13,35	3,12	13,45	0,5308	11,6	15,2
CK	U/L	8	168	165	207,9	41,12	74	427
LDH	U/L	7	19,9	12	20,84	2,559	13	32

Abbreviations: CK: Creatinine Kinase, GF: Gravid females; ICF: Intracapsular fluid; IU/ L: international units/litre; IQR: Interquartile range; K: Potassium; LDH: Lactate Dehydrogenase, mmol/L: millimole/litre; Min: minimum value; Max: maximum value, *n*: sample size; SEM: Standard Error of Mean, U/L: Units/litre; UF: Uterine fluid

5.4.2.2 Biochemical analytes (major stress analytes) in plasma, UF and ICF of GF

The major stress analytes CK (p = 0.47; **Table 5.3**), LDH (p = 0.007; **Table 5.3**) showed no significance between the plasma and UF. Only K showed a significant difference (p = 002; t(19.6) = 3.56; **Table 5.3**). AST is another major stress analyte but could not be compared therefore was left out. ICF (although present in the table) could not be compared due to sample size of one female.

5.4.2.3 Biochemical analytes (minor stress analytes) in ICF and UF of GF

The composition of UF was tabulated for all GF stages (**Table 5.4)** and the sub-groups (RS4-RS5D) (**APPENDIX M**) respectively. There were significant differences in Ca (RS4 vs. RS5: p = 0.0002, t(18.9) = 4.6; RS4 vs. RS5B: p =0.0004, t(3.84)= 11.32; RS4 vs, RS5C: p = 0.0005, t(3.72)=11.58; RS4 vs. RS5D: p = 0.008, t(11.46) = 3.15) and Mg (RS4 vs. RS5 p = 0.0012, t(18.1) = 3.83; RS4 vs. RS5A: p = 0.02, t(2.08) = 6.13; RS4 vs. RS5B: p = 0.01, t(2.2) = 8.0) in relevant GF sub-groups **(APPENDIX L-APPENDIX M).** This suggests the presence of minerals in the UF for developing

growing embryos as pregnancy proceeds. These minerals found in the UF could also be derived because of accumulative waste products from the embryo itself. No comparisons could be drawn on the descriptive statistics for ICF (**Table 5.5)**, as capsular fluid is only found in the pre-hatched females (RS5A).

Table 5.4: Descriptive statistics for the UF analytes in all *C. taurus* GF

Analytes	Unit	*n*	Median	IQR	Mean	SEM	Min-Max	
Sodium	mmol/l	18	343	47	338	11	265	440
Potassium	mmol/l	12	10	6	10	1	6	15
Na: K Ratio		12	32	18	33	4	7	54
Chloride	mmol/l	19	358	49	343	12	184	436
Anion Gap		20	-19	27	-19	4	-69	5
Urea	mmol/l	20	229	48	227	10	149	315
Creatinine	mmol/l	20	16	18	19	2	6	39
Calcium	mmol/l	21	10	5	9	1	1	17
Phosphate	mmol/l	13	1	1	1	0	0	4
Ca: P		14	9	21	15	4	0	43
Total Protein	g/L	20	4	3	5	0	2	9
Total Bilirubin	umol/L	15	4	6	5	1	1	11
Cholesterol	mmol/L	14	0	0.08	0	0	0	0
Magnesium	mmol/L	21	5	3	5	0	2	10
Triglyceride	mmol/L	17	0	1	0	0	0	1
ALP	U/L	19	194	264	179	30	3	414
ALT	U/L	17	6	16	14	4	2	47
LH	mIU/ml	15	0	0.2	0	0	0	0
FSH	mIU/ml	15	4	3.2	4	1	1	9
Progesterone	nmol/L	13	1	1	1	0	0	2
Oestradiol	nmol/L	7	139	0	124	15	37	139

Abbreviations: ALP: alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; Ca: P: Calcium: Phosphate ratio; Dist. : Distribution; FSH: Follicle stimulating Hormone; g/L: grams/litre; IQR: Interquartile Range; LH: Luteinising Hormone; mmol/L: millimole/litre; min: minimum; max: maximum; mIU/ ml: milli-international units /millilitre; *n*: sample size; nmol/L: nanomole/litre; Na: K: Sodium: Potassium ratio; SEM: Standard error of mean; U/L:units/litre; umol/L: micromole/litre.

Table 5.5: Descriptive statistics for the analytes found in the ICF of RS5A females (*C. taurus*).

	RS5A							
	Units	***n***	**Median**	**IQR**	**Mean**	**SEM**	**Min**	**Max**
Sodium	mmol/l	8	322	16	326	4	315	349
Potassium	mmol/l	8	13	3	13	1	12	15
Chloride	mmol/L	8	332	20	334	5	310	361
Na:K ratio	mmol/l	8	25	6	25	1	21	28
Anion Gap		8	-3	8	0	3	-9	16
Urea	mmol/l	7	234	24	239	5	222	258
Creatinine	mmol/l	8	14	7	13	1	8	20
Calcium	mmol/l	8	8	1	8	0	7	9
Phosphate	mmol/l	8	2	0	1	0	1	2
Ca:P ratio		8	5	1	5	0	4	7
Total Protein	g/L	8	5	2	5	0	4	6
Total Bilirubin	umol/L	8	2	2	2	0	1	4
Cholesterol	mmol/L	6	0	1	1	0	0	1
Magnesium	mmol/L	8	6	1	6	0	6	7
Triglyceride	mmol/L	8	1	4	2	1	0	6
ALP	U/L	7	7	2	6	1	4	8
AST	U/L	8	62	49	57	8	29	81
ALT	U/L	8	4	4	5	1	3	7
LH	mIU/ml	5	0	0	0	0	0	0
FSH	mIU/ml	5	2	2	2	0	1	3
Progesterone	nmol/L	5	2	2	1	0	0	3
Oestradiol	nmol/L	5	139	0	139	0	139	139

Abbreviations: ALP: alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; Ca: P: Calcium: Phosphate ratio; FSH: Follicle stimulating Hormone; g/L: grams/litre; ICF: Intracapsular fluid; IQR: Interquartile Range of the median; LH: Luteinising Hormone; mmol/L: millimole/litre; mIU/ ml: milli-international units /millilitre; min: minimum; max: maximum; nmol/L: nanomole/litre *n*: sample size; Na: K: Sodium: Potassium ratio; RS5A: pre-hatch; U/L:units/litre; umol/L: micromole/litre.

5.4.3 Reproductive hormones in the plasma of NGF and GF

No significant differences were noted for LH and FSH among the NGF and GF. The LH hormone levels remained similar in all NGF stages (~0.07 mIU/ml) (**Figure 5.1A**). The FSH showed no differences but there does seem to be a very slight increase for sexually active females (RS3) (**Figure 5.1B**). A peak was noticed in RS5A for LH **(Figure 5.1A**) and FSH (**Figure 5.1B**) however, this was based on one sample size. Another non-significant peak was noticed for LH in females carrying embryos going through intrauterine cannibalism (RS5C) (**Figure 5.1A**). Females in RS5B could not be assessed. This was due to haemolysis of samples that prevented endocrinology analysis. Both progesterone and oestradiol levels showed a steady increase with maturation **(Figure 5.1C-D).** Progesterone (p = 0.5, t(45.9) = 0.67; **Table 5.2**) and oestradiol (p = 0.53; **Table 5.2**) were non-significantly higher in the NGF than GF sharks. Progesterone was higher in concentration than oestradiol in most stages and peaked in the SS3 females. Oestradiol peaked in the RS4 females. Progesterone was significantly higher in the NGF RS3 sharks compared to RS1 (p = 0.001, t(6.59)=3.75)^{A}, RS2B (p = 0.003, t(17.23) = 4.45)^{C} (**Figure 5.1C, APPENDIX G-APPENDIX H**, RS5C (p = 0.02)^{E} and RS5D (p = 0.001, t(19.2) = 3.81)^{F} **(Figure 5.1C, APPENDIX J).** Oestradiol was significantly higher in the NGF RS3 sharks compared to RS1 (p = 0.01)^{H} and RS2B (p = 0.02), RS5C (p = 0.0014, t(2) = 26.27)^{P} and RS5D (p = 0.006, t(6) = 4.21)^{Q} (**Figure 5.1D; APPENDIX G-APPENDIX H** and **APPENIDX J).**

Progesterone showed a higher significant difference in GF stage RS4 compared to RS1 (p = 0.01, t(5.22) = 3.88)^{B}; RS2B (p = 0.001, t(9.15) = 4.82)^{D} (**Figure 5.1C, APPENDIX G)** and RS5D (p = 0.002, t(10.78) = 4.1)^{G} respectively **(Figure 5.1C, APPENDIX G and APPENDIX J).** Oestradiol was significantly higher in the RS4 compared to RS1 (p = 0.012)^{I}, RS2B (p < 0.0001)^{L} , RS3 (p < 0.0001, t(7) = 13.72)^{O}, RS5C (p = 0.02, t(8.66) = 2.89)^{R} and RS5D (p < 0.0001, t(11.28) = 9.57)^{S} **(Figure 5.1D).** Females in RS5B could not be assessed in this study. The females in RS5C was also significantly higher than RS5D (p < 0.0001, t(7.85) = 11.10^{T} **(Figure 5.1D, APPENDIX J).**

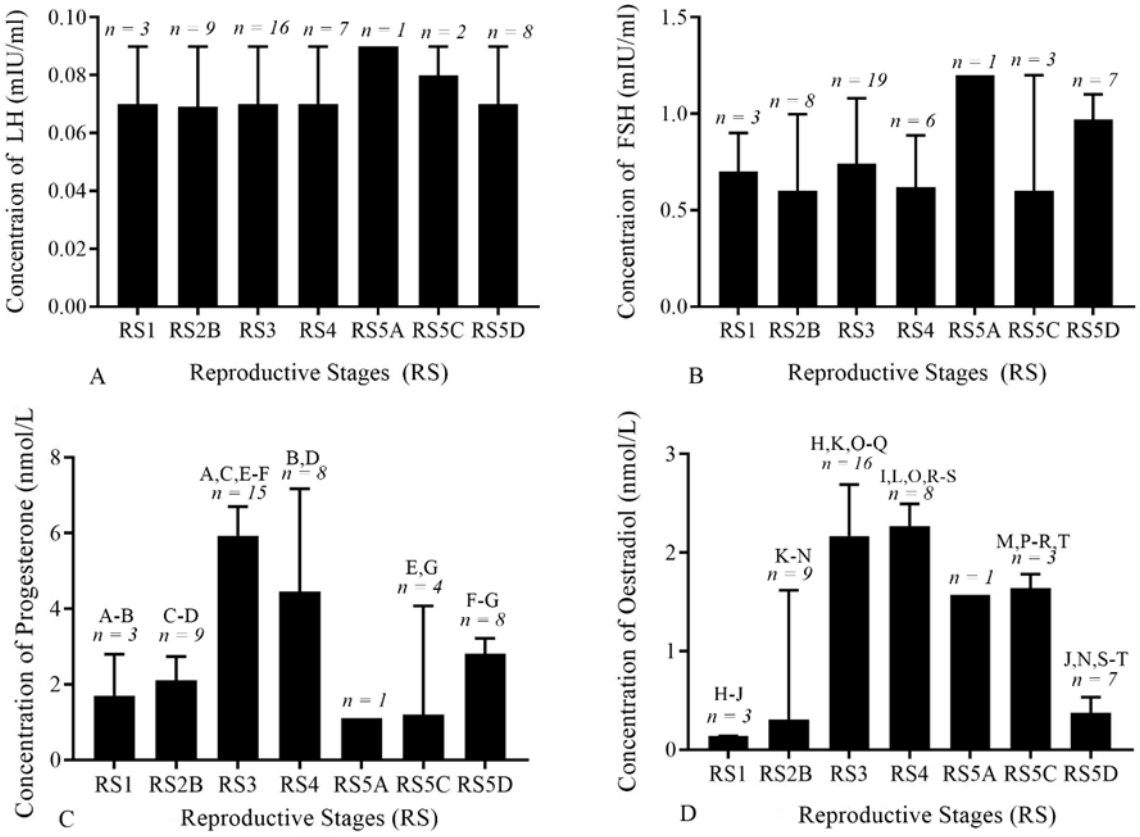


Figure 5.1: Plasma hormone levels of A) LH, B) FSH, C) progesterone and D) oestradiol trend within the plasma of all females. Letters *A-T* (in the graph) represents significance *p* values. Abbreviations: FSH: follicle stimulating hormone, LH: luteinising hormone; mIU/ml: milli-international units per millilitre; *n*: sample, nmol/L: nanomol/litre; RS 1-D: Reproductive Stage 1-5D.

5.4.4 Rank in plasma and UF analytes

The fluid compartments (i.e., plasma and UF) were ranked based on individual analytes ranked from highest to lowest concentrations in the GF *C. taurus* sharks (RS4 and RS5). This was summarised in (1) and (**Table 5.6**)**.** Rank comparisons could not be made between plasma, UF and ICF of the RS5A stage due to paucity of samples.

Table 5.6: Rank of analytes between the plasma and UF in *C. taurus* GF

Rank	Analytes	*p*
(1) UF > plasma	(A) Na, Cl, Ca: P, ALP, Mg, LH, FSH:	$p < 0.0001$
	(B) Na: K:	$p = 0.0006$
(2) Plasma > UF	(A) Anion Gap; Urea, Creatinine, Phosphorus, Total protein, Progesterone; Oestradiol:	$p < 0.0001$
	(B) K	$p = 0.003$
	(C) Triglycerides	$p = 0.026$

Abbreviations: ALP: alkaline phosphatase; Ca: P: Calcium: Phosphate ratio; Cl: Chloride; FSH: Follicle stimulating hormone; LH: Luteinising hormone; Mg: magnesium; Na: sodium; Na: K: sodium: potassium ratio; *p*: significant value < 0.05; UF: Uterine fluid.

5.5 Discussion

Studies have suggested that the vascularised uterine lamellae (UL) function as a respiratory membrane [5,34]. Our study confirmed this finding throughout all the gestating reproductive stages of *C. taurus* females. It's been shown that the increased surface area and location of BV could oxygenate the UF [5]. Studies on shark plasma [27,35-38] including the response to stress in *C. taurus* [39] has been achieved. The most recent biochemical plasma profile was undertaken for male and female *C. taurus* [1]. The composition of ICF, only found in early reproductive stage females, is influenced by the enclosed embryos and UF [13].The UF, has been found to increase during gestation [13] and has the ability to oxygenate the embryos [13,40,41]. Endocrine control major events in reproduction [42]. Steroids studies, with regards to reproduction, has been investigated in different reproductive modes [10,43-50]. Most endocrine studies in elasmobranchs has focused on progesterone and oestradiol [43,51]. Hormone levels were assessed in the serum of NGF *C. taurus* sharks [9,10,48] which showed the important role of progesterone and oestradiol play in the females reproductive cycles to promote maturity, pregnancy and support to the embryos. This study investigated 17 biochemical analytes that were grouped into stress, biochemical and reproductive hormone analytes.

5.5.1 Stress analytes

Stress factors (anthropogenic and natural events) faced by sharks [25-29] leads to physiological changes in their bodies that affects blood parameters [34,52,53] therefore only "freshly dead" females (i.e., within 24 hours) were sampled for maternal fluid analytes [34]. Although the stress analytes (lactate, CK and Potassium) showed no significant differences in all stages and across different fluid mediums (i.e., plasma and UF), they still were present in extremely higher concentrations to other analytes. Lactate is used as a tool to indicate stress to match the required energy demand during the fight response [27,52,54]. Stress studies have shown an increase in potassium and CK acts as indicators of stress due to the overall loss of cellular integrity. Potassium has also been used as indication of stress [32,34] with sharks experiencing cardiac arrhythmias and subsequent death with >7mmol/L [1,11]. Increased CK activity occurs in sharks experiencing capture stress [11,52,55,56]. This is an acknowledged drawback of the study but could not be helped due to the difficulty posed in attaining live females. However, the capture of the same species within 24 hours of net capture and stress levels that were not significantly different suggests the trend across the analytes could be assessed.

5.5.2 Biochemistry analytes

No conclusions to the health of these sharks can be inferred by the analysis of the analytes due to the levels of stress experience by deceased sharks in this study and its impact on biochemical analytes, but the composition, concentration and trends in the plasma (**Table 5.2**), UF **(Table 5.4**) and ICF (**Table 5.5**) in *C. taurus* females has been reported.

Electrolytes in serum and UF composition has been quantified for a few sharks [5,7,8,12,57,58] apart from *C. taurus*. It was interesting to note the two minerals important for embryo development, Ca (p = 0.0002, t(18,99) = 4.64) and Mg (p = 0.0012, t(18.07) = 3.83) were found in significantly higher concentrations in the UF of RS5 females compared to RS4 females (**APPENDIX K**). This would suggest that the presence of minerals in the UF for developing embryos appeared throughout the RS5

females, the stage where developing embryos are found. Additionally, the lack of significant difference in TP between the gravid reproductive stages was itself an interesting find due to the advanced stages in pregnancy requiring nutrition. However the TP concentration reported in this study (i.e., 26 g/L) was almost similar to the Sand Tiger concentration of TP (i.e., 30g/L) [1] as well as other elasmobranchs [11,52,55,56]. Other organic compounds were found amongst the composition of all three fluid components. The presence of the organic compounds in the plasma, ICF and UF can be taken up as an additional source of nourishment during embryo development [5,16,59-62]. The organic content found in the UF and ICF fluid, could suggest a form of nutrient provision to *C. taurus* embryos even though no secretory structures have been found in previous studies [4] and confirmed by our study (reported elsewhere, **CHAPTER 4**). Composition of the UF could be contributed by blood via hemotrophic transfer across the uterine lining, secretory activity of the lining and/or paraplacental sites [62]. It is possible that these compounds could serve as additional nutrition to *C. taurus* embryos through the gestation period [63]. The composition of the ICF can also be contributed by the secretory activity of the embryonic surfaces [62,64]. In addition, the pre-hatched embryos (RS5A) could have nutritional restriction due to the selective permeability of the egg capsule that controls intracapsular passage of nutrients [5]. The nutrient found in the fluids in this study, is probably a minimal form of nutrient supply compared to this species intrauterine cannibalism and oophagy nutrient strategy [3,4,65]. The presence of organic compounds in the RS4 females, where no embryos are found, could indicate spillage of yolk into the fluid due to possible mechanical damage of many capsules in a confined uterine space. On the other hand, the presence of organic compounds in the RS5 females, does not necessarily suggest that the embryos are absorbing these compounds, indicated by the lack of decrease in TP that remained rather consistent through all the gravid stages i.e. RS5A-RS5D **(APPENDIX J, APPENDIX L-APPENDIX M)** , even though it has been reported that post-hatched *C. taurus* embryos with gill filaments have been seen in the UF indicating both , the UF could function in nutrition and oxygenation in a species that is aplacental [7].

Physiological analysis has shown how GF are vulnerable to capture induced abortions [66]. As energy during this time is relocated to the maintenance of the embryos, less

energy is available to maintain homeostatic balance during this stressful experience. Blood chemistry of pregnant elasmobranchs indicates how vulnerable they are to reproductive disruption caused by fishing [67] where the rate of abortion induced by acute stress (i.e. few minutes to hours) (NW) can reduce the chances of maintaining the pregnancy as well as the female surviving (NW). Wosnick *et al* (2018) study documented that urea, K and phosphate can serve as physiological parameters to assess stress in relation to capture induced stress. It was noticed that these parameters were higher in G compared to NGF *C. taurus* sharks in this study. Although, no abortions were noted in late gestation (i.e., RS5D) due to the presence of both embryos (in each uterus) at the time of dissection after death, it is possible that the earlier GF stages of the sharks i.e. RS5A-5C could be susceptible to capture-induced abortions [66]. It was noticed that the both the mean urea and phosphate concentrations were higher while the K was lower in this study compared to the values reported in [66]. This can be investigated further by comparing these stages to live NGF and GF. If abortion is shown to occur in this species, tougher legalisation to manage this species, from capture-induced parturition especially during their reproductive season would need to be practised as the mortality of these embryos has implications on this elasmobranch population which is currently Vulnerable.

The differences in biochemical analytes were noted between the immature and mature dead NGF *C. taurus* sharks (in this study) versus the NGF *C. taurus* sharks (from America) from another study (**Table 5.7**) [1]). Analytes that were significantly different (Diff1 and Diff2; *p* and *t*(df) values) was indicated in **Table 5.7**. It was interesting to find TP was higher in *C. taurus* females from USA compared to SA. The difference in TP may be linked to the diet of the same species in different locations (i.e., SA and Australia) as different locations offer a variation in food/protein availability.

Table 5.7: Comparison of immature and mature NGF *C. taurus* species [i.e., Ragged-tooth female sharks (dead in this study) vs. Sand tiger females (live from Otway (2015)

		Dead immature Ragged-tooth females			Live immature Sand Tiger females					Dead mature Ragged-tooth females			Live mature Sand Tiger females					
	Units	*n*	Mean	SD	*n*	Mean	SD	*p*	*t*(df)	Diff[1]	*n*	Mean	SD	*n*	Mean	SD	*p*	*t*(df)	Diff[2]
Sodium	mmol/l										17	248	12	7	258	4	0,3	*t* (22) =2,22	10,1
Potassium	mmol/l										16	13	4	7	5	0	0,001	*t* (21) = 4,9	7,8
Chloride	mmol/l										16	224	14	7	244	9	0,02	*t* (21) = 3,6	20,4
Urea	mmol/l	3	328	48	8	376	11	0,17	*t* (9.00) = 2,87	48	31	388	23	7	380	9	0,9	*t* (36) = 0,85	7,7
Creatinine	mmol/l										17	37	17	7	30	10	0,9	*t* (22) = 0,97	6,82
Calcium	mmol/l	3	4	0	8	4	0	0,22	*t* (9.00) = 2,63	1	31	4	1	7	4	0	0,94	*t* (36) = 0,21	0,06
Phosphate	mmol/l										18	5	2	7	2	0	0,01	*t* (23) = 3,85	3,4
Total Protein	g/L	3	22	7	8	31	3	0,13	*t* (9,00) = 3,12	9	31	28	5	7	30	3	0,9	*t* (36) = 1,13	2,3
Total Bilirubin	umol/L	3	2	2	8	2	1	0,9	*t* (9,00) = 0,64	0	30	4	2	7	2	1	0,13	*t* (35) = 2,61	2
Cholesterol	mmol/L	3	1	0	8	1	0	0,3	*t* (9,00) = 2,15	0	30	1	0	7	2	0	<0,0001	*t* (35) = 8,88	1,1
Magnesium	mmol/L	2	2	0	8	2	0	0,26	*t* (8,00) = 2,50	0	31	3	1	7	2	0	0,71	*t* (36) = 1,49	0,7
Triglyceride	mmol/L	3	0	0	8	0	0	0,31	*t* (9,00) = 2,14	0	27	0	0	7	0	0	0,95	*t* (32) = 0,20	0,01
ALP	U/L	3	17	8	8	19	5	0,9	*t* (9,00) = 0,59	2	29	19	10	7	23	4	0,9	*t* (34) = 1,11	4,2
ALT	U/L	3	473	567	8	28	8	0,26	*t* (9,00) = 2,45	445	29	1568	1312	7	32	9	0,05	*t* (34) = 3,06	1536
AST	U/L	3	3	1	8	3	1	0,9	*t* (9,00) = 0,53	0	27	39	43	7	3	1	0,31	*t* (32) = 2,12	36,2
CK	U/L	3	88	99	8	43	23	0,63	*t* (9,00) = 1,31	45	24	1497	3562	7	44	23	0,9	*t* (29) = 1,06	1453

Abbreviations: ALP: alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase CK: Creatinine Kinase; Diff: Difference in means; *n*: sample size; ND: Not done; SD: Standard Deviation; *p* = significance level set at < 0.05. Diff[1]: mean difference of immature *C. taurus* females (i.e., immature Ragged-tooth - immature Sand tiger); Diff[2] = mean difference of *C. taurus* females (i.e., mature Ragged-tooth – mature Sand tiger); *p*[1]: significance for Diff[1]; *p*[2]: significance for Diff[2]. Units and their abbreviations as per Table 5.6.

5.5.3 Endocrinology

Punctuated breeders are females that undergo a year of pregnancy followed by a resting year. These species, *Cacharias taurus*, like the *P. glauca* [68] and *C. plumbeus* [69] are punctuated breeders that produce offspring every two years (biennial cycle). Far less is understood about luteinising (LH) and follicle stimulating hormones (FSH) [70,71] in reproduction compared to oestradiol and progesterone hormones which have been shown to play significant roles in pregnant females [48,49,51].

The consistent low (0.07 miUl/ml) LH concentrations in all the NGF and GF groups (RS1-RS5D) suggest that these could be indicative of baseline activity of LH for forthcoming ovulation periods for punctuated breeders that have superimposed ovulation in their pregnancy stages [51] (**Figure 5.1A**). Ovulation results from a surge in LH [51], which was observed in RS5A females (**Figure 5.1A),** however, this was based on a sample size of one female and due to caution will be subsequently ignored in this study.

Increasing FSH normally leads to the ovary increasing levels of oestradiol which leads to a surge of LH resulting in ovulation [51]. Under this definition of ovulation, analysis of the oestradiol with could suggest three possible stages where the rate of ovulation increases (i.e., one in the NGF and two in the GF stages). The FSH levels showed no significant difference at any of the reproductive stages (i.e. RS1-RS5D; *p* ranged from 0.2 - 0.9 mIU/ml) The RS3 females could indicate the first ovulatory period. The FSH appeared at its highest (0.7 mIU/ml) within NGF sharks at RS3. (**Figure 5.1B**). The progesterone (5.9 nmol/L; **Figure 5.1C)** and oestradiol (2.2nmol/L; **Figure 5.1D)** increased steadily as the female matured, also both peaking in the RS3 females, with progesterone being higher in concentration than oestradiol. High progesterone occurs in the peri-ovulatory phase (i.e., the phase between ovarian stimulation and ovulation) [47]. This phase is the RS3, where *C. taurus* female's mate (indicated by observation of skin lesions during our dissections), which could serve as the first trigger for ovulation with the encapsulation of ova in the next sexual phase (i.e., RS4). In addition, increased progesterone is known to reduce endogenous spontaneous myometrial activity in early pregnancy to prevent expulsion of uterine contents [43]. The oestradiol levels in RS3 would aid in

vitellogenesis (i.e., the process where the yolk precursors are sequestered from the liver to the ovary), ovulation (i.e., the release of mature ova from the ovary) and capsule (membranous sheath) production, by the O.G, to encapsulate ova enroute to the uterus [47]. It is possible that the high levels of progesterone could be limiting the levels of oestradiol thereby regulating the amount of ovulation and capsule production at a stage of pregnancy where embryos have not yet developed.

The second possible increase in ovulation is in the RS4 GF, possibly triggered by the presence of capsules in the uterus (103/106 capsules in the left and right uterus respectively). The concentration of FSH (**Figure 5.1B**) in the RS4 GF (0.62 mIU/ml) did drop compared to the NGF RS3 sharks (0.7 mIU/ml), while the oestradiol (**Figure 5.1D)** and progesterone (**Figure 5.1C)** were at its highest concentrations (2.27 nmol/L vs. 4.45 nmol/L respectively) in GF; with progesterone being higher than oestradiol suggesting a regulatory control to oestradiol function. This implies an energy saving system as embryos have not yet begun to develop in these females.

The third possible increase in ovulation may lie in the RS5C females, where intrauterine cannibalism occurs [3]. The FSH (**Figure 5.1B**) concentration does appear to drop. Oestradiol (**Figure 5.1D)** was found in higher levels (1.64 nmol/L) than progesterone (1.2 nmol/L) (**Figure 5.1C)** which would allow for the up-regulation of oocytes filled with yolk to become encapsulated. An increase in oestradiol in RS5C could result in production of unfertilised capsules being used as a food source for embryos breaking out of the capsules as well as for the embryo (per uterus) that survives and requires capsules for oophagous phase. This would explain why the capsule count is the highest in RS5C females (197/177 capsules per left and right uterus respectively) compared to other stages (i.e. RS5A: 132/169; RS5D: 50/48 left and right uterus respectively; no full complement of data for RS5B; **Table 3.6**). This middle pregnancy stage could challenge the previous notion that superimposed ovulation occurs in early stages of pregnancy [51]. The decline in progesterone also allows for myometrial activity which could assist with periodical flushing of the lumen with seawater [43] that can assist with increasing oxygenation and removal of waste products from the fluid in the uterus [3,11]. A LH peak **(Figure 5.1A**) was also observed in the RS5C females, but due to a sample set of

one female; no further elaboration will be made.

It is possible that the RS5A female could hold another stage for ovulation with increased levels of FSH and oestradiol and low levels of progesterone that would support nutrition for the embryos requiring them in the next RS5B (i.e. stage where embryos escape encapsulation). This latter stage could not be assessed in this study. However further sampling will be needed to determine this. The rate of ovulation appears to decrease in RS5D (oophagous stage) due to the lowest oestradiol level indicating a decrease in ovulation and capsule formation with a spike in FSH that could indicate few capsules being formed that were more laiden with yolk to serve as food source for the surviving embryos [72]. An increase in progesterone in RS5D females can inhibit uterine contraction, before parturition, and can inhibit oestradiol action.

The oestradiol and progesterone increase during the NGF maturation stages of the female. Trends in this study indicated a majority of lower oestradiol concentrations compared to progesterone. In contrast to our study, higher oestradiol was found, in immature (450 - 690 pg/ml) and mature (600-2000 pg/ml) captive *C. taurus* females [9,48] compared to immature (10.8-38.13pg/ml) and mature (10.89-890.7 pg/ml) wild species (in our study) **(Figure 5.1D**). These studies reported higher oestradiol than progesterone levels in mature captive females (*C. taurus*) [9,48]. Also, these studies observed higher oestradiol than progesterone levels in each single mature captive female (*C. taurus*), that was serially sampled over a few years in their respective study [9,48]. Closer inspection of the mature females, in these studies, suggests they are similar in staging to the mature, NGF sharks (i.e., RS2B and RS3) in our study based on length, maturity and lack of capsule presence. Further analysis revealed that four (RS2B) out of 10 females in the RS2B and RS3 mature NGF sharks showed higher levels of oestradiol compared to progesterone. However, the overall trend when females in our study are grouped is progesterone higher than oestradiol

FSH levels could suggest that there is egg maturation occurring during early pregnancy and that follicle development may not only be occurring after parturition and before the next pregnancy as stated for some punctuated breeders [51]. Although the presence of

LH and FSH has been verified further information is required on its function in elasmobranch studies looking into the pattern of these hormones during reproduction requires elucidation. Assumptions cannot be made of trend of LH and FSH because of the low sample number in this study and insufficient literature at the current time.

This study suggests that these oophagy species, that depend on the intermittent ovulation of eggs during their pregnancy to serve as nutrition for embryos in the uterus [63], may indicate possible ovulation occurring from RS3 females, with three main areas where the rate in these ovulation periods increase. The overall data may display the pattern required for oophagous species to continuously produce capsules filled with ova.

5.5.3 Post-mortem references

The impact of capture stress on plasma parameters is of concern, although many have tried to find alternative methods to minimise the stress the animal undergoes [28,73]. Normally a need for baseline references helps to maintain a shark's health by understanding how far away respective parameters are from the "normal/reference points". This has always proven challenging especially now with the current conservation pressure to reduce mortality in sharks. Wosnick *et al.* (2017) have proposed, for the first time, the use of post-mortem data to establish endpoint references of stress in a shark which would indicate how close to "death" a shark is reaching. The results of this study can serve as post-mortem reference values to assess when a female is reaching dangerously high levels of stress.

5.6 Conclusion

This chapter reported on the similar composition of the plasma, UF and ICF in both NGF and GF C. *taurus* sharks which have never been documented. Uterine fluid only appears to be present from early capsule stage (RS4) of the GF. Changes in hormone levels, especially in the plasma, indicated possible endocrine support especially noticed during mating periods and pregnancy which could result in three ovulation periods. These changes allow the uterine tissue to become specialised for respiration and

osmoregulation; which was investigated but reported elsewhere (see **CHAPTER 4;** Naidoo *et al,* in prep**).**

5.7 Acknowledgement

Immense gratitude to: the KZNSB research staff (Mr P Zungu, Mr E Makhatini, Mr B Zungu and Mrs AJ Ganesan) and the KZNSB operations crew for the tracking and proper handling of all sharks included in this study; The National Health Laboratory Services (NHLS) (Addington Hospital, Durban, SA) and Chemical Pathology Laboratory (Albert Luthuli Hospital, Durban, SA) for biochemical analysis; Dr K Dhuness, Mrs. R. Sobaren Singh and Ms. SP Wintner for their assistance in the editing process. Gratitude to the New South Wales Fisheries, Department of Primary Industries, for the scholarship, purchasing of necessary equipment and funding of analysis on this project. National Research Fund (Deutscher Akademischer Austauschdienst; DAAD) is also gratefully acknowledged.

5.8 Reference

1. Otway, N.M. (2015) *Serum biochemical reference intervals for free-living Sand Tiger sharks (Carcharias taurus) from east Australian waters.* Veterinary Clinical Pathology, **44**, 262-274.
2. Gilmore, R.G., Dodrill, J.W. and Linley, P.A. (1983) *Reproduction and embryonic development of the sand tiger shark, Odontaspis taurus (Rafinesque).* Fishery Bulletin, **81**, 201-225.
3. Gilmore, R.G., Putz, O. and Dodrill, J.W. (2005) In Hamlett WC (ed.), *Reproductive biology and phylogeny of Chondrichthyes: Sharks, Batoids and Chimaeras.* Science Publishers, Enfield, New Hampshire, USA, 435-462.
4. Hamlett, W.C. and Hysell, M.K. (1998) *Uterine specializations in elasmobranchs.* Journal of Experimental Zoology, **282**, 438-459.
5. Files, T. and Lombardi, J. (1993) *Free amino acids in the uterine fluids of four species of viviparous sharks (Squalus acanthias, Carcharhinus plumbeus, Mustelus Canis and Rhizoprionodon terraenovae).* Comparative Biochemistry and Physiology Part B: Comparative Biochemistry, **104**, 583-588.
6. Kormanik, G.A. (1992) *Ion and osmoregulation in prenatal elasmobranchs: evolutionary implications.* American Zoologist, **32**, 294-302.
7. Price, K.S. and Daiber, F.C. (1967) *Osmotic environments during fetal development of dogfish, Mustelus canis (Mitchill) and Squalus acanthias Linnaeus, and some comparisons with skates and rays.* Physiological Zoology, **40**, 248-260.
8. Thorson, T.B. and Gerst, J.W. (1972) *Comparison of some parameters of serum and uterine fluid of pregnant, viviparous sharks (Carcharhinus leucas) and serum of their near-term young.* Comparative Biochemistry and Physiology Part A: Physiology, **42**, 33-40.
9. Henningsen, A., Murru, F., Rasmussen, L., Whitaker, B. and Violetta, G. (2008) *Serum levels of reproductive steroid hormones in captive sand tiger sharks, Carcharias taurus (Rafinesque), and comments on their relation to sexual conflicts.* Fish Physiology and Biochemistry, **34**, 437-446.
10. Rasmussen, L. and Murru, F. (1992) *Long-term studies of serum concentrations of reproductively related steriod hormones in individual captive carcharhinids.*

Marine and Freshwater Research, **43**, 273-281.

11. Ellis, M.T. and Otway, N.M. (2011) *Uterine fluid composition of the dwarf ornate wobbegong shark (Orectolobus ornatus) during gestation.* Marine and Freshwater Research, **62**, 576-582.

12. Evans, D., Oikari, A., Kormanik, G.A. and Mansberger, L. (1982) *Osmoregulation by the prenatal spiny dogfish, Squalus acanthias.* Journal of Experimental Biology, **101**, 295-305.

13. Lombardi, J., Jones, K.B., Garrity, C.A. and Files, T. (1993) *Chemical composition of uterine fluid in four species of viviparous sharks (Squalus acanthias, Carcharhinus plumbeus, Mustelus canis and Rhizoprionodon terraenovae).* Comparative Biochemistry and Physiology Part A: Physiology, **105**, 91-102.

14. Hamlett, W. and Koob, T. (1999) In Hamlett WC (ed.), *Sharks, Skates and Rays:The Biology of Elasmobranch Fish*. Baltimore, MD: Johns Hopkins University Press, 398-444.

15. Castro, J.I., Sato, K. and Bodine, A.B. (2016) *A novel mode of embryonic nutrition in the tiger shark, Galeocerdo cuvier.* Marine Biology Research, **12**, 200-205.

16. Needham, J. (1942) *Biochemistry and morphogenesis.* Biochemistry and morphogenesis., pp 785

17. Sato, K., Nakamura, M., Tomita, T., Toda, M., Miyamoto, K. and Nozu, R. (2016) *How great white sharks nourish their embryos to a large size: evidence of lipid histotrophy in lamnoid shark reproduction.* Biology Open, **5**, 1211-1215.

18. Tomita, T., Nozu, R., Nakamura, M., Matsuzaki, S., Miyamoto, K. and Sato, K. (2017) *Live-bearing without placenta: Physical estimation indicates the high oxygen-supplying ability of white shark uterus to the embryo.* Scientific Reports, **7**, 11744.

19. Koob, T., Tsang, P. and Callard, I. (1986) *Plasma estradiol, testosterone, and progesterone levels during the ovulatory cycle of the skate (Raja erinacea).* Biology of Reproduction, **35**, 267-275.

20. Manire, C.A., Walsh, C.J., Rhinehart, H.L., Colbert, D.E., Noyes, D.R. and Luer, C.A. (2003) *Alterations in blood and urine parameters in two Florida*

manatees (Trichechus manatus latirostris) from simulated conditions of release following rehabilitation. Zoo Biology, **22**, 103-120.

21. Snelson, F.F., Rasmussen, L., Johnson, M.R. and Hess, D.L. (1997) *Serum concentrations of steroid hormones during reproduction in the Atlantic stingray, Dasyatis sabina.* General and Comparative Endocrinology, **108**, 67-79.
22. Cliff, G. and Dudley, S.F.J. (1992) *Protection against shark attack in South Africa, 1952-90.* Marine and Freshwater Research, **43**, 263-272.
23. Dudley, S. and Cliff, G. (1993) *Sharks caught in the protective gill nets off Natal, South Africa. 7. The blacktip shark Carcharhinus limbatus (Valenciennes).* South African Journal of Marine Science, **13**, 237-254.
24. Dudley, S.F. and Simpfendorfer, C.A. (2006) *Population status of 14 shark species caught in the protective gillnets off KwaZulu–Natal beaches, South Africa, 1978–2003.* Marine and Freshwater Research, **57**, 225-240.
25. Bakker, J., Wangensteen, O.S., Chapman, D.D., Boussarie, G., Buddo, D., Guttridge, T.L., Hertler, H., Mouillot, D., Vigliola, L. and Mariani, S. (2017) *Environmental DNA reveals tropical shark diversity in contrasting levels of anthropogenic impact.* Scientific Reports, **7**, 16886.
26. Fatoki, O. and Mathabatha, S. (2001) *An assessment of heavy metal pollution in the East London and Port Elizabeth harbours.* Water SA, **27**, 233-240.
27. Hoffmayer, E.R. and Parsons, G.R. (2001) *The physiological response to capture and handling stress in the Atlantic sharpnose shark, Rhizoprionodon terraenovae.* Fish Physiology and Biochemistry, **25**, 277-285.
28. Skomal, G.B. and Mandelman, J.W. (2012) *The physiological response to anthropogenic stressors in marine elasmobranch fishes: a review with a focus on the secondary response.* Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, **162**, 146-155.
29. Vermeulen, L. and Wepener, V. (1999) *Spatial and temporal variations of metals in Richards Bay Harbour (RBH), South Africa.* Marine Pollution Bulletin, **39**, 304-307.
30. Naidoo, K., Chuturgoon, A.A., Cliff, G., Ellis, M.T., Otway, N.M., Gregory, M.A., Singh, S.D. and Naidu, S.L. (2017) *Dentition facilitates the release of encapsulated Ragged-tooth Shark (Carcharias taurus) embryos.* Environmental

Biology of Fishes, **100**, 1343-1354.

31. Wikelski, M. and Cooke, S.J. (2006) *Conservation physiology.* Trends in Ecology & Evolution, **21**, 38-46.

32. Brooks, E.J., Mandelman, J.W., Sloman, K.A., Liss, S., Danylchuk, A.J., Cooke, S.J., Skomal, G.B., Philipp, D.P., Sims, D.W. and Suski, C.D. (2012) *The physiological response of the Caribbean reef shark (Carcharhinus perezi) to longline capture.* Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, **162**, 94-100.

33. Wosnick, N., Bornatowski, H., Ferraz, C., Afonso, A., Rangel, B.S., Hazin, F.H.V. and Freire, C.A. (2017) *Talking to the dead: using Post-mortem data in the assessment of stress in tiger sharks (Galeocerdo cuvier)(Péron and Lesueur, 1822).* Fish Physiology and Biochemistry, **43**, 165-178.

34. Manire, C., Hueter, R., Hull, E. and Spieler, R. (2001) *Serological changes associated with gill-net capture and restraint in three species of sharks.* Transactions of the American Fisheries Society, **130**, 1038-1048.

35. Arnold, J.E. (2005) *Hematology of the sandbar shark, Carcharhinus plumbeus: standardization of complete blood count techniques for elasmobranchs.* Veterinary Clinical Pathology, **34**, 115-123.

36. Cain, D.K., Harms, C.A. and Segars, A. (2004) *Plasma biochemistry reference values of wild-caught southern stingrays (Dasyatis americana).* Journal of Zoo and Wildlife Medicine, **35**, 471-476.

37. Pillans, R.D., Anderson, W.G., Good, J.P., Hyodo, S., Takei, Y., Hazon, N. and Franklin, C.E. (2006) *Plasma and erythrocyte solute properties of juvenile bull sharks, Carcharhinus leucas, acutely exposed to increasing environmental salinity.* Journal of Experimental Marine Biology and Ecology, **331**, 145-157.

38. Pillans, R.D., Good, J.P., Anderson, W.G., Hazon, N. and Franklin, C.E. (2005) *Freshwater to seawater acclimation of juvenile bull sharks (Carcharhinus leucas): plasma osmolytes and Na+/K+-ATPase activity in gill, rectal gland, kidney and intestine.* Journal of Comparative Physiology B, **175**, 37-44.

39. Haulena, M. (1999) *Plasma biochemical changes in response to transport handling and variation in water quality in Sand Tiger Sharks.* PhD Thesis. The University of Guelph, Canada.

40. Tomita, T., Cotton, C.F. and Toda, M. (2015) *Ultrasound and physical models shed light on the respiratory system of embryonic dogfishes.* Zoology, **119**, 36-41.

41. Tomita, T., Nakamura, M., Sato, K., Takaoka, H., Toda, M., Kawauchi, J. and Nakaya, K. (2014) *Onset of buccal pumping in catshark embryos: how breathing develops in the egg capsule.* PLoS ONE, **9**, e109504.

42. Henningsen, A.D. (1999) *Review. Levels of recirculating reproductively-related steroid hormones in female elasmobranchs. Implications for reproduction in a captive environment.* Aquarium Sciences and Conservation, **2**, 97-116.

43. Callard, I., George, J.S., Koob, T. and Hamlett, W. (2005) In BGM, J. (ed.), *Reproductive biology and phylogeny of chondrichthyes: sharks, batoids and chimeras*, 3, 283-300.

44. Callard, I., Giannoukos, G., Charnock-Jones, D., Benson, S. and Paolucci, M. (1994) *Hormone regulation of vitellogenin genes and the evolution of viviparity.* Perspectives in Comparative Endocrinology, **1994**, 325-332.

45. Callard, I.P., Fileti, L.A. and Koob, T.J. (1993) *Ovarian steroid synthesis and the hormonal control of the elasmobranch reproductive tract.* Environmental Biology of Fishes, **38**, 175-185.

46. Graham, J.D. and Clarke, C.L. (1997) *Physiological action of progesterone in target tissues 1*. Endocrine Reviews, **18**, 502-519.

47. Koob, T.J. and Callard, I. (1991) *Reproduction in female elasmobranchs.* Comparative Physiology, **4**, 155-200.

48. Rasmussen, L., Hess, D.L. and Gruber, S.H.(1992) In Hamlett WC (ed.), *Reproductive Biology of South American Vertebrates.*Springer,New York,19-42.

49. Rasmussen, L.E. and Gruber, S.H. (1993) *Serum concentrations of reproductively-related circulating steroid hormones in the free-ranging lemon shark, Negaprion brevirostris.* Environmental Biology of Fishes, **38**, 167-174.

50. Sumpter, J. and Dodd, J. (1979) *The annual reproductive cycle of the female lesser spotted dogfish, Scyliorhinus canicula L., and its endocrine control.* Journal of Fish Biology, **15**, 687-695.

51. Koob, T.J. and Callard, I.P. (1999) *Reproductive endocrinology of female elasmobranchs: lessons from the little skate (Raja erinacea) and spiny dogfish*

(Squalus acanthias). Journal of Experimental Zoology, **284**, 557-574.

52. Cliff, G. and Thurman, G. (1984) *Pathological and physiological effects of stress during capture and transport in the juvenile dusky shark, Carcharhinus obscurus.* Comparative Biochemistry and Physiology Part A: Physiology, **78**, 167-173.

53. Mandelman, J.W. and Skomal, G.B. (2009) *Differential sensitivity to capture stress assessed by blood acid–base status in five carcharhinid sharks.* Journal of Comparative Physiology B, **179**, 267-277.

54. Smith, H.W. (1929) *The composition of the body fluids of elasmobranchs.* Journal of Biological Chemistry, **81**, 407-419.

55. Harms, C., Ross, T. and Segars, A. (2002) *Plasma biochemistry reference values of wild bonnethead sharks, Sphyrna tiburo.* Veterinary Clinical Pathology, **31**, 111-115.

56. Wells, R., McIntyre, R., Morgan, A. and Davie, P. (1986) *Physiological stress responses in big gamefish after capture: observations on plasma chemistry and blood factors.* Comparative Biochemistry and Physiology Part A: Physiology, **84**, 565-571.

57. Jones, K., Wine, R., Garrity, C. and Lombardi, J. (1990), *American Zoologist*. American Society of Zoologists, **30**, A83-A83.

58. Stevens, B. (2002) *Uterine and oviducal mechanisms for gestation in the common sawshark, Pristiophorus cirratus.* Bachelor of Science (Honors) Thesis, University of Melbourne, Melbourne, Australia.

59. Hamlett, W.C., Kormanik, G., Storrie, M., Stevens, B. and Walker, T.I. (2005) *Chondrichthyan parity, lecithotrophy and matrotrophy.* Reproductive Biology and Phylogeny of Chondrichthyes: sharks, batoids and chimaeras, **3**, 394-434.

60. Lombardi, J., Wourms, J. and Hall, J. (1988) *Structural Organization of umbilical stalk appendiculae in the viviparous shark Rhizoprionodon Terraenovae.* American Zoologist, **28**, A80-A80.

61. Wourms, J.P. (1981) *Viviparity: the maternal-fetal relationship in fishes.* American Zoologist, **21**, 473-515.

62. Wourms, J.P., Grove, B.D. and Lombardi, J. (1988) *The maternal-embryonic relationship in viviparous fishes.* Fish Physiology, **11**, 1-134.

63. Gilmore, R.G. (1993) *Reproductive biology of lamnoid sharks.* Environmental Biology of Fishes, **38**, 95-114.

64. Hamlett, W.C. (1989) *Evolution and morphogenesis of the placenta in sharks.* Journal of Experimental Zoology Part A: Ecological Genetics and Physiology, **252**, 35-52.

65. Gilmore, R.G. (1983) *Observations on the embryos of the longfin mako, Isurus paucus, and the bigeye thresher, Alopias superciliosus.* Copeia, 375-382.

66. Wosnick, N., Awruch, C., Adams, K., Gutierre, S., Bornatowski, H., Prado, A. and Freire, C. (2018) *Impacts of fisheries on elasmobranch reproduction: high rates of abortion and subsequent maternal mortality in the shortnose guitarfish.* Animal Conservation, 10.1111/acv.12458.

67. Adams, K.R., Fetterplace, L.C., Davis, A.R., Taylor, M.D. and Knott, N.A. (2018) *Sharks, rays and abortion: the prevalence of capture-induced parturition in elasmobranchs.* Biological Conservation, **217**, 11-27.

68. Pratt, H. (1979) *Reproduction in the blueshark, Prionace glaauca.* Fish Bulletin., **77**, 455-470.

69. Wass, R.C. (1973) *Size, growth, and reproduction of the sandbar shark, Carcharhinus milberti, in Hawaii.* Pacific Science, **27**, 305-318.

70. Awruch, C. (2013) *Reproductive endocrinology in chondrichthyans: The present and the future.* General and Comparative Endocrinology, **192**, 60-70.

71. Buechi, H.B. and Bridgham, J.T. (2017) *Evolution of specificity in cartilaginous fish glycoprotein hormones and receptors.* General and Comparative Endocrinology, **246**, 309-320.

72. Sorbera, L. and Callard, I. (1995) *Myometrium of the spiny dogfish Squalus acanthias: peptide and steroid regulation.* American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, **269**, R389-R397.

73. Skomal, G., Lobel, P.S. and Marshall, G. (2007) *The use of animal-borne imaging to assess post-release behavior as it relates to capture stress in grey reef sharks, Carcharhinus amblyrhynchos.* Marine Technology Society Journal, **41**, 44-48.

BRIDGE

CHAPTER 5 TO CHAPTER 6

The presence of heavy metals in the tissues and body fluids of elasmobranchs has been gaining much attention in recent years. After demonstrating the importance of the maternal fluids for the successful gestation of *C. taurus* embryos in **CHAPTER 5** focus had to turn to the possible presence of heavy metals in these maternal fluids due to the length of time the aplacental embryos spend surrounded by them during gestation. **CHAPTER 6** dealt with the investigation of five heavy metals in the gravid female's plasma, ICF and UF of three specific *C. taurus* females where all three fluids presented which is rare find. This scenario provided an opportunity to determine if heavy metals, which could affect embryo development,

CHAPTER 6

Possible maternal offloading of metals in the plasma, uterine and capsule fluid of pregnant Ragged-tooth sharks (*Carcharias taurus*) on the east coast of South Africa

Kristina Naidoo[1], Anil Chuturgoon[1*], Geremy Cliff[2, 3], Sanil Singh[3], Megan Ellis[4], Nicholas Otway[5], Andre Vosloo[6] and Michael Gregory[7]

[1]Discipline of Medical Biochemistry, [3]Biomedical Resource Unit, [6]School of Laboratory Medicine and Medical Sciences and [7]School of Health Sciences, University of KwaZulu-Natal (UKZN), Durban, South Africa

[2]KwaZulu-Natal Sharks Board (KZNSB), Umhlanga, South Africa

[4]Gladstone Ports Corporation, Gladstone, Queensland, Australia

[5]New South Wales Department of Primary Industries, Port Stephens Fisheries Institute, Taylors Beach, New South Wales, Australia

*Corresponding author, email: chutur@ukzn.ac.za

Environmental Science and Pollution Research (2017) 24:16798-16805

DOI 10.1007/s11356-017-9281-1

6.1 Abstract

We studied the possible metal offloading onto the progeny of three pregnant female Ragged- tooth sharks (*Carcharias taurus)* (*C. taurus*). The presences of five metals aluminium (Al), arsenic (As), cadmium (Cd), lead (Pb) and selenium (Se) were validated by weight spectrometry in the maternal plasma as well as the intracapsular and uterine fluids (UF) in which embryos develop. Metals were ranked in a decreasing concentration as follows: Plasma: As>Al>Se>Pb>Cd; ICF: As>Se>Al>Cd>Pb and UF: As>Se>Al>Cd>Pb. As was present in the highest concentration in all three sharks. Al, Pb and Cd were found to be the highest within the plasma while concentrations of Se were similar in all three fluids. These results indicate that this species embryos are exposed to metals during early development, but the impact of this exposure remains unknown. To the best of our knowledge, this is the first investigation to confirm the presence of metals in the fluids that surround the developing *C. taurus* embryos, a species that is already listed as Vulnerable.

Keywords: *Carcharias taurus*, Fluid, Intracapsular, Metals, Plasma, Reproduction, South Africa, Uterus

6.2 Introduction

Elasmobranchs are apex predators known to accumulate both organic and metal environmental contaminants [1]. Factors that contribute to this bioaccumulation, allowing them to serve as environmental monitors, are their life history characteristics such as their trophic position, longevity and diet [2,3]. The presence of metals has been documented in elasmobranchs [1,3,4]. Maternal offloading is the process whereby a female transfers anthropogenic contamination to her offspring [5]. This process has been reported in animals [6,7] including elasmobranchs [5,8,9]. Metal contamination has been documented in elasmobranchs in South Africa (SA) [10,11].

The term "'metals" in this paper has replaced the widely used "heavy metals" [12]. This term represents both heavy (As, Cd, Se and Pb) and light (Al) density metals. The high-density metals, with the exception of Se, are non-essential metals that are toxic at low concentrations while essential metals, Se and Al, can be toxic at high concentrations [13]. This study focussed on these metals as they are known to accumulate in an organism as it ages; regardless of type of tissue or species [14] and chronic bioaccumulation can lead to impaired reproduction [13,15].

These metals can originate from anthropogenic and natural sources. Dispersion of these elements into the biosphere can occur through natural processes such as disruption of earth's crust and sediment erosion [16]. The largest source is numerous anthropogenic activities (increased urbanisation and industrialization) [17], mining and agriculture [18].

International [19,20] and local studies [10,11] on elasmobranchs focussed on shark species which served as a food source, thereby posing a possible risk to human health. The discovery of contamination in sharks led to the investigation of maternal offloading [11]. Watling *et al*. (1982) documented the presence of metals in tissues of the female Dusky shark (*Carcharhinus obscurus*) including her embryos. This study also validated the presence of metals in the tissue of the adult shortfin mako (*Isurus oxyrinchus*) and white shark (*Carcharodon carcharias*) caught in inshore waters off the east coast of SA.

Internationally the presence of metals in the tissue of both the female and her embryos has also been shown in the same species of sharks such as the *C. carcharias* and *I. oxyrinchus* [5]. This has also been demonstrated in the Thresher shark (*Alopias vulpinus*) [5] and Pacific sharpnose shark (*Rhizoprionodon longurio*) [8,21].

Few studies have investigated metals in the circulatory system of elasmobranchs such as the *C. obscurus* [11] and *R. longurio* [21]. The *A. vulpinus, C. carcharias* and *I. oxyrinchus* are Mackerel sharks (order Lamniformes) that display metal maternal offloading [5,22]. Their reproductive mode is one of oophagy, in which embryos consume unfertilised eggs *in utero* [23]. This has also been established as one of the routes for females to contaminate their young [22]. The, another lamniform shark, utilizes oophagy as well as intrauterine cannibalism in its aplacental reproductive mode [23]. *C. taurus* females, in stages of pregnancy, are caught in the bather protection nets deployed at beaches along the KZN-SA coast to provide protection against shark attacks [24], thereby providing an opportunity to investigate metal levels in this species.

C. taurus is a large, coastal species, listed as Vulnerable by the International Union Conservation of Nature (IUCN). Its characteristics of slow growth and late sexual maturity, a biennial reproductive cycle and low fecundity fuels the concern as to whether this species can sustain current increasing exploitation [25]. Initially *C. taurus* embryos develop within enclosed collagenous capsules, filled with ICF [23]. Each encapsulated embryo (EE) escapes from their capsule (~60 mm) and remain in the uterus, as a free-floating embryo (FFE) surrounded by uterine fluid (UF) [23]. Uterine modifications (increased vascularisation) and the continuous supply of capsules containing unfertilised eggs filled with yolk are part of the matrotrophic support to developing *C. taurus* embryos [23].

The unusual discovery of small, encapsulated embryos (mean 16 mm; range 9-71 mm) in three pregnant *C. taurus* initiated this investigation for the presence of metals in three fluid compartments: maternal plasma, intracapsular fluid (ICF) and UF. The plasma would be mainly influenced by the metal intake through the shark's diet [8,26]. The second and third fluid compartments were those surrounding the embryos within the

inner ICF and the outer UF which could become contaminated from maternal contributions [5]. In this study we showed that embryos develop in fluid which is contaminated. To the best of our knowledge this study is the first to describe the presence of metals in maternal plasma, ICF and UF of *C. taurus*.

6.3 Materials and methods

Three pregnant *C. taurus* sharks (**Table 6.1**), caught in bather protection nets along the KwaZulu-Natal coastline, were examined within 24 hours of capture. Institutional ethical clearance was granted from the University of KwaZulu-Natal (ethical approval no 076/10/animal). Total lengths (TL, mm) and weight (kg or g) were presented as mean ± 1SD and median ± interquartile range (IQR). Median values were used during the analysis of this study. Mean values were also included for comparison with other studies.

Blood was drawn from the female's lateral vein using an 18G needle attached to a 10 mL disposable syringe and immediately transferred into vacutainers coated with anticoagulant (BD Diagnostic). Vacutainers were placed on ice after standing at room temperature (RT, 23^{0}C) for 30 minutes and then centrifuged (4,000 *x*g, 10 min, RT) to obtain the plasma. The ICF (2 mL) was carefully removed with a 10 mL syringe (Terumo), from each intact capsule (n = 8). Each EE was carefully excised from its capsule. Although embryos and capsules were present in both uteri, only fluid from the right uterus was analysed. The plasma, ICF and UF were then stored at -20^{0}C until further analysis.

All fluid samples were analysed for five metals, aluminium (Al), arsenic (As), cadmium (Cd), lead (Pb) and selenium (Se), using inductively coupled plasma mass spectrophotometry (ICP- MS, Perkin Elmer SCIEX 6100) (Perkin Elmer, USA). Sample pre-treatment was done prior to analysis where organic matter was removed by HNO_3 diluent (10% *v/v* nitric acid). Samples were preserved in a 2% nitric acid solution to provide stability. Analysis of blanks gave readings at or below the detectable limits. A

calibration curve was established for a 1 ppm [1 mg/ml] multi-element standard (Perkin Elmer, USA) using the ICP-MS 1600. During this analysis we also determined the response correction factor relative to the 1 ppm standard. The correction factor was calculated as the measured concentration of each metal standard divided by the nominal concentration of that metal standard. Concentration values [mg/L], from ICP- MS were multiplied by both the dilution factor to correct for the tenfold dilution to the sample and the response correction factor relative to the 1 ppm standard. GRAPHPAD PRISM (GraphPad Software Inc.; Version 7) was used for statistical analysis (mean ± SD). The Shapiro-Wilk test showed that all data did not pass normality. Mann Whitney and Spearman correlation (r_s, p) were used for non-parametric data. This study compared concentrations of each metal within the three fluids. Partioning of metals was determined by calculating the ratio concentration between two fluid compartments i.e., UF: plasma ratio, the ICF:UF ratio and the ICF: plasma ratio. Ratios >1 suggested a preferential partionining in the target tissue (the capsule or the uterus). Ratios <1 indicated the possible impermeability of the tissue or possible detoxification process of that metal in the target. Levels of significance were set at $p < 0.05$, based on two-tailed tests.

Table 6.1: Details of the three captured *C. taurus* sharks (shark 1- shark 3) examined and their respective encapsulated embryos (EEs) and floating embryos (FFEs).

	Maternal details		Embryo details		
Reference no.	**TL (mm)**	**Weight (kg)**	**No of EE/FFE**	**TL (mm)**	**Weight (g)**
Shark 1	2640	169	7/0	9-21	0.1-0.6
Shark 2	2760	161	2[a]/7	14-71	0.1-3.6
Shark 3	2638	130	0/6	14-225	0.1-71

[a]Two embryos enclosed within one capsule.

6.4 Results

The size range and Weight of the pregnant females and their embryos are detailed in **Table 6.1** total of 22 embryos were found. Nine were EEs and 13 were FFEs. Twenty of

these embryos were smaller than 100 mm while two, from shark 3, were 221 and 225 mm.

All five metals were found in all three fluid compartments. **Table 6.2** shows the median and mean concentrations in decreasing order for each fluid compartment. The median values of a particular metal with the same superscripted letter indicated a significant difference ($p \leq 0.05$) between compartments. Due to the small sample size, significant relationships should be viewed with caution.

Of the five metals, As was found in the highest concentration in all three fluid compartments, while Pb and Cd were present in the lowest concentrations (**Table 6.2)** Arsenic, Al, Pb and Cd was significantly higher in the plasma than the ICF ($p = 0.024^{A}$; $p = 0.02^{B}$; $p = 0.012^{C}$; $p = 0.012^{D}$). The plasma concentrations of these four metals were also much higher than the UF, but these differences were not statistically significant. Se was an exception and was present in similar concentrations in all three fluids (**Figure 6.1**).

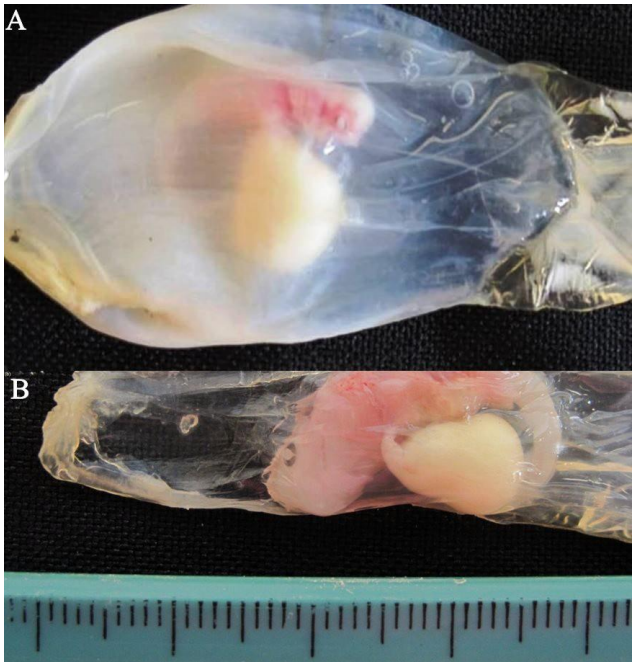


Figure 6.1: Encapsulated *C. taurus* embryos (EEs) found within ICF-filled, semi-translucent egg capsule. Both embryos showed the presence of eye structures as well as an attached yolk sac. The embryos measured A) 28 mm and B) 35 mm TL respectively.

Table 6.2: The median and mean concentrations [mg/L] of metals in the three fluid compartments i.e., maternal plasma, ICF and UF of three *C. taurus*.

Compartment	*n*	Metal	Median ± IQR [mg/L]	Mean± SD [mg/L]
Plasma	3	As	41.5 ± 19.61^{A}	35.39 ± 21.3
		Al	37.2 ± 11.76^{B}	31.36 ± 15.2
		Se	3.3 ± 0.73	3.16 ± 0.75
		Pb	2.3 ± 0.86^{C}	2.53 ± 0.77
		Cd	0.6 ± 1.97^{D}	1.71 ± 2.17
ICF	8	As	5.8 ± 1.78^{A}	6.76 ± 2.9
		Se	3.8 ± 0.63	4.05 ± 0.66
		Al	0.2 ± 2.53^{B}	3.97 ± 7.43
		Pb	0.04 ± 0.04^{D}	0.42 ± 0.76
		Cd	0.02 ± 0.48^{C}	0.05 ± 0.03
UF	3	As	4.5 ± 0.83	4.63 ± 0.87
		Se	3.3 ± 0.17	3.38 ± 0.18
		Al	0.3 ± 0.13	0.20 ± 0.18
		Cd	0.03 ± 0.02	0.04 ± 0.02
		Pb	0.001 ± 0.005	0.004 ± 0.004

The concentration (median ± IQR) within each fluid are ranked from highest to lowest. Medians with the same superscripted letters indicated a significant difference between compartments ($p \leq 0.05$).

With only three female sharks of approximately the same size, it was difficult to relate metal concentration to the shark's length (mm)/weight (kg). Further, all embryos both (EEs and FFEs), smaller than 100 mm TL, showed a significant positive correlation between embryo length and weight (r_s = 0.72, p < 0.0003). The EE alone (n = 9) showed a positive, yet nonsignificant correlation between length and weight (r_s = 0.28, p = 0.50). The EE length and weight (n = 9) both showed a nonsignificant, negative correlation with all five metals within the ICF (**Table 6.3**) Analysis of the intermetal correlation within the ICF showed the following significant positive correlations (**Table 6.3**). The concentration ratios (UF: plasma, ICF: UF and ICF: plasma) are shown in **Figure 6.2A-C.** With the exception of Se, the other four metals occurred in much lower concentrations (ratio < 1) in both the ICF and UF than in the plasma. Selenium was the only metal to be marginally higher in the ICF than the plasma (ratio >1) **(Figure 6.2A).** All the metals were higher in the ICF than UF (ratio >1; **Figure 6.2C**).

Table 6.3: Tabulated Spearman r_s and p values for metal correlations in the ICF of embryo length and weight against metal concentration.

	Metal	r_s	p
EE Length	Al	-0.61	0.12
	As	-0.06	0.89
	Cd	-0.38	0.34
	Pb	-0.47	0.24
	Se	-0.42	0.30
EE weight	Al	-0.29	0.49
	As	-0.60	0.13
	Cd	-0.73	0.04
	Pb	-0.28	0.53
	Se	-0.22	0.59
Intermetal correlation	Al vs. Se	0.95	0.001
	Se vs. Pb	0.89	0.006
	Al vs. Pb	0.87	0.008
	As vs. Cd	0.88	0.007

Abbreviations: EE: encapsulated embryos; r_s: spearman relationship value; p: significant value < 0.05.

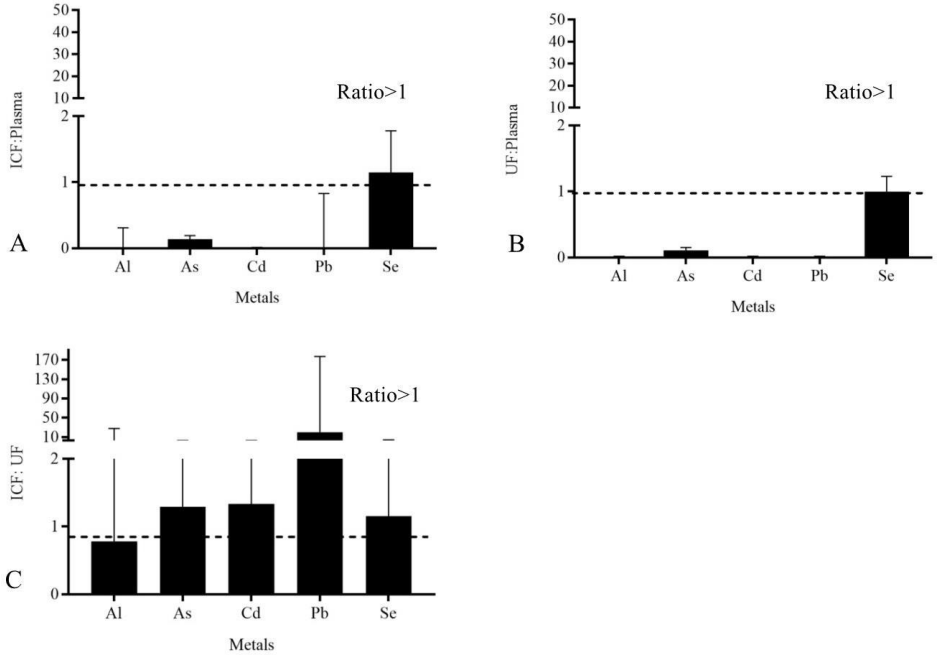


Figure 6.2: The ratio of each metal in the different compartments: A) ICF: Plasma, B) UF: Plasma and C) ICF: UF. Abbreviation: Al: Aluminium; As: Arsenic; Cd: cadmium; ICF: intracapsular fluid; Pb: Lead; Se: Selenium; UF: uterine fluid.

6.5 Discussion

In elasmobranchs, muscle and liver were the preferred tissues for metal studies [4,10], with few studies evaluating metal concentrations within the plasma [11]. To date, there are no studies on ICF or UF, possibly because of the delicate nature of these tissues and their highly transient nature which renders them far more difficult to obtain and extract than muscle and liver. These fluid compartments provide support and nourishment to *C. taurus* embryos [23]. This study showed that all five metals (Al, As, Cd, Pb and Se) were found in the three fluid compartments of the sharks investigated. Accumulation of these metals in the female is believed to be largely through the diet [22]. The breakdown of this diet can result in metal contamination being passed through the body via the plasma [21]. As a result, the plasma would be expected to have the highest concentration of these metals. This study showed that As, Al, Pb and Cd were found in higher concentrations in the plasma, which could suggest that the wall of the capsule and uterus may play a role as a barrier, impeding the entrance to certain metals (**Figure 6.2A-B)**.

Nonessential metals (i.e., As, Cd and Pb) serve no established biological function [13]. Arsenic is environmentally widespread [27] and was the highest occurring metal in all three fluid compartments, with concentrations in the plasma 5-7 times higher than the other two fluids. Arsenic was the predominant metal in muscle tissue [28.3 mg/L] of another local shark, *Mustelus mustelus* in the Western Cape (SA) [10]. Although accumulation in marine fish is often high, much of it is present as the nontoxic compound, arsenobetaine, accounting for 94% of the soluble As in white sharks [28]. In *C. taurus* the plasma and ICF concentrations of Al, Cd, Pb and Se were higher than those previously recorded in muscle tissue of *M. mustelus* [10].

Nonessential metals (Cd and Pb) can be toxic at low concentrations [13]. In this study they were the lowest of the five metals in all compartments. Vas *et al*. (1987) showed a similar trend of low concentrations of these metals within the aplacental School shark (*Galeorhinus galeus*). Plasma levels of Cd and Pb in *C. taurus* were one and two orders of magnitude higher, respectively, than in the blood of *I. oxyrinchus* [Cd: 0.06 mg/L and

Pb: 0.02 mg/L], also sampled on the east coast of SA [11]. *C. taurus* is a shallow water, coastal species and is more likely to be exposed to industrial run-off than the more pelagic, open water *I. oxyrinchus*. In maternal *R. longurio* these metals were found in the muscle [Cd: 0.03 mg/L and Pb: 4.96 mg/L] and liver [Cd: 1.67 mg/L and Pb: below limit of detection] compared to embryo muscle [Cd:0.08 mg/L and Pb: 2.08 mg/L] and liver [Cd: 0.18 mg/L and Pb: 1.43 mg/L] [8]. This indicates metal transfer between the female and her embryos [8]. Pb, like the other metals investigated in this study, was shown to be higher in the ICF than the UF (ratio>1) (**Figure 6.2C)**. Pb was the only metal to hyperaccumulate in the ICF suggesting it had greater permeability than the rest.

The essential metals (Al and Se) are required for normal metabolic activity but can be toxic at high concentrations. Se has a detoxifying effect on mercury (Hg) in sharks [29]. It is therefore possible that Se could counteract the toxicity of other metals, such as Al and Pb, which could explain the significant positive correlation between Se and both Al and Pb. Selenium is an exception as it is the only metal found in similar concentrations in the three fluid compartments (ratio >1) (**Figure 6.2A** and **C**), suggesting that it can readily pass from one compartment to another; this is advantageous in any detoxification process.

The high concentrations of metals in the plasma in ovulating *C. taurus* suggests the potential for plasma to contaminate other tissues, including ova and fluids such as ICF and UF, resulting in the embryos being exposed to these elevated levels [30,31]. This metal transfer into the fluids from the plasma can occur through increase in vasculature in the uterine mucosa during gestation [23,32].

The presence of metals in the ICF indicates that embryos developing within the capsules are exposed to these potentially toxic metals during a critical stage of embryo growth, which encompasses development of the oral cavity, jaws and alimentary tract [23]. The high concentrations in the ICF could be mainly derived from the encapsulated yolk in the ova. These ova develop into EEs and growing embryos maintain a yolk sac that will eventually be used for nutrition [23]. It has been shown that the maternal yolk-filled oocytes could be a source of contamination [22]. Evidence of metal-contaminated yolk in aplacental Thresher sharks (*A. vulpinus*) was previously reported [22]. It is therefore

likely a similar form of contamination occurred in the aplacental *C. taurus*. The yolk-granule precursor, vitellogenin (Vtg), is produced in the liver and taken up by the oocytes [33]. Studies show that Cd and Se can bind to Vtg [15] while As and Al can reduce Vtg production [34,35]. Metals can affect the supply of yolk to the developing progeny by contaminating the yolk or reducing its production. In addition, the embryos epidermal surface could have secretory activity thereby further contaminating the ICF environment during development [31,36].

Embryos use their dentition to escape encapsulation [23] into less contaminated UF environment for the remainder of their gestation. In this study, the plausible reasons for lower metal concentrations in the UF as compared to the ICF are: (1) the female's natural metal clearance process [37] ,(2) the transfer of metals into surrounding embryo tissue [36,38]and (3) possible uterine flushing [39,40]. The first hypothesis originates from the presence of protein structures called metallothionein which serve as a group of metal-binding proteins in elasmobranchs [37,41]. These proteins can serve as protection against metal toxicity by binding to various metals. The second hypothesis originates from studies that showed that the body of the embryos can incorporate components from surrounding fluids *in vitro* [36,38]. The third hypothesis introduces the possibility of uterine flushing being able to reduce contamination in the UF. Uterine flushing is the phenomenon whereby the female periodically flushes her uterine environment with seawater, to assist sharks with respiratory exchange and waste removal [39,42,43]. Uterine flushing has been shown to occur in the *Squalus acanthias* and the *Orectolobus ornatus* [39,40,44] but is still to be elucidated in *C. taurus*.

Apart from the fluids, *C. taurus* embryos can become further contaminated from their *in utero* diet that comprise of: 1) cannibalising their siblings [23] and 2) reliance on maternal yolk-filled oocytes (oophagy) throughout their gestation period [23]. The oocytes themselves can be contaminated [22]. Metals could accumulate in the ovarian tissue from binding to Vtg [15] and also reduce egg production via inhibiting Vtg in oophagous species [34,35] .The embryos themselves, from the stage of encapsulation, can accumulate metals in their tissues [3,45]. Therefore, they are contaminated during their intrauterine cannibalistic phase. The negative correlation between the EE size and the

ICF concentration of metals indicate that the embryos take up the metals as they develop, or the metals diffuse out of the capsule. There have been many reports of embryonic deformity [9,46,47]. Currently there is no study that can provide clear evidence that metals or any form of contamination is the cause for the deformities reported [9,46,47]. However, studies by Zaera and Johnsen (2011) that investigated the presence of metals in the tissue of a deformed *M. mustelus* embryo does provide evidence that environmental contamination cannot be discarded as a possible cause.

Watling *et al*. (1982) referred to the east coast of SA as being "relatively unpolluted with respect to metals". Recent research suggests otherwise [48]. Several species of large sharks, including 30 *C. taurus*, all caught in the bather protection nets, had higher total mercury (THg) levels than conspecifics sampled from coastal waters of the North Atlantic and North Pacific but with similar levels to sharks from the Mediterranean Sea, which is considered to have anomalously high Hg levels. Heavy metal contamination was recorded in the Palmiet river catchment area located in KZN (SA), which is urbanised but comprises of industries and residential areas [49]. Greenfield *et al*. (2011) documented the presence of metals in the sediment records around Richards Bay (SA), which has an industrial harbour specializing in coal export [50]. All three sharks, used in this study, were captured in Richards Bay. *C. taurus* females undertake a biennial migration along the east coast of SA of approximately 1000 km from their gestation grounds to their pupping grounds in the south, so they are not exposed to any single point source of pollution such as a marine outfall or an industrialised harbour for lengthy periods.

6.6 Conclusion

The metal burden on *C. taurus* embryos could be increased further by their unique employment of embryophagy where the surviving embryo accumulates all the metals passed on from the mother to her other offspring. At this stage there is no evidence that the concentrations recorded could adversely affect pregnancy, development of embryos and parturition, but the potentially negative impact on the reproduction of this

threatened species is concerning. A limitation of this study was that the muscle and liver tissue of both the mothers and the embryos were not analysed to confirm maternal offloading in this study.

6.7 Acknowledgements

We wish to thank Mrs. Naicker (Department of Geology, University of KwaZulu-Natal) for the metal analysis and dedicated KwaZulu-Natal Sharks Board Operations staff for the careful handling of all sharks in this study. Financial support in the form of a UKZN Doctoral grant and a scholarship from the New South Wales Fisheries, Department of Primary Industries (NSW DPI) is gratefully acknowledged.

Compliance with ethical standards: Institutional ethical clearance was granted from the University of KwaZulu-Natal (ethical approval no. 076/10/animal)

Conflict of Interest: The authors declare that they have no conflict of interest.

6.8 References

1. Gelsleichter, J. and Walker, C.J. (2010) In Carrier JC, Musick JA and Heithaus MR (eds.), *Sharks and their relatives II: biodiversity, adaptive physiology and conservation*. CRC Press, Boca Raton, 491-537.
2. Hornung, H., Krom, M., Cohen, Y. and Bernhard, M. (1993) *Trace metal content in deep-water sharks from the eastern Mediterranean Sea.* Marine Biology, **115**, 331-338.
3. Mull, C.G., Blasius, M.E., O'Sullivan, J.B. and Lowe, C.G. (2012) In ML, D. (ed.), *Global perspectives on the biology and life history of the White Shark.* CRC Press, Boca Raton, Florida, 59-75.
4. Vas, P. (1987) *Observations on trace metal concentrations in a carcharhinid shark, Galeorhinus galeus, from Liverpool Bay.* Marine Pollution Bulletin, **18**, 193-194.
5. Lyons, K., Carlisle, A., Preti, A., Mull, C., Blasius, M., O'Sullivan, J., Winkler, C. and Lowe, C.G. (2013) *Effects of trophic ecology and habitat use on maternal transfer of contaminants in four species of young of the year lamniform sharks.* Marine Environmental Research, **90**, 27-38.
6. Borrell, A., Bloch, D. and Desportes, G. (1995) *Age trends and reproductive transfer of organochlorine compounds in long-finned pilot whales from the Faroe Islands.* Environmental Pollution, **88**, 283-292.
7. Peng, H., Zhang, K., Wan, Y. and Hu, J. (2012) *Tissue distribution, maternal transfer, and age-related accumulation of dechloranes in Chinese sturgeon.* Environmental Science & Technology, **46**, 9907-9913.
8. Frías-Espericueta, M., Cardenas-Nava, N., Márquez-Farías, J., Osuna-López, J., Muy-Rangel, M., Rubio-Carrasco, W. and Voltolina, D. (2014) *Cadmium, copper, lead and zinc concentrations in female and embryonic pacific sharpnose shark (Rhizoprionodon longurio) tissues.* Bulletin of Environmental Contamination and Toxicology, **93**, 532-535.
9. Zaera, D. and Johnsen, E. (2011) *Foetal deformities in a smooth-hound shark, Mustelus mustelus, from an oil exploited area in Angola.* Cybium, **35**, 231-236.
10. Bosch, A.C., O'Neill, B., Sigge, G.O., Kerwath, S.E. and Hoffman, L.C. (2016) *Heavy metal accumulation and toxicity in smoothhound (Mustelus mustelus)*

shark from Langebaan Lagoon, South Africa. Food Chemistry, **190**, 871-878.

11. Watling, R., Watling, H., Stanton, R., McClurg, T. and Engelbrecht, E. (1982) *The distribution and significance of toxic metals in sharks from the Natal Coast, South Africa.* Water Science and Technology, **14**, 21-30.

12. Hodson, M.E. (2004) *Heavy metals—geochemical bogey men?* Environmental Pollution, **129**, 341-343.

13. Govind, P. and Madhuri, S. (2014) *Heavy metals causing toxicity in animals and fishes.* Research Journal of Animal, Veterinary and Fishery Sciences, **2**, 17-23.

14. Eisler, R. (1984) *Trace metal changes associated with age of marine vertebrates.* Biological Trace Element Research, **6**, 165-180.

15. Ghosh, P. and Thomas, P. (1995) *Binding of metals to red drum vitellogenin and incorporation into oocytes.* Marine Environmental Research, **39**, 165-168.

16. Kunito, T., Kubota, R., Fujihara, J., Agusa, T. and Tanabe, S. (2008) *Arsenic in marine mammals, seabirds, and sea turtles.* Reviews of Environmental Contamination and Toxicology, **195**, 31-69.

17. Vermeulen, L. and Wepener, V. (1999) *Spatial and temporal variations of metals in Richards Bay Harbour (RBH), South Africa.* Marine Pollution Bulletin, **39**, 304-307.

18. Fatoki, O. and Mathabatha, S. (2001) *An assessment of heavy metal pollution in the East London and Port Elizabeth harbours.* Water SA, **27**, 233-240.

19. Stevens, J. and Brown, B. (1974) *Occurrence of heavy metals in the blue shark Prionace glauca and selected pelagic in the NE Atlantic Ocean.* Marine Biology, **26**, 287-293.

20. Turoczy, N., Laurenson, L., Allinson, G., Nishikawa, M., Lambert, D., Smith, C., Cottier, J., Irvine, S. and Stagnitti, F. (2000) *Observations on metal concentrations in three species of shark (Deania calcea, Centroscymnus crepidater, and Centroscymnus owstoni) from southeastern Australian waters.* Journal of Agricultural and Food Chemistry, **48**, 4357-4364.

21. Frías-Espericueta, M.G., Zamora-Sarabia, F.K., Márquez-Farías, J.F., Osuna-López, J.I., Ruelas-Inzunza, J. and Voltolina, D. (2015) *Total mercury in female Pacific sharpnose sharks Rhizoprionodon longurioand their embryos.* Latin American Journal of Aquatic Research, **43**, 534-538.

22. Lyons, K. and Lowe, C.G. (2013) *Mechanisms of maternal transfer of organochlorine contaminants and mercury in the common thresher shark (Alopias vulpinus).* Canadian Journal of Fisheries and Aquatic Sciences, **70**, 1667-1672.

23. Gilmore, R.G., Putz, O. and Dodrill, J.W. (2005) In Hamlett WC (ed.), *Reproductive biology and phylogeny of Chondrichthyes: Sharks, Batoids and Chimaeras.* Science Publishers, Enfield, New Hampshire, USA, 435-462.

24. Cliff, G. and Dudley, S.F.J. (1992) *Protection against shark attack in South Africa, 1952-90.* Marine and Freshwater Research, **43**, 263-272.

25. Pollard, D. and Smith, A. (2009) *Carcharias taurus. Version 2012.2: IUCN Red List of Threatened Species.* e.T3854A10132481. http://www.iucnredlist.org. Accessed 23 March 2010.

26. Mathews, T. and Fisher, N.S. (2009) *Dominance of dietary intake of metals in marine elasmobranch and teleost fish.* Science of the Total Environment, **407**, 5156-5161.

27. Keil, D.E., Berger-Ritchie, J. and McMillin, G.A. (2011) *Testing for toxic elements: a focus on arsenic, cadmium, lead, and mercury.* Laboratory Medicine, **42**, 735-742.

28. Hanaoka, K.i., Fujita, T., Matsuura, M., Tagawa, S. and Kaise, T. (1987) *Identification of arsenobetaine as a major arsenic compound in muscle of two demersal sharks, shortnose dogfish Squalus brevirostris and starspotted shark Mustelus manazo.* Comparative Biochemistry and Physiology. B, Comparative Biochemistry, **86**, 681-682.

29. Branco, V., Vale, C., Canário, J. and dos Santos, M.N. (2007) *Mercury and selenium in blue shark (Prionace glauca, L. 1758) and swordfish (Xiphias gladius, L. 1758) from two areas of the Atlantic Ocean.* Environmental Pollution, **150**, 373-380.

30. Kormanik, G.A. (1993) *Ionic and osmotic environment of developing elasmobranch embryos.* Environmental Biology of Fishes, **38**, 233-240.

31. Lombardi, J. and Files, T. (1993) *Egg capsule structure and permeability in the viviparous shark, Mustelus canis.* Journal of Experimental Zoology Part A: Ecological Genetics and Physiology, **267**, 76-85.

32. Hamlett, W.C. and Hysell, M.K. (1998) *Uterine specializations in elasmobranchs.* Journal of Experimental Zoology, **282**, 438-459.

33. Hamlett, W. and Koob, T. (1999) In Hamlett WC (ed.), *Sharks, Skates and Rays:The Biology of Elasmobranch Fish*. Baltimore, MD: Johns Hopkins University Press, 398-444.

34. Boyle, D., Brix, K.V., Amlund, H., Lundebye, A.-K., Hogstrand, C. and Bury, N.R. (2008) *Natural arsenic contaminated diets perturb reproduction in fish.* Environmental Science & Technology, **42**, 5354-5360.

35. Hwang, U.-K., Kang, H.-S., Lee, Y. and Shon, J.-K. (2011) *Aluminum Inhibits Vitellogenin Production via Toxic Effects on Hepatocytes in the Rockfish Sebastes schlegelii.* Fisheries and Aquatic Sciences, **14**, 355-361.

36. Lombardi, J., Wourms, J. and Hall, J. (1988) *Structural Organization of umbilical stalk appendiculae in the viviparous shark Rhizoprionodon Terraenovae.* American Zoologist, **28**, A80-A80.

37. Planas, J., Tort, L., Torres, P. and Flos, R. (1991) *Cadmium induction of metallothioneins in several dogfish organs.* Revista espanola de fisiologia, **47**, 75-80.

38. Wourms, J.P., Grove, B.D. and Lombardi, J. (1988) *The maternal-embryonic relationship in viviparous fishes.* Fish Physiology, **11**, 1-134.

39. Ellis, M.T. and Otway, N.M. (2011) *Uterine fluid composition of the dwarf ornate wobbegong shark (Orectolobus ornatus) during gestation.* Marine and Freshwater Research, **62**, 576-582.

40. Evans, D., Oikari, A., Kormanik, G.A. and Mansberger, L. (1982) *Osmoregulation by the prenatal spiny dogfish, Squalus acanthias.* Journal of Experimental Biology, **101**, 295-305.

41. Cho, Y.S., Choi, B.N., Ha, E.-M., Kim, K.H., Kim, S.K., Kim, D.S. and Nam, Y.K. (2005) *Shark (Scyliorhinus torazame) metallothionein: cDNA cloning, genomic sequence, and expression analysis.* Marine Biotechnology, **7**, 350-362.

42. Burger, J.W. and Loo, T.L. (1959) *Bromination of phenol red by the dogfish, Squalus acanthias.* Science, **129**, 778-779.

43. Sato, K., Nakamura, M., Tomita, T., Toda, M., Miyamoto, K. and Nozu, R. (2016) *How great white sharks nourish their embryos to a large size: evidence*

of lipid histotrophy in lamnoid shark reproduction. Biology Open, **5**, 1211-1215.

44. Price, K.S. and Daiber, F.C. (1967) *Osmotic environments during fetal development of dogfish, Mustelus canis (Mitchill) and Squalus acanthias Linnaeus, and some comparisons with skates and rays.* Physiological Zoology, **40**, 248-260.

45. Otway, N.M. (2015) *Serum biochemical reference intervals for free-living Sand Tiger sharks (Carcharias taurus) from east Australian waters.* Veterinary Clinical Pathology, **44**, 262-274.

46. Carmo, W.P.D.d. and Fávaro, L.F. (2015) *Teratogenic processes in an embryo without gills and low yolk absorption of Zapteryx brevirostris (Elasmobranchii: Rhinobatidae).* Brazilian Journal of Oceanography, **63**, 497-500.

47. Ehemann, N., Marín-Sanz, J. and Barany-González, M. (2016) *Two cases of two-head shark embryos, smalleye smooth-hound Mustelus higmani and the blue shark Prionace glauca.* Boletín de Investigaciones Marinas y Costeras-Invemar, **45**, 149-153.

48. McKinney, M.A., Dean, K., Hussey, N.E., Cliff, G., Wintner, S.P., Dudley, S.F., Zungu, M.P. and Fisk, A.T. (2016) *Global versus local causes and health implications of high mercury concentrations in sharks from the east coast of South Africa.* Science of the Total Environment, **541**, 176-183.

49. Moodley, K., Pillay, S., Pather, K. and Ballabh, H. (2014) *Heavy Metal Contamination of the Palmiet River: KwaZulu Natal South Africa.* International Journal of Scientific Research in Environmental Sciences, **2**, 397.

50. Greenfield, R., Wepener, V., Degger, N. and Brink, K. (2011) *Richards Bay Harbour: Metal exposure monitoring over the last 34 years.* Marine Pollution Bulletin, **62**, 1926-1931.

BRIDGE

CHAPTER 6 TO CHAPTER 7

Whilst determining if heavy metals were present in the maternal fluids of *C. taurus* females in **CHAPTER 6,** we came across an opportunistic find of a few embryos that appeared to escaped encapsulation at a far shorter length (i.e., < 60 mm TL) than previously described in literature (i.e. ≥ 60 mm TL). **CHAPTER 7** examined the structures in the embryo jaw to determine if they were dental in composition to serve as embryonic teeth.

Investigating the presence of heavy metals in fluids that surround the embryos during gestation, and the examination of embryos found outside their respective capsules at a shorter length than previously recorded increases the knowledge and the possible consequences to *C. taurus* reproductive strategy that is currently not documented.

CHAPTER 7

Dentition facilitates the release of encapsulated Ragged-tooth shark (*Carcharias taurus*) embryos

Kristina Naidoo[1], Anil A. Chuturgoon[1*], Geremy Cliff[2, 6], Megan T. Ellis[3], Nicholas M. Otway[4], Michael A. Gregory[5], Sanil D. Singh[6] and Subashen L. Naidu[7]

[1]Discipline of Medical Biochemistry, [5]School of Health Sciences and [6]Biomedical Resource Unit, University of KwaZulu-Natal, Durban, South Africa [2]KwaZulu-Natal Sharks Board, Umhlanga, South Africa [3]Gladstone Ports Corporation, Gladstone, Queensland, Australia [4] New South Wales Department of Primary Industries, Port Stephens Fisheries Institute, Taylors Beach, New South Wales, Australia [7]Microscopy and Microanalysis Unit (MMU), University of KwaZulu-Natal (Pietermaritzburg), South Africa

*Corresponding author email: chutur@ukzn.ac.za

Environmental Biology of Fishes

DOI 10.1007/s10641-017-0647-5

7.1 Abstract

The capture of four in the early stages of pregnancy in the bather protective nets along the KwaZulu-Natal coastline provided an opportunity to investigate embryonic development. A total of 31 embryos, 8-225 mm total length, were found. Of these, 15 were encapsulated and 16 were found free-floating in the uterus. Six embryos, three of which were encapsulated (35-50 mm) and three free-floating (36-52 mm), were examined under both light and scanning electron microscopy. The embryos possessed tooth-like structures. Spectral analysis of these structures revealed the presence of calcium, phosphorus, fluoride and oxygen, which supports the hypothesis that they are teeth. These teeth would enable embryos to escape encapsulation. These free-floating embryos are the smallest on record; with the previous smallest being a 40 mm embryo. These findings would now amend the current literature of *C. taurus* embryology. These results could affect the current understanding of *C. taurus* reproduction and biology and may impact any current breeding programs that are attempting to increase the fecundity of these species.

Keywords: *Carcharias taurus*, dentition, embryos, encapsulation, Ragged-tooth, South Africa

7.2 Introduction

The reproductive biology of *C. taurus*, known in SA as the Ragged-tooth shark, has been well documented, mainly in the United States of America, where it is known as the Sand tiger shark. Embryo nourishment of this aplacental species is oophagous, a trait found in all lamnoid sharks. *C. taurus*, however appears to be the only species that also follows the additional unique nutrient and survival strategy of intrauterine cannibalism that results in a single well-developed embryo being conceived from each uterus [1-3]. This extremely low fecundity, with only two embryos born biennially, in conjunction with poorly managed fisheries, has resulted in classified as Vulnerable by the International Union for Conservation of Nature (IUCN) [4].

Gilmore *et al*. (1983) and Gilmore (1993) categorised *C. taurus* embryo development into six stages; encapsulation (pre-hatching) (stage I-II):13-60 mm TL; post-hatching (stage III): 60-100 mm; intrauterine cannibalistic phase (stage IV): 100-335 mm; oophagous phase (stage V): 335-1000 mm and pre-parturition (stage VI); 900-1000 mm. This study focused on stages I-IV.

The “peg-like” dentition of embryonic lamnoid sharks [5], becomes functional during the encapsulated phase and enable each encapsulated embryo (EE) to break free from its protective membranous collagen sheath termed a capsule [6,7]. Upon emergence the free-floating embryo (FFE) initially derives nourishment from its own external yolk sac, possibly supplemented by uterine fluid (UF) absorbed by the external gill filaments [1]. Embryos, between 100-335 mm, can cannibalise their siblings. Thereafter the single surviving embryo in each uterus enters an oophagous phase, ingesting many capsules containing infertile ova, which allows rapid growth immediately prior to parturition.

The capture of sharks in the bather protection nets along the KwaZulu-Natal (KZN) coastline (see Cliff and Dudley (1992) for a detailed description of this operation [8] provided an opportunity to investigate early-stage embryo development. This study employed stereomicroscopy, SEM and energy dispersive X-ray microanalysis (EDX), focusing on stages I-IV. The dentition of adult *C. taurus* has been well documented [7]. Elemental composition of shark tooth has also been documented [9]. Scarcity of all lamnoid embryos [5] has made investigating and reporting on dentition of *C. taurus*

embryos incredibly difficult. By contrast, this is the first study to report on the escape of *C. taurus* embryos from their capsules at a far earlier than reported stage documented 28 years ago by Gilmore *et al*. (1983) as well as Hamlett (1983) and 36 years ago by Bass *et al*. (1975).

7.3 Materials and Methods

Embryo specimens were obtained from four sharks (shark 1-shark 4; **Table 7.**1), caught in the KZN bather protection nets, and were dissected 24 hours after death, with ethical approval of the University of KwaZulu-Natal (076/10/Animal). Thirty-one embryos were found and according to Gilmore *et al*. (1983) classification system these embryos corresponded to stages I-II, stage III and stage IV. The embryos were either EE (n = 15) or FFE (n = 16) (**Table 7.1**). Each EE was carefully excised from its capsule and immediately fixed in ethanol (70%). All measurements, expressed as total length (TL), were presented as mean (± 1SD) using Microsoft Excel (2010). GraphPad Prism (Version 7.02) was used to determine correlations between embryo total length and the chemical compositions of the embryonic teeth.

A digital camera was used to take full body images of the embryos on the day of dissection. Thereafter, the dentition and overall structures were closely examined under a Nikon AZ100 stereo photomicroscope at 5x and 10x magnifications.

The SEM images were obtained using the ZEISS EVO LS15 Variable Pressure (VP) SEM with SmartSEM software (Version 5.04). Further SEM analysis was carried out on six embryos from shark 4. The KZNSB accession no and (TL) of these six embryos (E1-E6) are E1: UMT12004_R1 (50 mm), E2: UMT12004_R2 (46 mm), E3: UMT12004_L1 (52 mm), E4:UMT12004_L2 (36 mm), E5:UMT12004_A1 (35 mm) and E6: UMT12004_A2 (35 mm). Further details of E1-E6 can be found in **Table 7.1.** These embryos were referred to as E1-E6 respectively throughout the manuscript. The Zeiss EVO LS 15 SEM is capable of Environmental SEM (ESEM) which allows for the imaging of hydrated samples with no preparation other than allowing a few minutes for the embryos to dry. The software allowed further detailed examination and measurements (µM) of the embryo dentition **Table 7.2.** However, a tooth numbering system had to be

created prior to any further measurement or analysis. The dental terminology used in this study was based on the tooth numbering and jaw labelling system used by Tomita *et al*. (2017) but it was modified to differentiate between the left and right sides of the upper jaw (paratoquadrate, pq) and lower jaw (Meckel's cartilages, Mc) jaw. The position of each tooth was recorded based on 1) ID of the embryo; 2) its jaw position (pq or Mc);3) the left or right-side position of the jaw and 4) its location along the gum; moving in a distal direction from the centre of the jaw (**Figure 7.1).** The location of each tooth on the jaws was at times difficult to view using the SmartSEM software which produced a grey scale image. This was overcome by referring to the corresponding stereo photomicroscope images (**Figure 7.1**). Dental measurements (tooth height: TH; tooth width: TW) were taken on the labile side of the pq and Mc jaw in E1-E6. Tooth height equalled the crown height while the width was measured from end-end of the widest part of the crown **Table 7.2** teeth could not be measured as they were broken off (represented by a dash in **Table 7.2**).

In addition, of an Oxford X-Max 80 mm Silicon Drift Detector (SDD) through Energy Dispersive X-ray (EDX) and INCA analysis software (Version 4), was used the evaluate the elemental composition of the outer enameloid layer of the teeth present on the pq and Mc jaws of E1-E6. The EDX analysis was restricted to maximum analysis to a depth of 1µM. At this depth was measuring the shiny layer enameloid (SLE) was measured. Element detection at the gum line of each embryo provided control values. The elemental composition of a single tooth from two mature *C. taurus* females (shark 5: R. B10020 and shark 6: R.B09014; **Table 7.1**) were also investigated for comparative purposes. A vertical section of each tooth allowed for the element analysis of the enameloid layer (exposed on the top and the sides of the vertical sectioned tooth covering the inner oesteodentine layer) in the adult *C. taurus* tooth. The embryo and adult teeth used for spectral analysis in this study have been deposited in the KZNSB tissue collection (Umhlanga, SA).

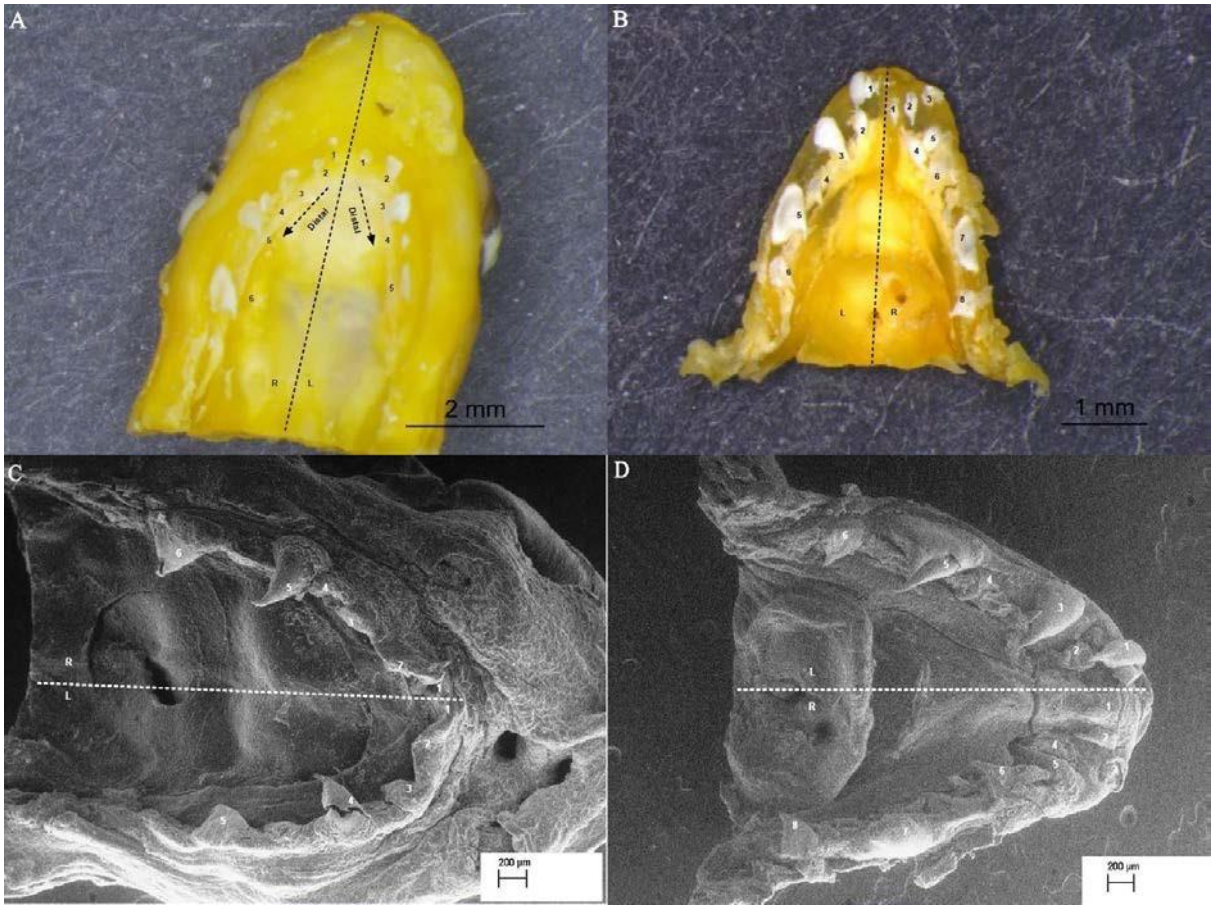


Figure 7.1: Stereo (A-B) and SEM images (C-D) of the tooth locations, measured on the labial side, of the upper jaw (pq) (A, C) and lower jaw (Mc) (B, D) of E3 (52 mm FFE) from shark 4. Image B) shows the naming the teeth from the centre of the jaw moving distally.

7.4 Results

Table 7.1 presents the length and weight of each of the four-pregnant *C. taurus* sharks (shark 1-shark 4) together with as well as the length, weight count and sex of their associated embryos. A total of 31 embryos were found, with litter sizes of 7, 9, 6 and 9 between the respective uteri of shark 1- shark 4 (**Table 7.1)**. The 15 EEs ranged from 8 to 50 mm (mean = 23.4±12.2 mm), The 16 FFEs ranged from 14 to 225 mm (mean = 58.3±65.7); 14 of these FFEs were 71 mm TL and smaller (mean = 34.8±14.6 mm). Shark 1 had seven embryos, all EEs, 9-21 mm. Shark 2 had nine embryos, with two EEs in the same capsule (14-15 mm) and the remaining seven (22-71 mm) were FFEs. Of these seven, one FFE (71 mm) was larger than the other four FFEs (22-36 mm) present in the same uterus. Shark 3 had six FFEs, with one embryo in each uterus (221 and 225

mm) far larger than their two free-floating siblings (14-28 mm). Shark 4 had nine embryos, of which six were EEs (29-50 mm), including three (28-35 mm that were aborted, as well as three FFEs (36-52 mm) (**Figure 7.4-Figure 7.7**).

The distribution of embryos between uteri (right: left) for the four females were 4:3 (shark 1), 4:5 (shark 2), 3:3 (shark 3) and 2:4 (shark 4; excluding four aborted EEs). All four females contained embryos smaller than 71 mm that were too small to be sexed, except for shark 2) which had 1 male and shark 3 that had 1 female: 1 male. All the FFEs (**Figure 7.2)** and EEs (**Figure 7.6A)** possessed external gill filaments.

Initial visual inspection of the mouths of the FFEs, and some EEs, revealed that the tooth-like structures on both upper and lower jaws were pointed and sturdy (**Figure 7.2**). Both light and electron microscopy confirmed that these structures resembled teeth (**Figure 7.3-Figure 7.7)**. The embryos studied lacked lateral cusplets which is a typical dental feature of *C. taurus* embryos [5,7].

The TH and TW did not show any trend in relation to the embryo length (**Table 7.2**). The average length (TH) of the teeth were E1 (mean = 473.87µM±128.13; range = 198-818; *n* = 29); E2 (mean = 308.9 µM±95.69; range = 175.2-379.8; *n* = 4); E3 (mean = 376.61µM±123; range = 173.7-589; *n* =18); E4 (mean = 346, 53 µM±80.6; range = 241-439; *n* = 5); E5 (mean =224.4 µM±5.34; range = 220.5-228.2; *n* = 2) and E6 (mean = 285.73µM±125.50; range = 108.6-483.6; *n* = 12). E1 had a longer TH mean than its siblings. **Table 7.2** shows the results from the spectral analysis. It confirmed the presence of calcium (Ca), fluorine (F), phosphate (P) and oxygen (O), which are the constituents of the dental mineral fluoroapatite ($Ca_5 (PO_4)_3F$). **Table 7.3** also shows the variation between the Ca (%), P (%), F (%) and O (%) in each tooth as well as between the teeth in each embryo. E1 also had a higher element percentage. Similarly, smaller E2 had higher element percentages compared to larger E3. The concentration of these elements in the embryo enameloid dentition was far lower than compared to the adult teeth.

Table 7.1: The total length (TL), sex (S), status (EE vs. FFE) and location (right or left uterus or aborted) of each embryo found in four pregnant *C. taurus* (shark 1-shark 4).The total length (TL), sex (S), status (EE vs. FFE) and location (right or left uterus or aborted) of each embryo found in four pregnant *C. taurus* (shark 1-shark 4).

No	ID	TL	Weight (kg)	Right Uterus		Left Uterus		Aborted
				EE TL/S	FF TL/S	EE TL/S	FF TL/S	EE TL/S
Shark 1	R. B11005	2640	169	9/ U 12/U 21/U 21/U	-	8/U 19/U 16/U	-	-
Shark 2	R. B11006	2760	161	14/U 15/U	34/U 39/U	-	71/M 22/U 32/U 35/U 36/U	-
Shark 3	R. B11007	2638	130	-	221/F 22/U 28/U	-	225/M 14/U 20/U	-
Shark 4	UMT12004	2562	140	50/U (E1)	46/U (E2)	29/U 39/U	36/U (E4) 52/U (E3)	28/U 35/U (E5) 35/U (E6)
Shark 5	R. B10020	2580	118	NA	NA	NA	NA	NA

Shark 5 and shark 6 represent details for the additional mature *C. taurus* that were used for comparative dental analysis. Abbreviations: EE: encapsulated embryo; FFE: free-floating embryo; ID: KZNSB shark identification; kg: kilogram; U: unidentified sex; M: Male; F: Female; NA: not applicable in this study.

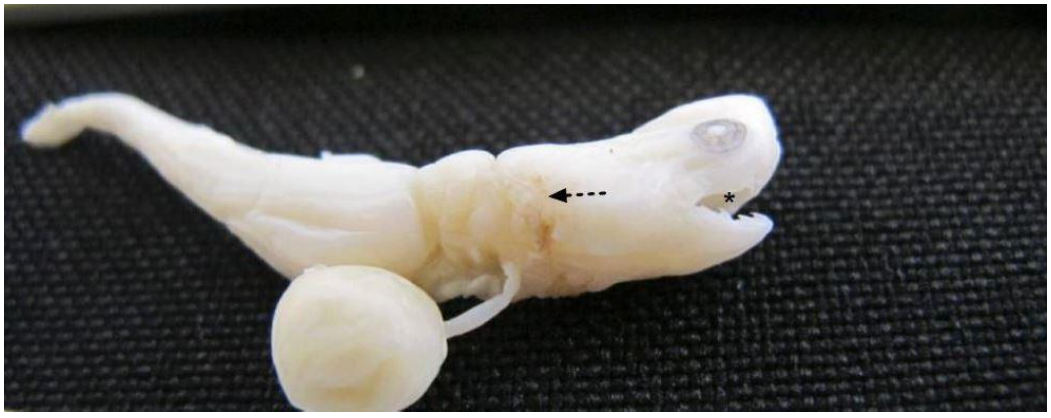


Figure 7.2: Digital image of a 34 mm FFE in shark 2. Asterisks indicate the rudimentary tooth-like structures. Arrow indicates remnants of forming gill filaments

Table 7.2: Median measurements (µm) of the uterine tissue (the epithelium and wall) of *C. taurus* NGF (RS1-RS3) and GF (RS4-RS5D) stages.

ID	TH	TW	ID	TH	TW	ID	TH	TW	ID	TH	TW	ID	TH	TW	ID	TH	TW	ID	TH	TW
E1_pq	507.4	187.4	E1_Mc	L1	597.8	257.6	E3_pq	R1	-	-	E3_Mc	L1	405.8	179.2	E2_pq	R1	190.5	133.9	E2_Mc	R1	379.8	175.2
R2	436.6	251.9		L2	488.3	159.9		R2	212.4	495.3		L2	-	--		L1	327.9	163.2		L1	415	202.2
R3	-	-		L3	818.3	348.4		R3	187.2	279.6		L3	522.9	275.5		L2	231.7	124.3				
R4	471.2	273.9		L4	650.8	224.5		R4	173.7	139		L4	-	-	E4_pq	L1	301.4	161.4	E4_Mc	L1	241	116.7
R5	339.9	297.9		L5	498.5	218		R5	589.6	296.3		L5	553.5	330.9		R1	439.8	152.5		L2	-	-
R6	628.3	339		L6	485.2	232.6		R6	449.2	497		L6	335.9	185.5		R2	410.2	217.2		R1	340.3	213.7
R7	528.9	467.8		L7	-	-		L1	359.3	110.5		R1	-	-	E5_pq	R1	228	62.4	E5_Mc		-	-
L1	-	-		L8	331	297.5		L2	-	-		R2	313.5	122.2		L1	220.5	160			-	-
L2	198.5	66.4		R1	517.6	293.8		L3	356.5	210		R3	-	-	E6_pq	R1	287.3	141.8	E6_Mc	L1	308.4	173.1
L3	516.2	198.3		R2	501.3	216.5		L4	508.5	264.1		R4	-	-		R2	483.6	208.5		L2	367.4	270.5
L4	370.9	272.4		R3	474.3	241.3		L5	275.3	361.8		R5	355.8	157.7		R3	108.6	251.2		L3	238.3	124
L5	381.9	187.1		R4	-	-						R6	486.8	237		R4	218.1	145.7		R1	441.7	369.2
L6	310.1	228.1		R5	413.6	255.7						R7	371.4	256.3		R5	138.5	94.8		R2	179.4	104.1
L7	520.4	288.3										R8	321.7	216.6		L1	446.5	174.1				
L8	539.6	327.2														L2	210.8	354.4				
L9	320	289.1																				
												Avg	E1	473.8±128.13	256.8±75.42	Range	E1	:198-818	n	E1	: 29	
shark 5	17.1	2.8											E2	308.98±95.69	159.77±31.50		E2	:175.2-379.8		E2	:4	
shark 6	37	3.8											E3	376.61±123.15	256.37±111.69		E3	:173.7-589.6		E3	:18	
													E4	346.53±80.57	172,32±42.80		E4	:241-439.8		E4	:5	
													E5	224.4±5.34	111.18±69.05		E5	:220.5-228		E5	:2	
													E6	285.73±125.50	200.93±92.38		E6	:108.6-483.6		E6	:12	

Tooth measurements from shark 5 and shark 6 are presented (mm) at the bottom left corner of the table. Averages of the total TH and TW for each embryo (E1-E6) appears at the bottom right corner of the table. Abbreviations: TH: total height; TW: total width; µM: micrometre; L: left side of the jaw; R: right side of the jaw; E: embryo; Avg: mean average ± SD of TH and TW; Range: range of TH; *n*: teeth count; dash (-) indicates tooth could not be measured

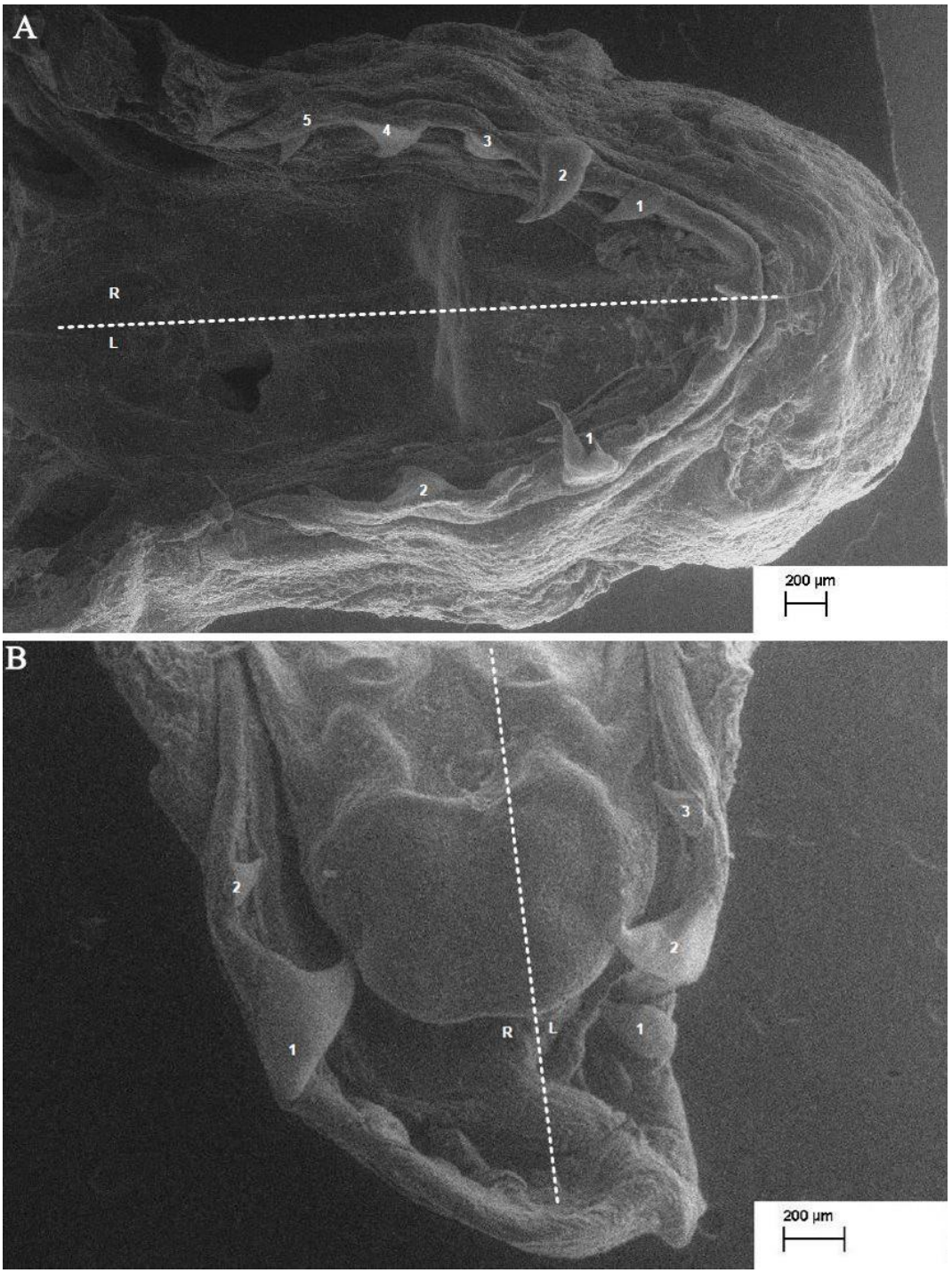


Figure 7.3: a) Stereo image of teeth location on the left and right sides of the A) upper jaw (pq) and B) lower jaw (Mc) of E6 (aborted 35 mm EE) from shark 4.

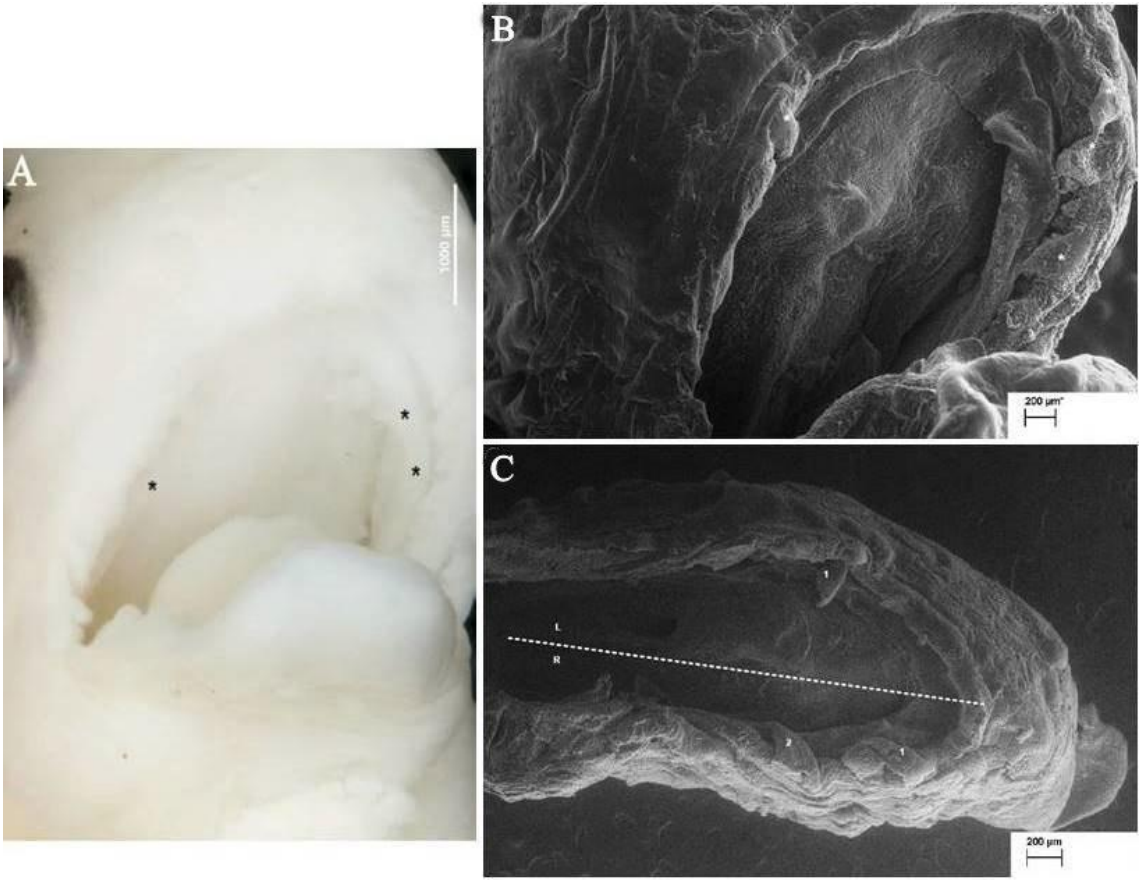


Figure 7.4: Stereo image A) shows the dental like structures (indicated by asterisks) in E4 (36 mm FFE) from shark 4. SEM images B) show clearer dentition (indicated by the asterisks). Teeth location shown on the left and right sides of the upper jaw (pq) SEM image.

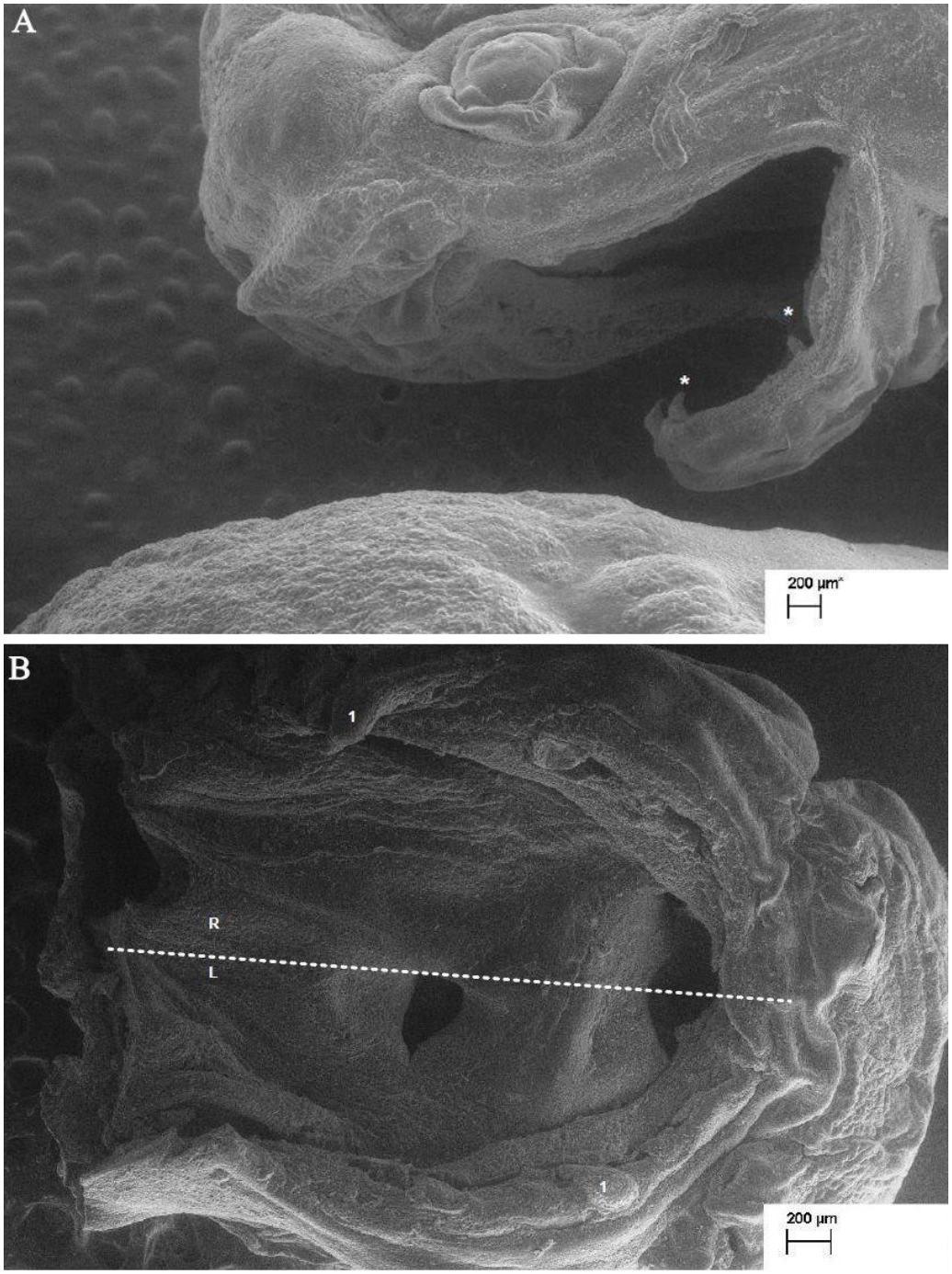


Figure 7.5: SEM image A) of E2 (46 mm FFE) from shark 4. The asterisks indicate the small peg-like structures along the gum line. SEM image B) shows teeth location the left and right sides of the upper jaw (pq). The lower jaws (Mc) did show a few teeth but tissue appeared damaged therefore not shown.

Figure 7.6: Stereo image (A) and SEM images (B and C) are based on the E1 (50 mm TL EE(from shark 4. The asterisks in stereo image A) shows the embryo peg-like tooth structures. The arrow in A) also indicates the presence of gill filaments. SEM images (B-C) shows teeth.

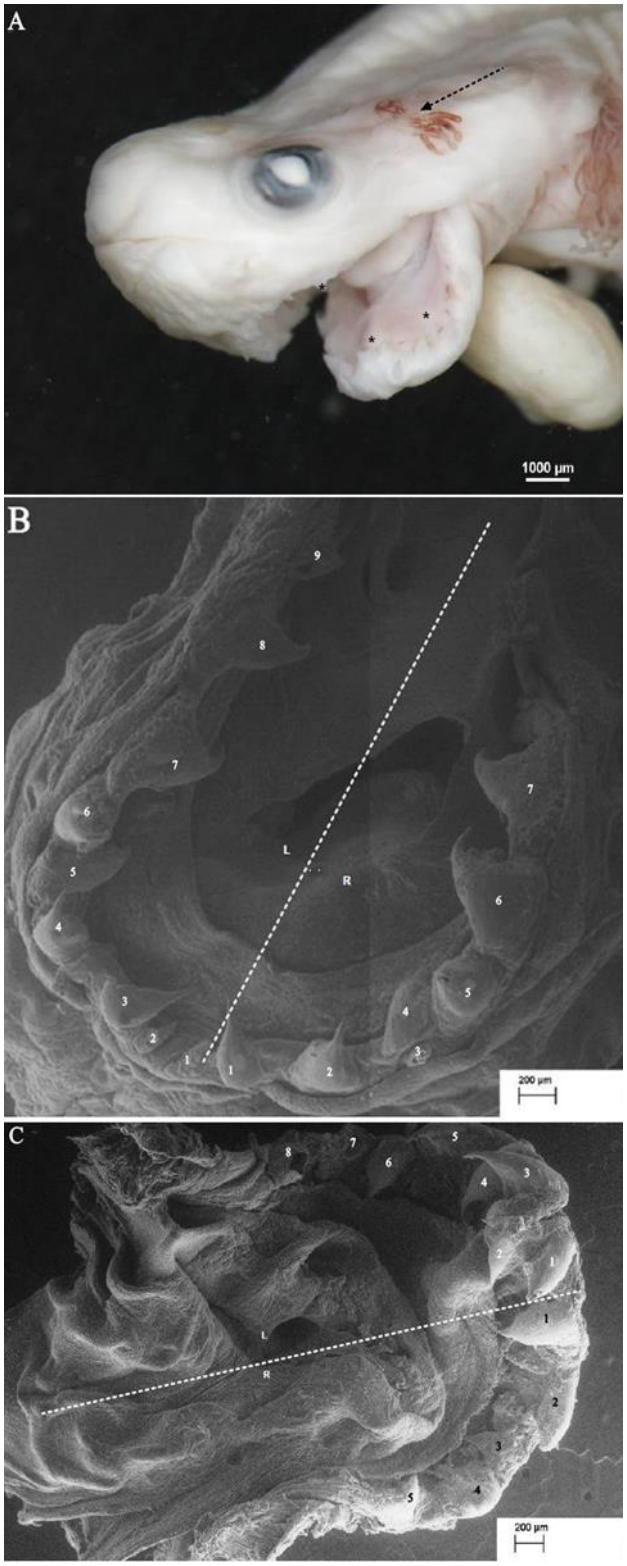


Figure 7.7: SEM images of E3 (52 mm FFE) fromshark 4. Image (B) is an enlargement of the boxed area 1 in Image A. Image C is an enlargement of the boxed area 2 in Image A. Asterisks indicates tooth- like structures. Teeth location on the upper (pq) and lower (Mc)

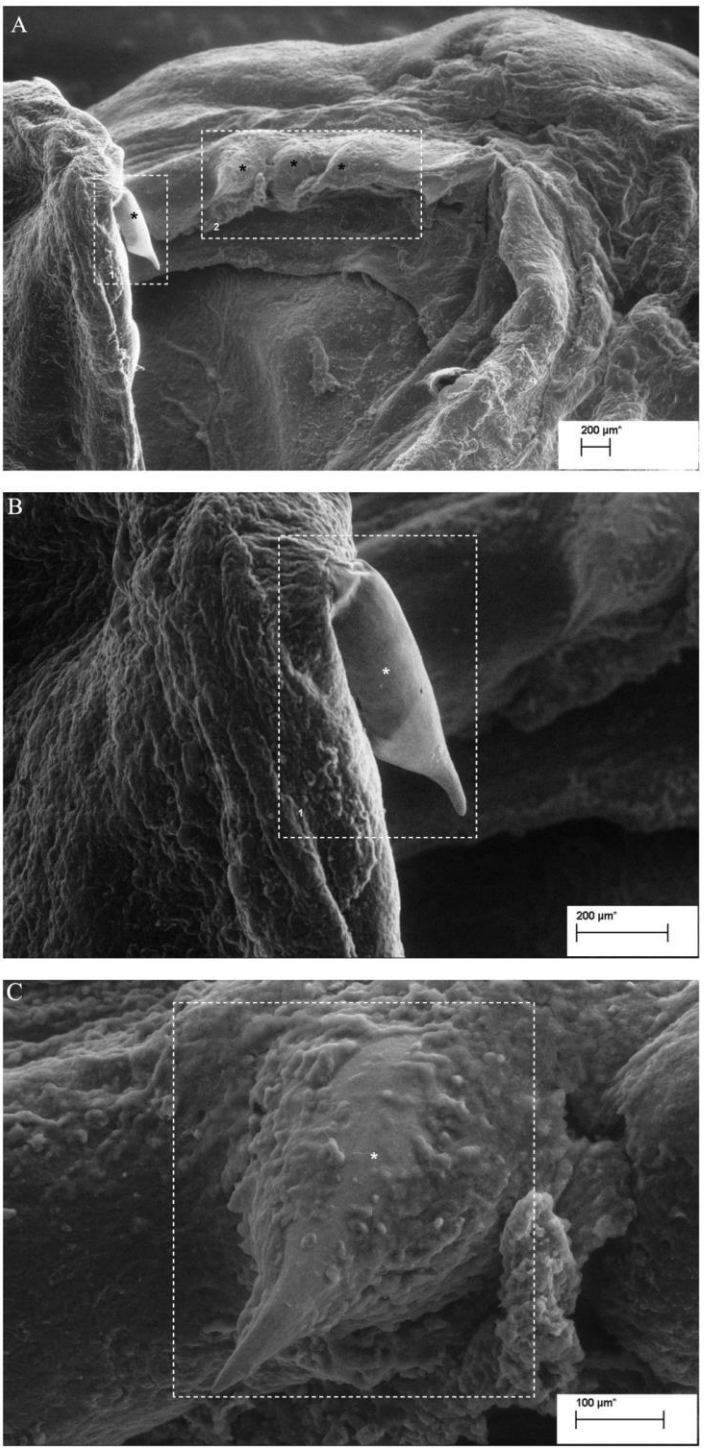


Table 7.3: The Mean, Standard Deviation and Range of the relative percentages of elements calcium (Ca), phosphorus (P), fluorine (F) and oxygen (O) that were analysed on four separate areas of the enameloid layer (SLE layer) of each tooth on the upper and lower jaws of E1-E6

	TL	Calcium			Oxygen			Phosphorus			Fluorine		
		Mean	SD	Range	Mean	SD	Range	Mean	SD	Range	Mean	SD	Range
E1	50	9.38	6.20	0.8-27.13	29.29	6.00	19.39-43.07	4.75	3.28	0.47-13.23	0.88	0.80	0.01-3.6
E2	46	9.04	5.97	0.85-21.73	29.34	6.12	21.66-40.58	4.66	3.06	0.68-9	0.86	0.69	0.04-2.67
E3	52	7.50	5.52	0.22-18.26	27.49	5.10	19.29-41.49	4.07	2.92	0.42-9.89	0.68	0.66	0.02-2.75
E4	36	7.07	4.35	0.49-16.01	25.76	4.09	19.27-37.49	2.77	2.33	0.55-7.55	0.47	0.61	0.05-2.22
E5	35	8.34	3.38	3.15-12.99	28.11	6.41	21.57-38.4	4.06	2.49	0.91-8.32	0.63	0.76	0-1.95
E6	35	9.19	4.91	0.76-19.19	29.13	5.36	19.83-41.48	4.39	2.99	0.38-10.24	0.79	0.86	0.03-3.55
Shark 5	2580	20.60	2.60	15.76-24.48	44.40	1.21	42.6-46.4	12.60	1.60	9.9-14.7	7.10	0.50	6.2-7.9
Shark 6	2674	23.40	3.40	16.7-27.6	28.60	1.60	26.8-29.9	11.23	1.48	9.4-12.9	2.90	0.40	2.6-3.32
Shark 5_EI	-	28.50	1.60	26.3-30.0	38.30	3.20	33.2-41	14.20	1.20	12.6-16.7	0.74	0.79	0.07-2.28
Shark 6_EI	-	21.40	3.25	15.8-25.6	41.80	0.54	41-42	12.70	1.50	11.4-14.5	0.40	0.23	0.2-0.7

The elemental composition for two mature female teeth also measured on the enameloid layer is presented. Additional testing is presented on the inner enameloid and oesteodentine layer of the same mature female teeth. Other elements were simultaneously detected for each tooth to equal a 100% total weight of the tested sample, but these elements were omitted from this table. Abbreviations: ID: embryo ID; TL: total length; E: Embryo; SD: Standard deviation; EI: enameloid layer exposed through a vertical section of the adult teeth

7.5 Discussion

This study documented the size and condition (EE vs. FFE) of 31 early-stage embryos found in four pregnant *C. taurus* sharks (**Table 7.1)**. These details were compared to the first four stages (I-IV) of embryo development as described by Gilmore *et al*. (1983), Gilmore (1993) and Gilmore *et al*. (2005).

All 15 EEs were 8 to 52 mm, which conforms to the documented size range (13-60 mm) of encapsulated embryos (stage I-II). Nine of the 16 FFEs were all smaller than the 60 mm, which is the lower observed limit of the post-hatch (stage III). Although [1,6] has stated that embryo dentition is required for the EE to escape from within the capsule, it is possible that the capsule can also be ruptured by a larger sibling which has entered its cannibalistic phase (stage IV), thereby allowing the encapsulated embryo to escape. It is therefore conceivable that the four FFEs found in shark 3, which include the smallest FFE of the entire study at only 14 mm may have escaped encapsulation because of far larger siblings (221 and 225 mm) attacking the capsules. By contrast, the post-hatched (stage III) 71 mm embryo which was found together with of four smaller FFEs (22-36 mm TL), in shark 2, was too small to be become cannibalistic according to documented literature [1,7]. In this case four embryos must have escaped encapsulation of their own accord, by using dentition. This supposition was substantiated by the presence of tooth-like structures, in several FFEs (34-52 mm TL) which appeared to be sufficiently sturdy and functional to enable these embryos to escape encapsulation.

Literature is scant on *C. taurus* regarding their embryonic dentition. The presence of teeth in EEs of 40-60 mm has been noted [1,5,7]. Hamlett (1983) reported on rudimentary but non-functional dentition in 30-35 mm embryos; advanced embryonic teeth in a 40 mm individual; both upper and lower jaw teeth by 45 mm and double rows of teeth forming at 55 mm. Unfortunately, there was no mention of whether these were EEs or FFEs. Gilmore (1983) documented a 49 mm TL FFE in which “erect, wide, triangular teeth, lacking basal denticles, were clearly visible”. Bass *et al*. (1975) described a 40 mm embryo found in the stomach of a 170 mm sibling as being in excellent condition with external gill filaments and a small external yolk sac. There was no mention of a capsule, but based on its size and the results of the current study it is assumed that this was a FFE

[10]. The presence of external gill filaments in both the FFEs (**Figure 7.2)** and EEs (**Figure 7.6A**) supported previous studies that suggested the use of these gills for embryo respiration and possible absorption of nutrients from the fluid wherein they are submerged [1]. The results of the present study indicate that there appears to be an overlap between the encapsulated and post-hatch stages. This study dictates that the lower size range of the post-hatched (stage III) [1], be reconsidered and reduced from 60 mm TL to at least 36 mm, based on the presence of apparently functional teeth in specimens of this size. Should further detailed examination of the mouths of the smaller FEEs (the 14-32 mm) reported above, show the presence of tooth-like structures that prove to be embryonic teeth; the lower range of stage III may come down even further.

Spectral analysis of the elementary composition of the embryonic teeth structures in this study confirmed the presence of calcium (Ca), phosphate (P), fluorine (F) and oxygen (O) (**Table 7.3**) amongst other elements of the periodic table (not listed in this study) in *C. taurus* dentition These elements are the constituents of the dental tooth mineral, fluoroapatite (Ca_5 $(PO_4)_3$ F) previously documented in a variation of sharks [9,11]. Following Whitenack *et al*. (2012) reporting on the enameloid layer being 200-900 µM and the definition of the outmost enameloid third layer being the shiny layer enameloid (SLE) [12,13], the elemental composition in this study was determined at a maximum depth of 1 µm within the outer enameloid (i.e., SLE layer) of the embryo as well as the enameloid layer, exposed in a sectioned adult *C. taurus* tooth. Apart from protecting the teeth from bacterial attack and mechanical stressors associated with predation through possible lubrication of the teeth [14], the true function of these minerals is still to be fully elucidated.

This pilot study showed wide ranges in the percentage of elements for each embryo (E1-E6). Analysis of the element composition showed no correlation to embryo TW or embryo TH (mean and SD for both). An interesting observation was that the larger pre-hatch E1 (50 mm) still enclosed in its capsule while its smaller pre-hatch sibling in the opposite uterus, E2 (46 mm), was found free-floating. Both these embryos appear to have similarly sturdy and pointed teeth **(Figure 7.5-Figure 7.6**). However, E1 had higher percentages in Ca, P and F **(Table 7.3)** compared to E2 and remaining embryos including E3 (52 mm). In addition, E1 had a higher TH (mean = 473.8µM ±128.13) compared to

the E2 (mean = 308.98 µM ± 95.69 and E3 (mean = 376.61 µM ±123.15) **(Table 7.2)** Additionally E2 (46 mm), which was smaller than the pre-hatched E3 (52 mm), also had higher Ca, O, P and F compared to the larger E3. These results may suggest that the puncturing capability of embryonic teeth could have more to do with the heterogeneous distribution of these minerals [9]than the size of the teeth or the actual percentage of elements used to create the fluoroapatite crystallites that compose the enameloid layer [13]. These embryos are small and still in development therefore it is possible that these teeth could be less mineralised and prone to more fluctuated distribution than teeth in older embryos.

The element percentage of the enameloid layer (i.e., SLE layer) in the adult *C. taurus* females (shark 5 and shark 6) showed higher Ca, O, P and F percentages compared to the SLE layer of the embryo dentition. The chemical composition of shark teeth has been documented [9,11]. One such species was the adult *Isurus oxyrinchus*, also a lamnoid species [15], which showed a lower F percentage (3.08%) but a higher percentage in Ca (37.8%) and P (54.3%) [9,11] compared to our adult *C. taurus* females (**Table 7.3)**. This study also looked at the inside of the teeth through a vertical section of both teeth from shark 5 and shark 6. Moyer *et al*. 2015 showed that the lamnoid tooth has an enameloid layer (made of three sub layers i.e., the outermost shiny layer enameloid (SLE); parallel-fibered enameloid and the tangle-fibered enameloid) and an inner oesteodentine layer (OD). The vertical section gave the opportunity to measure the element composition of the enameloid layer exposed on the top and sides of the sectioned adult tooth. Results showed that the percentages of the elements, except for fluorine, in the enameloid layer were like the SLE layer of the adult teeth **(Table 7.3).** The fluorine percentage was lower in the inner enameloid layer; like the fluorine levels seen in the embryos enameloid layer. The OD layer was also not measured as this layer is made up of hydroxyapatite rather than fluoroapatite which was not the current focus of this study. The comparison of teeth at different maturities of *C. taurus* and other species has not been documented; and is to still be explored. Further investigation will need to determine these elements through other techniques such as additional infrared spectroscopy and X-ray diffraction. A limitation of this study was that the embryonic teeth could not be removed from the jaws and viewed due to their small size and fragility. This meant that a profile of embryonic *C. taurus* tooth, could not be achieved as seen with previous studies on

larger embryos and adult teeth [10,16,17]. However, the tooth measurements TH and TW (**Table 7.2**) presented in this study adds to the embryonic teeth data initiated by Tomita *et al.* (2017). Rows of replacement teeth in the jaw could not be identified with our sample set. We also confirmed the absence of lateral cusplets, in these embryonic teeth, which is a typical dental feature of *C. taurus* embryos [7,18].

7.6 Conclusion

Scarcity of lamnoid embryos suggests that more captures and further investigations may see the alteration of previous documented literature. This study showed that *C. taurus* embryos could hatch as small elsewhere. It is not surprising that there is some variation in the size at which *C. taurus* embryos escape from their capsules. This is made possible by the precocious development of dentition. It could suggest the embryos ability to escape encapsulation as early as possible to begin feeding on the other contents of the uterus, including its siblings. This could suggest these embryos experiencing an increase in growth rate and eliminating most of the *in-utero* competition through early cannibalisation. Changes to the cannibalistic stage of these embryos would affect current studies intending on increasing the fecundity of these Vulnerable labelled species.

7.7 Acknowledgement

We are indebted to the team of the Microscopy and Microanalysis Unit, at the University of KwaZulu-Natal for all their assistance and support. This study was funded by the National Research Fund and by the New South Wales Department of Primary Industries in New South Wales (Australia). All procedures performed in this study involving animals were in accordance with the ethical standards of the University of KwaZulu-Natal and KwaZulu-Natal Sharks Board (076/10/Animal).

7.8 References

1. Gilmore, R.G., Putz, O. and Dodrill, J.W. (2005) In Hamlett WC (ed.), *Reproductive biology and phylogeny of Chondrichthyes: Sharks, Batoids and Chimaeras.* Science Publishers, Enfield, New Hampshire, USA, 435-462.
2. Hamlett, W.C. (1983) *Maternal-fetal relations in elasmobranch fishes*. PhD Thesis, University of Clemson, USA.
3. Springer, S. (1948) *Oviphagous embryos of the sand shark, Carcharias taurus.* Copeia, 153-157.
4. Pollard, D. and Smith, A. (2009) *Carcharias taurus. Version 2012.2: IUCN Red List of Threatened Species.* e.T3854A10132481. http://www.iucnredlist.org. Accessed 23 March 2010.
5. Shimada, K. (2002) *Teeth of embryos in lamniform sharks (Chondrichthyes: Elasmobranchii).* Environmental Biology of Fishes, **63**, 309-319.
6. Gilmore, R.G. (1993) *Reproductive biology of lamnoid sharks.* Environmental Biology of Fishes, **38**, 95-114.
7. Gilmore, R.G., Dodrill, J.W. and Linley, P.A. (1983) *Reproduction and embryonic development of the sand tiger shark, Odontaspis taurus (Rafinesque).* Fishery Bulletin, **81**, 201-225.
8. Cliff, G. and Dudley, S.F.J. (1992) *Protection against shark attack in South Africa, 1952-90.* Marine and Freshwater Research, **43**, 263-272.
9. Enax, J., Prymak, O., Raabe, D. and Epple, M. (2012) *Structure, composition, and mechanical properties of shark teeth.* Journal of Structural Biology, **178**, 290-299.
10. Bass, A.J., Aubrey, J.D. and Kistnasamy, N. (1975). Oceanographic Research Institute, Durban, South Africa, 1-100.
11. Lübke, A., Enax, J., Loza, K., Prymak, O., Gaengler, P., Fabritius, H.O., Raabe, D. and Epple, M. (2015) *Dental lessons from past to present: ultrastructure and composition of teeth from plesiosaurs, dinosaurs, extinct and recent sharks.* Royal Society of Chemistry Advances, **5**, 61612-61622.
12. Gillis, J.A. and Donoghue, P.C. (2007) *The homology and phylogeny of chondrichthyan tooth enameloid.* Journal of Morphology, **268**, 33-49.
13. Moyer, J.K., Riccio, M.L. and Bemis, W.E. (2015) *Development and*

microstructure of tooth histotypes in the blue shark, P rionace glauca (C archarhiniformes: C archarhinidae) and the great white shark, C archarodon carcharias (L amniformes: L amnidae). Journal of Morphology, **276**, 797-817.

14. Gajic, A. (2013) *Spiny dogfish shark secretion openings for fluorapatite, fluoride content in the enameloid of their teeth.* Editor-Chefe, 136.
15. Joung, S.J. and Hsu, H.H. (2005) *Reproduction and embryonic development of the shortfin mako, Isurus oxyrinchus Rafinesque, 1810, in the northwestern Pacific.* Zoological Studies Taipei, **44**, 487.
16. Applegate, S.P. (1965) *Tooth terminology and variation in sharks with special reference to the sand shark Carcharias taurus Rafinesque.* Contributions in Science, Los Angeles County Museum, **86,** 1-18.
17. Shimada, K. (2002) *Dental homologies in lamniform sharks (Chondrichthyes: Elasmobranchii).* Journal of Morphology, **251**, 38-72.
18. Sadowsky, V. (1969) *On the dentition of the sand shark, Odontaspis taurus, from the vicinity of Cananeia, Brazil.* Boletim do Instituto Oceanografico, **18**, 37-44.

BRIDGE

CHAPTER 7 TO CHAPTER 8-10

The female morphometric indices **(CHAPTER 3**), uterine histology (**CHAPTER 4)** and biochemistry of the maternal fluids (**CHAPTER 5**) were examined separately to give an in-depth understanding to how the female adapts to supporting her aplacental embryos. In addition, **CHAPTER 6** and **CHAPTER 7** described new avenues of research that could result in further adaptations for both the female and her embryos. **CHAPTER 8**, joins all the important results from the previous **CHAPTER 3** in a discussion showing the interplay between all aspects that leads to the female supporting her aplacental embryos. The study ends in **CHAPTER 9** which offers a conclusion to this study as well as suggestions/need for future work on aspects still requiring further investigation. **CHAPTER 10** contains all extra information that could not be presented in the relevant chapters.

CHAPTER 8

DISCUSSION

This study serves as the first record of the adaptations made to the uterus and associated reproductive indices in supporting a successful maternal-embryo relationship from NGF (RS1-RS3) to GF (RS4-5D) *C. taurus* sharks.

8.1 *C. taurus* NGF

Migration patterns of the NGF sharks (RS1-RS3) indicated large numbers getting caught in spring (September-November) (**Table 3.4**). Females in the RS2A group could not be assessed. The mating females (RS3) however were captured between October-December, an observation that has been recorded before [1, G Cliff, Natal Sharks Board, unpublished data].

Length and weights of the female in the NGF sharks (RS1-RS3) generally increased as the female matured (**Table 3.2**). The mature and sexually active females (RS3) represented the last NGF stage where mating occurred. The liver and ovary mass increased in size as indicated by the increase in HSI% and GSI% throughout the NGF stages. The heaviest liver weight was recorded in the mature, mating females (RS3) females. The slight increase in FSH **(Figure 5.1B**) suggested action on the ovary to mature follicles that will result in ovulation. Oestradiol and progesterone showed its highest levels in RS3 females; with progesterone (**Figure 5.1C**) being higher than oestradiol (**Figure 5.1D**). This suggests that progesterone regulates oestradiol functions i.e. promoting vitellogenesis, ovulation and capsule production [2]. High progesterone is also known to occur in the peri-ovulatory phase (i.e., the phase between ovarian stimulation and ovulation). This phase would likely include the RS3 females as ovulation has already occurred in RS4 females (evident by the released capsules). *Cacharias taurus* females displays a punctuated reproductive cycle (one-year pregnancy, one-year rest) [2] as well as a superimposed ovulatory cycle in the early-stage of pregnancy [2]. The consistent baseline levels of LH **(Figure 5.1A**) throughout all the stages could indicate this superimposed ovulation in their gravid stages but requires clarification. Therefore, ovulation occurring in RS3; prior to the first stage of pregnancy would corroborate with the literature. Previous studies on NGF *C. taurus*

sharks have shown that oestradiol is usually higher than progesterone [3,4]. Closer examination revealed that some individual NGF sharks showed higher oestradiol than progesterone. It was also noticed that these females also had a GSI of 1%, which could be the reason for the profile of oestradiol being lower in concentration than progesterone in this study. The GSI concentration in the studies in question could not be assessed to due to them being live sharks.

The epithelial tissue in NGF sharks (RS1-RS3) (**Figure 4.3-Figure 4.6)** transformed into increased protruding UL with an increase in BV in RS3 while the thickening of the UW wall **(Table 4.1**) increased through all NGF stages. The thickening of the wall could be an adaptation to handle large volumes of capsules of varying weight that will be entering the uterus after ovulation i.e. (in RS4 females).

8.2 *C. taurus* GF:

Gravid females with capsules (RS4) were captured between November-December while females with embryos (RS5) were captured between February-July (**Table 3.4**). This observation has been recorded [1] and creates a predictive pattern of migration of *C. taurus* along KZN shores.

Length and weight appeared to increase overall with fluctuations (**Table 3.2**). The gravid stages showed a liver mass decrease from RS4-RS5D as indicated by the HSI%. The heaviest ovary mass was found in RS5A females and thereafter decreased as indicated by the GSI%. This suggests that the liver is spent providing precursors needed for yolk supply and ovary begins to lose ova from the RS5A stage, that become encapsulated. Capsule count increases from pre-hatched stage (RS4) (103/106 capsules per left and right uterus respectively) (**Table 3.6**) to intracannibalistic stage (RS5C) (197/177 capsules per left and right uterus respectively), but these capsules are smaller and light weight. The highest capsule count, at RS5C, also corresponds to a low GSI (**Figure 3.1B**). The capsule count decreased in in RS5D (50/48 capsules per left and right uterus respectively) (**Table 3.6**) females with larger, heavier capsules. The presence of capsules serves as an indication to the rate of follicle ovulation and/or

capsule formation. It indicates that follicle release/capsule formation is initially slow and reaches its maximum at the cannibalistic phase of the female (RS5C) as both the GSI% (**Figure 3.1B**) and capsule count decreased (**Figure 3.2)** in the next RS5D stage. This suggests that a high embryonic nutritional demand (i.e., unfertilised ova and intrauterine cannibalism) is being met in the cannibalistic phase of the RS5C females. The rate of ovulation increase, in the gravid stages, RS5C as well as in the RS54, is controlled by hormones. The RS4 females showed a decrease in FSH (**Figure 5.1B**) from RS3 concentrations but an increase in both oestradiol (**Figure 5.1D**) and progesterone **(Figure 5.1C**); were progesterone concentration was higher than oestradiol causing regulation of oestradiol function. Ovulation here will allow for further eggs to develop in capsules, at the RS5A, also indicating embryos developing at different rates. In the RS5C females, again FSH (**Figure 5.1B**) was lower but oestradiol (**Figure 5.1D**) was higher in concentration than progesterone (**Figure 5.1C**) allowing for the slower release of unfertilised eggs to the surviving embryo in RS5D. The increased oestradiol in RS5A and RS5C females **(Figure 5.1D**) allowed for increased rate of ova encapsulation, corroborated by the highest capsule count found in RS5C females. The females in RS5D, had the lowest oestradiol and highest progesterone which inhibits uterine contraction and modulates the frequency of uterine contraction allowing for some uterine flushing to occur without initiating early parturition [5]. A decrease in progesterone would activate myometrial activity that allows for the contraction of the wall close to parturition, needed during the birthing process. It will also assist in the increase in UF, resulting from the periodical flushing of the lumen with seawater [6] only observed during pregnancy [7,8]. An illustration (**Figure 8.1**) summarising the trends for the hormones described in this study has been included at the end of this section.

Uterine tissue of GF (RS4-RS5D) showed an 1) increase in epithelial folds (UL) projecting into the lumen creating an increased surface area [4,9,10], 2) increased by cable network of MR (containing BVs) lining the accordion fashion structure of the UL, 3) close proximity of BV's near the UF (**Figure 4.7-Figure 4.10**) and 4) a decrease in UW thickness (**Table 4.1**) as embryos developed (RS5A-RS5D). All these characteristics of the tissue supported the exchange of respiration and osmoregulation in the developing aplacental embryos [11-13]. The

thickness of the wall at RS4 (**Table 4.1**) could indicate the presence of capsules and absence of embryos prevents the thinning of the wall. The MR's (**Figure 4.12**) have been shown to effectively increase the oxygen supply of the uterus surface to around 56 times higher than if the uterus had a smooth surface [14]. This is the first evidence of MR structures on the uterine surface of *C. taurus*, which was inferred by Gilmore *et al.* (1993) due to its presence in a similar *I. oxyrinchus*. Hamlett and Hysell (1998) also described the presence of blood vessels in the UL of a GF. The tissue description in our study, confirms and extends Hamlett and Hysell (1998) findings of BV formation by suggesting the authors were describing a female in early- to mid-pregnancy due to the images in this study depicting blood vessels in the middle of the UL as blood vessels in late pregnant females are distributed along the periphery of the UL. The increase in vascularisation in the uterine tissue from mating females (S3) to late stage pregnant females (RS5D) indicated the important role plasma played in the embryo's reproductive development. The similarity in composition of the biochemical analytes in plasma, UF and ICF presented here is to our knowledge the only data that exists for wild *C. taurus* females of different reproductive stages. The presence of similar composition of analytes in different fluids suggests diffusion is occurring allowing for osmoregulation. Organic material concentrations were low and did not appear to change across the different embryo growth stages suggesting embryos may not be in position to take these nutrients up [15-17]. If there is a nutrition support, it would be minimal at best, in comparison to the nutrient strategy of intracannibalism and oophagy [8,18,19]. This does need further investigation.

The smallest FEE encountered in this study was only 14 mm (**Table 7.1**) [20]. This is far smaller than previously reported [18,19] which could suggest an adaptive characteristic to survive cannibalistic attack or this study could be simply be reporting on a higher sample count than previously experienced in other reported studies. Also this study showed that these *C. taurus* embryos were developing in fluid mediums (i.e., ICF and UF) or affected by maternal plasma heavily contaminated with metals (**Table 6.2**) [21]. This is of concern as it could affect their normal development although no physical deformities were observed in the embryos examined. However, such a restriction could also limit nutrients linked to harmful chemicals.

This study showed through morphological, histological and biochemical analysis that *C. taurus* female is well adapted to provide the nurturing environment to develop her embryos to full term predators; with the possibility that the embryos are creating adaptive ways to survive. All these characteristics serve as critical *in utero* components to model *in vitro* when attempting to increase these species numbers through breeding programmes [22,23]. In addition, a concerning factor that this study highlighted is the presence of heavy metals which the females appear to be offloading onto her progeny. This could have devastating consequences over time for an already vulnerable species.

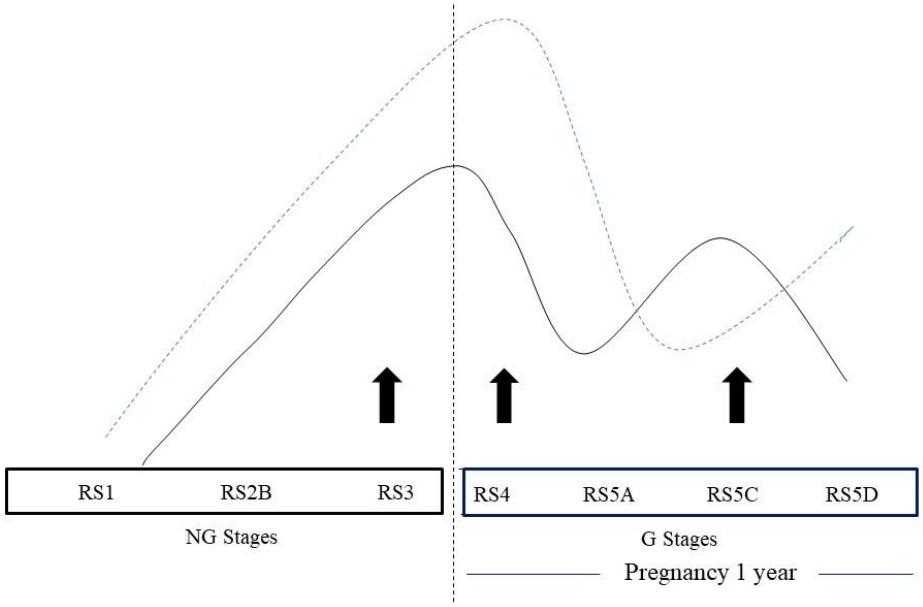


Figure 8.1: Illustration of hormone trends for progesterone (dashed blue curve) and oestradiol (solid curve) with three possible ovulatory periods where rate of ovulation increases (indicated by arrows) through the non-gravid (NGF) and gravid (GF) stages. Image adapted from reproductive cycle illustration [24]

8.3 References

1. Dicken, M.L., Booth, A.J. and Smale, M.J. (2008) *Estimates of juvenile and adult raggedtooth shark (Carcharias taurus) abundance along the east coast of South Africa.* Canadian Journal of Fisheries and Aquatic Sciences, **65**, 621-632.
2. Koob, T.J. and Callard, I. (1991) *Reproduction in female elasmobranchs.* Comparative Physiology, **4**, 155-200.
3. Henningsen, A., Murru, F., Rasmussen, L., Whitaker, B. and Violetta, G. (2008) *Serum levels of reproductive steroid hormones in captive sand tiger sharks, Carcharias taurus (Rafinesque), and comments on their relation to sexual conflicts.* Fish Physiology and Biochemistry, **34**, 437-446.
4. Rasmussen, L.E. and Gruber, S.H. (1993) *Serum concentrations of reproductively-related circulating steroid hormones in the free-ranging lemon shark, Negaprion brevirostris.* Environmental Biology of Fishes, **38**, 167-174.
5. Sorbera, L. and Callard, I. (1995) *Myometrium of the spiny dogfish Squalus acanthias: peptide and steroid regulation.* American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, **269**, R389-R397.
6. Callard, I., George, J.S., Koob, T. and Hamlett, W. (2005) In BGM, J. (ed.), *Reproductive biology and phylogeny of chondrichthyes: sharks, batoids and chimeras*, 3, 283-300.
7. Ellis, M.T. and Otway, N.M. (2011) *Uterine fluid composition of the dwarf ornate wobbegong shark (Orectolobus ornatus) during gestation.* Marine and Freshwater Research, **62**, 576-582.
8. Gilmore, R.G., Putz, O. and Dodrill, J.W. (2005) In Hamlett WC (ed.), *Reproductive biology and phylogeny of Chondrichthyes: Sharks, Batoids and Chimaeras.* Science Publishers, Enfield, New Hampshire, USA, 435-462.
9. Jollie, W.P. and Jollie, L.G. (1967) *Electron microscopic observations on accommodations to pregnancy in the uterus of the spiny dogfish, Squalus acanthias.* Journal of Ultrastructure Research, **20**, 161-178.
10. Storrie, M.T., Walker, T.I., Laurenson, L.J. and Hamlett, W.C. (2009) *Gestational morphogenesis of the uterine epithelium of the gummy shark (Mustelus antarcticus).* Journal of Morphology, **270**, 319-336.
11. Hamlett, W.C. (1983) *Maternal-fetal relations in elasmobranch fishes*. PhD

Thesis, University of Clemson, USA.

12. Hamlett, W.C. and Hysell, M.K. (1998) *Uterine specializations in elasmobranchs.* Journal of Experimental Zoology, **282**, 438-459.

13. Hamlett, W.C., Kormanik, G., Storrie, M., Stevens, B. and Walker, T.I. (2005) *Chondrichthyan parity, lecithotrophy and matrotrophy.* Reproductive Biology and Phylogeny of Chondrichthyes: sharks, batoids and chimaeras, **3**, 394-434.

14. Tomita, T., Nozu, R., Nakamura, M., Matsuzaki, S., Miyamoto, K. and Sato, K. (2017) *Live-bearing without placenta: Physical estimation indicates the high oxygen-supplying ability of white shark uterus to the embryo.* Scientific reports, **7**, 11744.

15. Hamlett, W.C. (1989) *Evolution and morphogenesis of the placenta in sharks.* Journal of Experimental Zoology Part A: Ecological Genetics and Physiology, **252**, 35-52.

16. Lombardi, J., Jones, K.B., Garrity, C.A. and Files, T. (1993) *Chemical composition of uterine fluid in four species of viviparous sharks (Squalus acanthias, Carcharhinus plumbeus, Mustelus canis and Rhizoprionodon terraenovae).* Comparative Biochemistry and Physiology Part A: Physiology, **105**, 91-102.

17. Wourms, J.P. (1977) *Reproduction and development in chondrichthyan fishes.* American Zoologist, **17**, 379-410.

18. Gilmore, R.G. (1993) *Reproductive biology of lamnoid sharks.* Environmental Biology of Fishes, **38**, 95-114.

19. Gilmore, R.G., Dodrill, J.W. and Linley, P.A. (1983) *Reproduction and embryonic development of the sand tiger shark, Odontaspis taurus (Rafinesque).* Fishery Bulletin, **81**, 201-225.

20. Naidoo, K., Chuturgoon, A.A., Cliff, G., Ellis, M.T., Otway, N.M., Gregory, M.A., Singh, S.D. and Naidu, S.L. (2017) *Dentition facilitates the release of encapsulated Ragged-tooth Shark (Carcharias taurus) embryos.* Environmental Biology of Fishes, **100**, 1343-1354.

21. Naidoo, K., Chuturgoon, A., Cliff, G., Singh, S., Ellis, M., Otway, N., Vosloo, A. and Gregory, M. (2017) *Possible maternal offloading of metals in the plasma, uterine and capsule fluid of pregnant ragged-tooth sharks (Carcharias taurus)*

on the east coast of South Africa. Environmental Science and Pollution Research, **24**, 16798-16805.

22. Otway, N.M. (2015) *Serum biochemical reference intervals for free-living Sand Tiger sharks (Carcharias taurus) from east Australian waters.* Veterinary Clinical Pathology, **44**, 262-274.

23. Venton, D. (2011) *Baby Sharks Birthed in Artificial Uterus.* Wired. http://www.wired.com/2011/09/artificial-shark-uterus. Accessed 23 June 2014.

24. Koob, T.J. and Callard, I.P. (1999) *Reproductive endocrinology of female elasmobranchs: lessons from the little skate (Raja erinacea) and spiny dogfish (Squalus acanthias).* Journal of Experimental Zoology, **284**, 557-574.

CHAPTER 9

CONCLUSION AND PROSPECTIVE FUTURE WORK

The aim of this study was to document *C. taurus* female reproductive strategy. The reason for documentation was to increase the knowledge across the different reproductive stages in *C. taurus* females and serve as vital *in utero* components to model in prospective *in vitro* breeding programmes aimed to increase their numbers by increasing their fecundity.

Uterine epithelial and uterine wall transformation supported respiration and osmoregulation in the developing aplacental embryos through the, increased vascularisation on UL, close proximity of BV's to UF, increased surface area and decrease in UW in females with embryos. No secretory structures were located in the uterus indicating that the uterus plays no role in embryo nourishment. Organic compounds were detected in the composition of the UF, where embryos develop, however this needs further investigation as it could also be sourced from the embryos themselves. Even if nutrition was secreted, it would be minimal compared to the intrauterine cannibalism and oophagy nutrient strategy that this species utilises.

The requirement of yolk and its importance to the nutritive development of these embryos is vital. This indicated the vital relationship between the liver and the ovary in providing the yolk precursor (vitellogenin) that are sequestered by the growing follicles within the ovary. The female is regarded as a punctuated breeder due to the year of rest between pregnancies after maturity. Assessment of the reproductive hormones in the maternal fluids may have confirmed that ovulation does occur in the early pregnant females as stated in previous literature. However, this study also showed that ovulation could also be occurring in females that are in the middle stage of their pregnancy i.e., females pregnant with cannibalistic embryos, which was indicated by the highest count of capsules in this stage. This suggests continuous ova support to the embryos from convincement within the capsules to their free-swimming gestation in the uterus.

Apart from the adaptations both in the uterus and associated reproductive indices to main a successful aplacental pregnancy, this study made two further discoveries. The first being *C. taurus* embryos are escaping from their capsules at a smaller size than

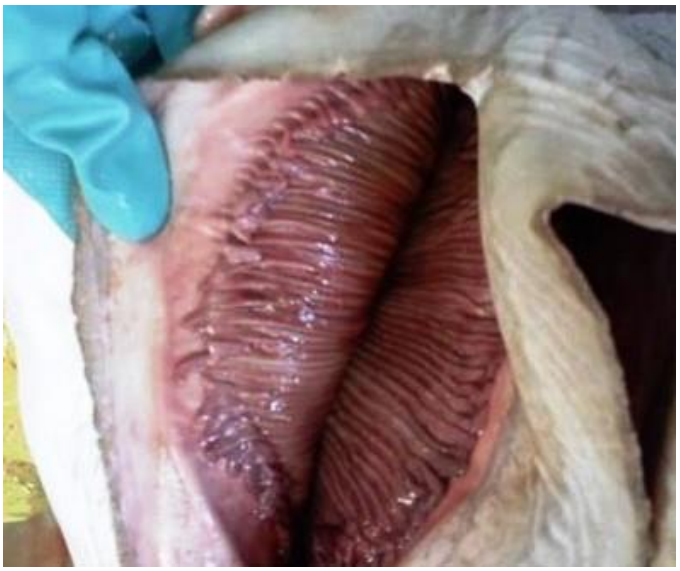
previously documented. This could provide a better opportunity to defend themselves during intrauterine cannibalism by not being contained in a capsule during the attack. The second is the discovery that high levels of heavy metals were detected in the maternal fluid (i.e. plasma, ICF and UF). The extent to which these heavy metals might be detrimental to embryo development and the ability of them to reproduce once it reaches its own maturity remains unknown. It is concerning as *C. taurus* spends its entire life in shallow coastal waters. Much of the pollutants which are derived from land-based anthropogenic activities are discharged into these waters. This study showed the intricate paths both the uterus and associated reproductive indices adapt to provide a sustained maternal-embryonic relationship. Further adaptations may be called upon with regards to the embryos early release and heavy metal exposure. In addition, pregnant *C. taurus* females, in early-middle stages of pregnancy, would be more susceptible to capture-induced abortion. The possibility of abortion in species that reproduce every two years does require further investigation especially from a conservation point of view.

This study created many future research avenues. The use of TEM to investigate the cellular structure of the UL, blood vessels and walls of the uterine tissue functionality. in healthy *C. taurus* females. In addition, it would be important to investigate the effects of testosterone, an important steroid derived from the ovary during reproductive cycles in female elasmobranchs. The RS2A and RS5B that could not be assessed. In addition, assessments into the capture-induced abortions needs to be investigated especially in the early stages of live R5A-RS5C females.

The results from this study may prove useful to the NSW DPI for the development of an AU for the propagation of *C. taurus* embryos. The “Vulnerable” status of *C. taurus* in SA caused by overexploitation, late maturity and low fecundity suggests that this species could benefit from an AU breeding intervention to increase their numbers should our species reach the Endangered status. However, this study also confirms the complexity of the maternal-embryonic relationship and all facets in this study that needs to be considered when trying to mimic the female’s physiological environment to increase the number of these species (via the AU breeding technology)

CHAPTER 10
APPENDIX

APPENDIX A: Redness of the gill structures indicates freshness of the shark



APPENDIX B: Depth of the recession of both eyes indicates freshness of the shark

APPENDIX C: Dissection Form

KZN SHARKS BOARD DISSECTION FORM

SPECIES: Sex:
Dissec. date: TAG No.
Length: **Weight:**
Field: mm Total: kg
Standard: mm Both livers: kg
Fork: mm Heart: g
Total: mm Gut content: g
Stretched: mm
Upper caudal TAGGINGF INFO:
(only if intact): mm Shark condition (1-4): Liver condition (1-4):
Jaw width: mm
Girth: mm Maturity (1-3): Activity (1-3): Juveniles: umbilical slit stage (1-5):

ID No. | | | | | | | | |

Date caught: _____
Place: _____
Net no. _____
Dissector's name: _____

MALES:
Sexual stage (1-6)
Clasper length mm
Pelvic fin length: mm
Testes Weight: g
Clasper condition (1-4):
Siphon sac (1-4):
Epididymes (1-4):
Seminal vesicles (1-4):
Testes length: mm
Testes width: mm

FEMALES:
Virgin (1=yes; 2=no):
Sexual stage (1-6):
No. of mature eggs:
Ovary Weight: R g L _gT g
Range of mature eggs: to mm
R to mm L to mm
Uterus condition (1-6):
Uterus shape (1-4):
Uterus length: mm
Uterus width: mm

REMARKS:

SAMPLES:	Collect	Done	FOR
NSB vert	☐	☐	
NSB finclip	☐	☐	
PR jaw	☐	☐	
PR teeth	☐	☐	
	☐	☐	_____
	☐	☐	_____
_____	☐	☐	_____
_____	☐	☐	_____
_____	☐	☐	_____
_____	☐	☐	_____
_____	☐	☐	_____
_____	☐	☐	_____
Photos	☐	☐	

Gut item	No.	Cap	Con.	St.	Length (mm)	Weight (g)	Kept	Remarks

REMARKS:

Path | Pup

Right uterus:

Sex Length (mm) Caudal (mm) Mass (g) Liver (g)

1._____
2._____
3._____
4._____
5._____
6._____
7._____
8._____
9._____
10._____
11._____

Include details of aborted pups in the above rows.

Number of capsules: R_____L_____

Number of placental scars: R:_____L_____

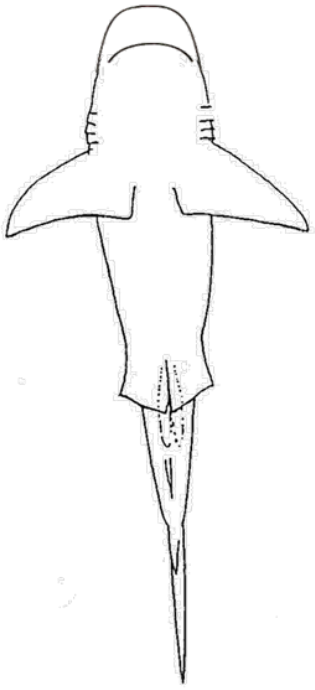
Left uterus:

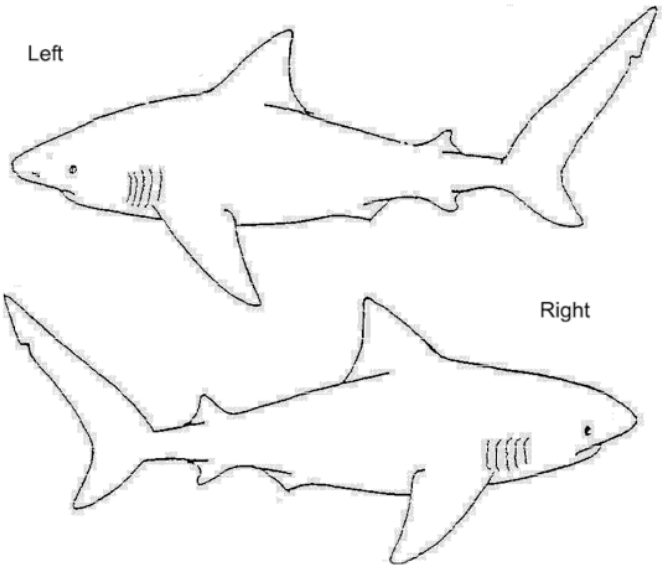
Sex Length (mm) Caudal (mm) Mass (g) Liver (g)

1._____
2._____
3._____
4._____
5._____
6._____
7._____
8._____
9._____
10._____
11._____

Pups: Teeth present: _____

Golden membrane/uterine eggs: R_____ L_____





APPENDIX D: Capsule Form

Dissection Form – Ragged-tooth

Date of Dissection: _____ ID No.: _____

Right Uterus								
Capsule No.	Total Length (mm)	Length of Head (mm)	Width (where head joins tail) (mm)	Max Width of tail (mm)	Weight (g)	Method of preservation	No. of eggs	Comments

Left Uterus								
Capsule No.	Total Length (mm)	Length of Head (mm)	Width (where head joins tail) (mm)	Max Width of tail (mm)	Weight (g)	Method of preservation	No. of eggs	Comments

Diameters of 3 largest eggs (follicles) in ovary

Other samples taken: (tick if samples where taken)

	Right	Left
Pieces of uterus		
Oviducal Gland		

NATAL ANTI-SHARK MEASURES BOARD

KEY SCIENTIFIC DISSECTION FORM

SHARK CONDITION

1. Fresh
2. Dehydrated
3. Decomposing
4. Rotten

LIVER CONDITION

1. Healthy
2. Unhealthy
3. Scarred
4. Piece missing

MATURITY

1. Juvenile
2. Adolescent
3. Mature

JUVENILES (Umbilicus)

1. Open
2. Muscle Closing
3. Skin Closing
4. Faint Scar
5. Not Visible

SEXUAL STAGES

	MALES	FEMALES
1.	Testes Undeveloped	Ovary
2.	Testes Developing	Ovary
3.	Mature Testes	Eggs in Ovary
4.	Sperm Sacs Full	Mating
5.	Mating	Pregnant
6.	Testes Regressed	Pupped

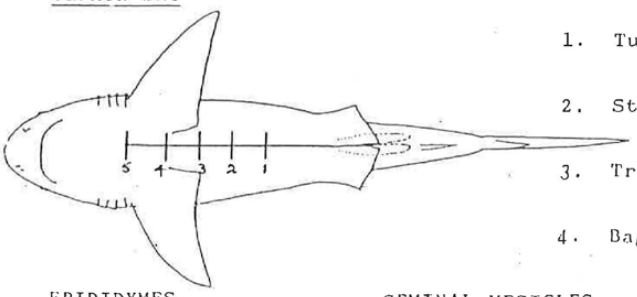
CLASPER CONDITION

1. Soft/Elongated
2. Stiff
3. Stiff, sperm present
4. Stiff, bleeding

EXPRESSION OF LARGEST EGGS

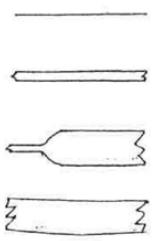
1. Not easy
2. Fairly easy
3. Dropping out

SIPHON SAC



SHAPE

1. Tubular
2. Straplike
3. Trumpet
4. Baglike



EPIDIDYMES

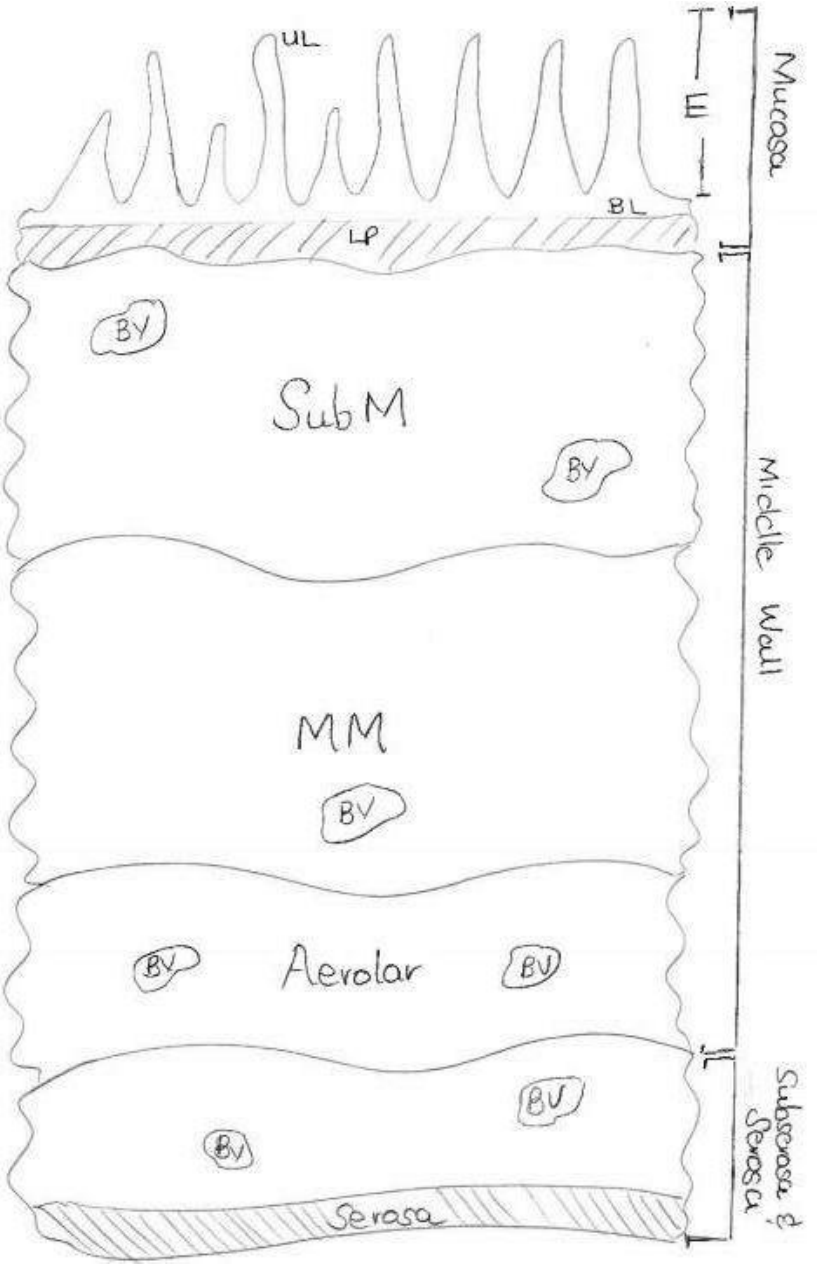
1. Flat
2. Slightly swollen
3. Fairly swollen
4. Bulging

SEMINAL VESICLES

1. Flat, empty
2. Small amount of fluid
3. Full of sperm
4. Empty, Flaccid, bloody

UTERUS CONDITION

1. On dorsal wall
2. Thin walled
3. Thick walled
4. Spongy
5. Well vascularised
6. Placental scars



A hand drawn diagram of the epithelium and the layers of the wall of the uterus. This is a schematic representation of *C. taurus* GF uterus. The epithelium and wall changes throughout the reproductive stages of the females as discussed in CHAPTER 4. Abbreviations: BV: Blood vessel E: Epithelium, LP: Lamina propria, UL: uterine lamellae.

APPENDIX F: Description of sexual staging of *C. taurus* NGF (RS1-RS3) and GF (RS4-RS5D). Data originated from the KNZSB database

SS	Definition	Description
RS1	NGF (Immature, inactive females)	These females contain ovary still developing. They will be captured in January, March and October. Morphometric measurements for this female are: • PCL_x (n = 166):1687±8.67 mm (PCL_{xd}: 1692±158 mm; R: 1332-2050 mm) • TL_x (n = 160): 2221±10.8 mm (TL_{xd}: 2229±178 mm; R: 1754-2647 mm) • $Weight_x$ (n = 166): 85.27±1.53 kg ($Weight_{xd}$:82.5±26 kg; R: 41-158 kg) • HSI_x (n = 159): 8.87±0.21 % (HSI_{xd}: 8.79±3.4%; R: 2.63-16.3 %) • GSI_x (n = 119): 0.143±0.02 % (GSI_{xd}: 0.11±0.03 %; R: 0.03-1.22 %) • UW_x (n = 155): 34.7±1.18 mm (UW_{xd}: 33±14 mm; R: 3-112 mm)
RS2	NGF (Mature, nonvirgin/virgin and inactive females)	This female contains developed ovary, but female is either not a virgin/virgin. Captured in March, May-July, October-November. Morphometric measurements are: • PCL_x (n = 486): 1932±5.07 mm (PCL_{xd}:1935±154 mm; R:1661-2290 mm) • TL_x (n = 466): 2509±6.05 mm (TL_{xd}: 2516±174 mm; R: 2180-2906 mm) • $Weight_x$ (n = 483): 136.3±.29 kg ($Weight_{xd}$: 138±40 kg; R:50-240 kg) • HSI_x (n = 440): 11.48±0.17 % (HSI_{xd}: 11.620±3.45 %; R: 1.54-58.13 %) • GSI_x (n = 457): 0.84±0.04% (GSI_{xd}: 0.77±1.05 %; R: 0.06-5.95 %) • UW_x (n = 461): 73.86±1.87 mm (UW_{xd}: 68±38 mm; R: 10-550)
RS2A	NGF (Mature, virgin and inactive females)	This female contains a developed ovary but has not mated. They will be captured in March-July, October November. Morphometric measurements for this female are: • PCL_x (n = 9): 1847±24.45 mm (PCL_{xd}: 1861±145 mm; R:1750-1930 mm) • TL_x (n = 8): 2410±32.7 mm (TL_{xd}: 2408±191 mm; R: 2290-2532 mm) • $Weight_x$ (n = 9):112±4.63 kg ($Weight_{xd}$: 106±24 kg; R:94-134 kg) • HSI_x (n = 8):10.22±0.86 % (HSI_{xd}: 9.85±4.58 %; R: 6.82-13.63 %) • GSI_x (n = 8): 0.13±0.01 % (GSI_{xd}: 0.13±0.05 %; R: 0.1-0.19 %) • UW_x (n = 9): 107.9±55.39 mm (UW_{xd}: 58±25.5 mm; R: 36-550 mm)

RS2B	NGF**:** Mature, non-virgin but inactive females	August. Morphometrics are: • PCL_x (*n* = 477): 1933±5.12 mm (PCL_{xd}: 1940±155 mm; R: 1661-2290 mm) • TL_x (*n* = 458): 2511±6.09 mm (TL_{xd}: 2516±176 mm; R: 2180-2906 mm) • $Weight_x$ (*n* = 474): 136.8±1.30 kg ($Weight_{xd}$: 138±40 kg; R: 50-240 kg) • HSI_x (*n* = 432): 11.50 ±0.17 % (HSI_{xd}: 11.64 ±3.45 %; R: 1.54-58.13 %) • GSI_x (*n* = 449): 0.86±0.04% (GSI_{xd}: 0.77±1.06%; R: 0.06-5.95 %) • UW_x (*n* = 452): 73.18±1.58 mm (UW_{xd}: 68 ± 37.7 mm; R:10-550 mm)
RS3	NGF**:** Mature, non-virgin and sexually active females	This female contains a developed ovary and is mating with fresh lesions. They will be captured in October-December. Morphometric measurements for this female are: • PCL_x (*n* = 25): 2004±16.6 mm (PCL_{xd}: 2020±112 mm; R: 1840-2170 mm) • TL_x (*n* = 25): 2596±19.57 mm (TL_{xd}: 2629±165 mm; R: 2418-2757 mm) • $Weight_x$ (*n* = 25): 153.7±3.07 kg ($Weight_{xd}$: 160±28.5 kg; R: 125-176 kg) • HSI_x (*n* = 25): 11.84±0.24% (HSI_{xd}: 12 ±1.84 %; R: 9.71-15.09 %) • GSI_x (*n* = 24): 1.98±0.11% (GSI_{xd}: 2.08 ±0.54 %; R: 0.55-2.65 %) • UW_x (*n* = 361): 96.81±1.34 mm (UW_{xd}: 99±38mm; R: 33-185 mm)
RS4	GF (with capsules only)	This female is pregnant with capsules only. They will be captured in December-January. Morphometric measurements for this female are: • PCL_x (*n* = 68): 1991±11.35 mm (PCL_{xd}:1990±136 mm; R: 1810-2230 mm) • TL_x (*n* = 66): 2577±14.08 mm (TL_{xd}: 2566 ±163 mm; R: 2354-2900 mm) • $Weight_x$ (*n* = 66): 142.4±2.571 kg ($Weight_{xd}$: 140±25.5 kg; R: 94-198 kg) • HSI_x (*n* = 64): 10.59±0.24% (HSI_{xd}: 10.51±2.11%; R: 6-16.75%) • GSI_x (*n* = 63): 2.74 0.13% (GSI_{xd}: 2.67±0.82%; R: 0.25-6%) • Capsule $\sum/F_n$ LU = 1333/66 females; $Capsule_x$: 20.2±3.47 ($Capsule_{xd}$: 14.5±16.25; R: 1-179) $\sum/F_n$ RU = 1248/65 females; Capsulex: 19.2 ± 3.18 ($Capsule_{xd}$: 13±15; R: 1-187) • Capsule Length: CL_x LU (*n* = 120 capsules):88.6±1.241mm (CL_{xd}: 87.5±18.8 mm; R: 45-120 mm) CL_x RU (*n* = 140 capsules): 85.4±1.06 mm (CL_{xd}: 86±16 mm; R: 43-114 mm) • Capsule Weight: CM_x LU (*n* = 120): 6.53±0.3 g (CM_{xd}: 6±4.4 g; R: 1-18 g) • CM_x RU (*n* = 138): 7.78±0.38 g (CM_{xd}: 7±5 g; R: 0.8-19 g)

		• The UW_x (*n* = 57): 106±3.59 mm (UW_{xd}:100.5±38 mm; R: 12-204mm)
RS5	GF (All stages RS5A-RS5D)	Females are pregnant (either with capsules and/or embryos). Captured in January-September • PCL_X (*n* = 286): 2000±5.23 mm (PCL_{xd}: 2000±120 mm; R:1730-2250mm) • TL_X (*n* = 267): 2555±16.02 mm (TL_{xd}: 2578±142 mm; R: 600.8-3022mm) • $Weight_x$ (*n* = 283):138.3 ±1.15 kg ($Weight_{xd}$:137±26 kg; R: 92-190kg) • HSI_x (*n* = 213): 7.18±0.124 % (HSI_{xd}: 7.143±2.54 %; R:0.96-13.33%) • GSI_x (*n* = 224): 2.638±0.147 % (GSI_{xd}: 2.168 ±4.35%; R: 0.134-7.92%) • Capsule Count: $\sum/F_n$ LU = 1427/67 females; $Capsule_x$: 21.3±2.526 ($Capsule_{xd}$: 14±39; R:1-60) $\sum/F_n$ RU = 1567/71 females; $Capsule_x$: 22.07±2.406 ($Capsule_{xd}$: 20±38; R:1-62) • Capsule Length: CL_x LU (*n* = 703): 85.2±0.44 mm (CL_{xd}: 85±14 mm; R: 10.8-121 mm); CL_x RU (*n* = 630): 83.08±0.53 mm (CL_{xd}: 83±16 mm; R: 9-126mm) • Capsule Weight: CM_x LU (*n* = 690): 9.7±0.31g (CM_{xd}: 8±3.9 g; R:1- 64g) CM_x RU (*n* = 627 capsules):9.52±0.45 g (CM_{xd}: 7±4.8 g; R: 0.4-178 g) • E Count: ELU (*n* = 310); Sex ratio: 138F:133M: 39U ERU (*n* = 300); Sex ratio: 136F:131M: 33U EUU (*n* = 3); Sex ratio: U • E Total Length: ETL_x LU (*n* =275/310) 680.4±14.46 mm (ETL_{xd}: 731.6±375; R:17.67 -1064 mm) ETL_x RU (*n* = 275/300): 676.9±14.53mm (ETL_{xd}: 710±376; R:15.75 -1035mm) • E Weight: EM_x LU (*n* = 265/310) 3404 ±147.4 g (EM_x: 3200±4400 g; R: 0 - 8500 g) EM_x RU (*n* = 268/300) 3399 ±148.4 g (EM_x: 3290±4509 g; R: 0.5 - 8300g) • UW_x (*n* =19*): 165.6 ±7.46 mm (UW_{xd}: 166±70 mm; R: 7-350mm)
RS5A	GF (Pre-hatch Stage: RS5A)	Female are pregnant with embryos encased in the capsules. Capsules are present with either fertilised or unfertilised ova. Captured in January-March, September. Morphometric measurements are: • PCL_x (*n* =11): 1988±25.46 mm (PCL_{xd}: 2000±140 mm; R:1840-2080mm) • TL_x (*n* = 11): 2576±32.5 mm (TL_{xd}: 2576±182 mm; R: 2368-2694mm) • $Weight_x$ (*n* = 10): 146.6±5.946 kg ($Weight_{xd}$: 145.3±33.5 kg; R: 119-178kg) • HSI_x (*n* = 9): 9.404 ±0.414 % (HSI_{xd}: 8.876±1.53 %; R: 7.47-11.72%) • GSI_x (*n* = 9): 4.498 ±0.269 % (GSI_{xd}: 4.692 ±1.07 %; R: 3.02-5.67%) Capsule Count: $\sum/F_n$ LU: 268/6; Capsulex :44.67±2.86 ($Capsule_{xd}$: 43 ±14; R: 37-54)

		$\sum/F_n$ RU: 346/8; $Capsule_x$: 43.3±2.64 ($Capsule_{xd}$: 41±11; R: 36-58) • Capsule Length: CL_x LU (*n* = 4): 93 ±1.92 mm (CL_{xd}: 92±7 mm; R: 90-98 mm) CL_x RU (*n* = 4 capsules): 80.25±7 mm (CL_{xd}: 85±24.8 mm; R: 60-91 mm) • Capsule Weight: CM_x LU (*n* = 4): 6.65±0.88 g (CM_{xd}: 6.3±3.3; R: 5.2-8.8 g) CM_x RU (*n* = 4): 4.35±1.52 g (CM_{xd}: 3.9±5.8 g; R: 1.2-8.4 g) • E count: ELU (*n* =32 females); from 11 adult females; Sex ratio: U ERU (*n* =27 females); from 11 adult females; Sex ratio: U EUU (*n* =3 females); from 1 adult female; Sex ratio: U • E total length: ETL_x LU (*n* = 10): 36.54±3.68 mm (ETL_{xd}: 38.88±15.58; R: 17-52 mm) ETL_x RU (*n* = 10): 38.52 ±4.39 mm (ETL_{xd}: 41.5±24.33; R: 15.75-58.2 mm) • E Weight: EM_x LU (*n* = 4): 0.34±0.10 g (EM_x: 0.34±0.38 g; R: 0.1-0.6 g) EM_x RU (*n* = 6): 0.842±0.30 g (EM_x: 0.7±1.29 g; R: 0.15-2 g) • UW_x (*n* = 7*): 173.9±4.6 mm (UW_{xd}: 166±55 mm; R: 130-210 mm)
RS5B	GF (Post-hatch stage: RS5B)	Females are pregnant with embryos hatched from their capsules. Maximum of three embryos could hatch from one capsule. Embryos (E) are between 60-100 mm TL. Captured in February and July. Measurements are: • PCL_x (*n* = 3):1953±58.4 mm (PCL_{xd}: 1900±180 mm; R: 1890-2070 mm) • TL_x (*n* = 2): 2576±118 mm (TL_{xd}: 2576±236 mm; R: 2458-2694 mm) • $Weight_x$ (*n* = 2):137±24 kg ($Weight_{xd}$: 137±48 kg; R: 113 -161 kg) • HSI_x (*n* = 2): 8.09±0.66% (HSI_{xd}: 8.096±1.324 %; R: 7.43-8.76 %) • GSI_x: The GSI in this stage could not be calculated. • Capsule Count: $\sum/F_n$ LU = 90/2; $Capsule_x$:45±0 ($Capsule_{xd}$: 45±0; R: no range) $\sum/F_n$ RU = 38/1; $Capsule_x$: 38± 0 ($Capsule_{xd}$: 38±0; R: no range) • Capsule Length: CL_x LU (*n* = 1):75 mm CL_x RU (*n* =1): 72 mm • J. Capsule Weight: CM_x LU (*n* = 1): 6.8 g CM_x RU (*n* = 1): 4.2 g • E count: ELU (*n* = 3) from 3 females; Sex ratio: 1F:0M: 2U ERU (*n* = 2) from 2 females: Sex ratio: 1F:0M: 1U • E total length: ETL_x LU (*n* = 3); 68.67±1.86 mm (ETL_{xd}: 70±6; R: 65-71 mm)

		ETL_x RU (n = 2); 66 ±2 mm (ETL_{xd}:66±4; R: 64-68 mm) • E Weight: EM_x LU (n = 2): 3±1 g (EM_x: 3±2g; R: 2-4 g) EM_x RU (n = 1*): 2 ± 0g (EM_x: 2±0 g; R: 2-2 g) • UW_x (n = 1) and UW_{xd} could not be calculated for this stage.
RS5C	GF (Intra-cannibalism: RS5C)	Females are pregnant with embryos in the intrauterine cannibalistic phase. Embryos are between 100-335 mm TL. Capsules (with unfertilised ova) are present to provide nutrition. Captured in February-April. Measurements: • PCL_x (n = 15): 1987±20.48 mm (PCL_{xd}: 1988±144 mm; R: 1860-2120 mm) • TL_x (n = 14): 2565±22.6 mm (TL_{xd}: 2578±134 mm; R: 2436 -2707 mm) • $Weight_x$ (n = 15): 140.8±4.40 kg ($Weight_{xd}$:138±25 kg; R: 119-178 kg) • HSI_x (n = 13): 9.07±0.32 % (HSI_{xd}: 9.5±2.28 %; R: 7.42-10.67 %) • GSI_x (n = 15): 4.69±0.183 % (GSI_{xd}: 4.69±0.67 %; R: 2.93-5.83 %) • Capsule Count: $\sum/F_n$ LU = 617/9; $Capsule_x$:68.51±18.29; ($Capsule_{xd}$: 54±15; R:39-215) $\sum/F_n$ RU = 999.2/11; $Capsule_x$: 90.8±28.2; ($Capsule_{xd}$: 57±19; R: 34-334) • Capsule Length: CL_x LU (n = 6): 82.2±7.23 mm (CL_{xd}: 85.5±28; R: 51-101) CL_x RU (n = 5): 90±5.3 mm (CL_{xd}: 86±21 mm; R: 76-107 mm) • J. Capsule Weight: CM_x LU (n = 6): 7.93±1.49 g (CM_{xd}: 9.3±4.8 g; R:1-11g) CM_x RU (n = 4): 6.9±1.27 g (CM_{xd}: 7±4.65g; R: 3.6-9.8 g) • E_x LU (n = 13); Sex ratio: 4F: 7M: 2U E_x RU (n = 13): Sex ratio: 6F: 6M: 1U • ETL_x LU (n = 13); 239.6±23.1 mm (ETL_{xd}:240±175.2; R: 115-335.2 mm) ETL_x RU (n = 13); 254.2±19.58 mm (ETL_{xd}: 280±136.1; R: 145-334 mm) • EM_x LU (n = 12);125.2 ±27.84 g (EM_{xd}:85.5±156.05 g; R: 14-308 g) EM_x RU (n = 13); 271.8±150.1 g (EM_{xd}: 100±187.5 g; R: 21-2050 g) • UW_x (n = 6*): 154.2±12.5 mm (UW_{xd}: 145±75 mm; R: 104-210 mm).

RS5D	GF (Oophagous stage: RS5D)	Females are pregnant with one embryo in each uterus. Embryos are between 335 - 900 mm TL. Capsules are present providing food during this oophagous phase. Captured in January-September. Measurements are: • PCL_x (*n* = 258): 2001±5.556 mm (PCL_{xd}: 2000±120 mm; R: 1730-2250 mm) • TL_x (*n* = 243): 2553±17.52 mm (TL_{xd}: 2578±144 mm; R: 600.8-3022 mm) • $Weight_x$ (*n* = 257): 137.9±1.223 kg ($Weight_{xd}$: 136.2 ±27 kg; R: 92-190 kg) • HSI_x (*n* = 189): 7.013±0.126 % (HSI_{xd}: 6.935 ±7.55 %; R: 0.956-13.3 %) • GSI_x (*n* = 203): 2.493±0.156 % (GSI_{xd}: 1.42±4.35 %; R: 0.134-7.92 %) • Capsule Count: $\sum/F_n$ LU = 746/52; $Capsule_x$: 14.35±2.43; ($Capsule_{xd}$: 3.5±27; R:1-60) $\sum/F_n$ RU = 754/52; $Capsule_x$: 14.5±2.385; ($Capsule_{xd}$: 3±24; R: 1-60) • Capsule Length: CL_x LU (*n* = 28): 87±2.5 mm (CL_{xd}: 84.5±21; R: 66-116 mm) • CL_x RU (*n* = 30): 79.1±4.2 mm (CL_{xd}: 81.5±22; R: 9-126 mm) • Capsule Weight: CM_x LU (*n* = 26): 25.42±3.45 g (CM_{xd}: 18.5±31 g; R:2-62g) CM_x RU (*n* = 29): 31.32 ± 6.3 g (CM_{xd}: 19.8±36 g; R: 4-178g) • E_x LU (*n* = 255); Sex ratio: 128F: 124M: 2U E_x RU (*n* = 254): Sex ratio: 128F: 121M: 2U • ETL_x LU (*n* = 254); 724±11.86 mm (ETL_{xd}: 760.2±330.5; R: 327.8-1064 mm) ETL_x RU (*n* = 243): 3612±147.9 mm (ETL_{xd}: 3600±4305; R: 144-8500 mm) • EM_x RU (*n* = 253); 722.2 ±11.94 g (PM_x: 759.6 ±342.3 g; R: 326.6-1035 g) EM_x LU (*n* = 243); 3612±149.7 g (PM_x: 3650±4338 g; R: 156-8300 g) • UW_x (*n* = 9): 154.2 ±28.5 mm (UW_{xd}: 168±219 mm; R: 7-350 mm)
RS6	Post-partum	• Few females from this stage have been captured however they are usually rare in KZN as the pupping ground is to the south. SS6 females present with embryos close to parturition. Captured mainly in July-August. Smaller numbers caught in January, May-June, September-November. • PCL_x (*n* = 26): 1935±38.69 mm (PCL_{xd}: 1955±157 mm; R: 1080-2200 mm) • TL_x (*n* = 24): 2522±41.34 mm (TL_{xd}: 2561±186 mm; R: 1712-2808 mm) • $Weight_x$ (*n* = 29): 137.2± 4.27 kg ($Weight_{xd}$: 134.4±29.4 kg; R: 103.6-202.6 kg) • HSI_x (*n* = 11): 7.9 ±0.55 % (HSI_{xd}: 8.36 ±2.93 %; R: 5-10.71 %) • GSI_x (*n* = 20): 0.554±0.109 % (GSI_{xd}: 0.381±0.55 %; R: 0.08-2.15%)

	• UW_x (*n* = 10): 132±21.08 mm (UW_{xd}: 116±61.25 mm; R: 59-300 mm). The larger UW would distinguish these females from the mature females preparing to mate in November (Stage 3 no G)

Abbreviations: CL: Capsule Length; CM: Capsule Weight; E: Embryo; ETL: Embryo Total length; EM: Embryo Weight; F: Female; GF: Gravid females; GSI: gonado-somatic index; HSI: hepatosomatic index; LU: left uterus; M: Male; mm: millimetre; NGF: non-gravid females; PCL: precaudal length; RU: right uterus; TL: total length; UW: uterine width; UU: Unidentified uterus; l50: length at 50% maturity; x: mean ± SEM (Standard Error of Mean); xd: median ± IQR; R: range; *n*: female sample size; * repeated ID's in these stages.

APPENDIX G: Descriptive statistics (Median ± IQR, Mean ± SEM and range) of plasma analytes in *C. taurus* NGF sub-groups (RS1 and RS2B).

		RS1							RS2B						
	Unit	***n***	**Median**	**IQR**	**Mean**	**SEM**	**Min**	**Max**	***n***	**Median**	**IQR**	**Mean**	**SEM**	**Min**	**Max**
Sodium	mmol/l	0	NE	NE	NE	NE	NE	NE	3	258	19	264	6	257	276
Potassium	mmol/l	0	NE	NE	NE	NE	NE	NE	3	8,9	4	10	1	8	12,2
Na:K		0	NE	NE	NE	NE	NE	NE	3	31	11	28	4	21	32,5
Chloride	mmol/l	0	NE	NE	NE	NE	NE	NE	2	245	20	245	10	235	255
Anion Gap		3	-5	0	-5	0	-5	-5	9	-5	28	33	31	-5	279,9
Urea	mmol/l	3	308	90	327	0	292	383	9	366,4	35	373	7	350	408,6
Creatinine	mmol/l	0	NE	NE	NE	27,9	NE	NE	3	36	17	34	5	24	41
Calcium	mmol/l	3	5	1	4	NE	3,9	4,6	9	4,1	1	4	0	3	5,4
Phosphate	mmol/l	0	NE	NE	NE	0	NE	NE	3	4,04	1	4	0	4	4,8
Ca: P		0	NE	NE	NE	NE	NE	NE	3	1,01	1	1	0	1	1,5
Total Protein	g/L	3	19	12	22,3	NE	18	30	9	26	11	27	2	19	36
Total Bilirubin	umol/L	3	1	3	2	4	1	4	9	4	5	4	1	1	7
Cholesterol	mmol/L	3	1	1	1	1	1	1	8	0,4	1	1	0	0	1
Magnesium	mmol/L	2	2	0	2	0	2	2	9	4,5	3	4	1	2	5,7
Triglyceride	mmol/L	3	0,2	0	0	0	0	0	8	0,34	0	0	0	0	0,9
ALP	U/L	3	13	15	17	0	11	26	8	22,5	7	23	2	15	31
AST	U/L	3	188	1022	473	5	104	1126	8	2100	3621	2193	604	95	4380
ALT	U/L	3	3	1	3	328	3	4	8	33	49	32	9	2	64
LH	mIU/ml	3	0	0	0	0	0	0	9	0,1	0	0	0	0	0,1
FSH	mIU/ml	3	1	1	1	57	0	1	8	0,6	1	1	0	0	1,1
Progesterone	nmol/L	3	1,7	3	2	524	0	3	9	2,1	1	2	0	1	3,3
Oestradiol	nmol/L	3	0,1	0	0	0	0	0	9	0,3	2	1	0,4	0	3,2

Abbreviations: ALT: Alanine aminotransferase; ALP: alkaline phosphatase; AST: Aspartate aminotransferase; Ca:P: Calcium: Phosphate ratio; FSH: Follicle stimulating Hormone; g/L: grams/litre; IQR: Interquartile Range of the median; LH: Luteinising Hormone; mmol/L: millimole/litre; mIU/ml: milli-international units /millilitre; min: minimum; max: maximum; *n*: sample no; nmol/l: nanomole/litre; NG: non-gravid females; Na: K: Sodium: Potassium ratio; SEM: Standard error of mean; RS: Reproductive Stage; RS1: immature (adolescent)females; RS2B: mature but sexually not active females; U/L:units/ litre; umol/L: micromole/litre.

APPENDIX H: Descriptive statistics (Median ± IQR, Mean ± SEM and ranges) of plasma in *C. taurus* NGF sub-group (RS3)

	RS3							
	Unit	***n***	**Median**	**IQR**	**Mean**	**SEM**	**Min**	**Max**
Sodium	mmol/l	14	244	9	244,5	2,4	226	264
Potassium	mmol/l	13	13,1	7,4	13,3	1,2	6,5	18,9
Na: K		13	18,8	12	20,8	2,4	12,6	38,9
Chloride	mmol/l	14	222	11,5	220,6	2,9	200	239
Anion Gap		22	24,9	38,2	18,2	4,1	-8	56,5
Urea	mmol/l	22	397,3	30,7	393,6	4,7	352,9	436,2
Creatinine	mmol/l	14	35,5	25	37,5	5	11	75
Calcium	mmol/l	22	4,1	0,6	4,1	0,1	3,1	4,9
Phosphatase	mmol/l	15	5,1	4,7	5,4	0,6	2	10,1
Ca: P		15	0,8	0,9	0,9	0,1	0,4	1,9
Total Protein	g/L	22	27,5	6,2	28,1	1	20	38
Total Bilirubin	umol/L	21	3	3	3,5	0,4	0	7
Cholesterol	mmol/L	22	0,4	0,3	0,5	0,1	0,1	1,1
Magnesium	mmol/L	22	2.3	0,8	2,5	0,2	0,9	4,7
Triglyceride	mmol/L	19	0,3	0,3	0,4	0	0,1	0,7
ALP	U/L	21	17	18	17,2	2,3	3	46
AST	U/L	21	1460	1280,5	1330	236,1	132	3995
ALT	U/L	19	9	94	42,5	11,3	3	122
LH	mIU/ml	16	0,1	0,02	0,1	0	0,1	0,1
FSH	mIU/ml	19	0,7	0,7	0,8	0,1	0,2	1,8
Progesterone	nmol/L	15	5,9	3,1	5,37	0,7	1,2	10,6
Oestradiol	nmol/L	16	2,2	1,2	2,1	0,3	0,1	3,9

Abbreviations: ALP: alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; Ca:P: Calcium: Phosphate ratio; FSH: Follicle stimulating Hormone; IQR: Interquartile Range of the median; LH: Luteinising Hormone; mmol/L: millimole/litre; mIU/ml: milli-international units/millilitre; min: minimum; max: maximum *n*: sample no; nmol/L: nanomole/litre; NG: non-gravid females; Na:K: Sodium: Potassium ratio; SEM: Standard error of mean; RS: Reproductive Stage; RS3: mature and sexually active females. g/L: grams/litre; U/L: units/litre; umol/L: micromole/litre.

APPENDIX I: Descriptive statistics (Median ± IQR, Mean ± SEM and range) of the plasma analytes in *C. taurus* GF sub-groups (RS4 and RS5)

		RS4							RS5						
	Unit	***n***	**Median**	**IQR**	**Mean**	**SEM**	**Min**	**Max**	***n***	**Median**	**IQR**	**Mean**	**SEM**	**Min**	**Max**
Sodium	mmol/l	6	239	39	236	11	190	266	10	239	29	236	7	187	256
Potassium	mmol/l	6	21	24	23	5	9	42	11	16	11	18	3	8	39
Na: K ratio		6	12	19	15	4	5	29	7	15	8	15	2	5	25
Chloride	mmol/l	7	209	45	211	8	182	240	14	211	28	215	4	198	241
Anion Gap		6	35	29	39	6	24	58	9	34	19	38	4	25	57
Urea	mmol/l	8	388	36	391	6	365	411	13	383	48	390	9	327	444
Creatinine	mmol/l	7	79	155	105	31	26	229	13	70	62	69	9	24	129
Calcium	mmol/l	5	4	1	4	0	4	5	11	4	0	4	0	4	5
Phosphate	mmol/l	7	9	12	9	2	3	18	14	8	5	7	1	2	12
Ca: P ratio		6	1	1	1	0	0	2	12	1	0	1	0	0	2
Total Protein	g/L	7	28	6	27	1	21	32	14	27	4	26	1	21	30
Total Bilirubin	umol/L	8	5	3	5	1	2	7	12	3	6	4	1	1	9
Cholesterol	mmol/L	8	0	1	0	0	0	1	14	0	0	0	0	0	1
Magnesium	mmol/L	8	3	2	3	0	1	5	14	3	2	3	0	2	4
Triglyceride	mmol/L	8	0	1	1	0	0	1	14	1	0	1	0	0	1
ALP	U/L	7	7	2	7	1	5	11	13	8	7	7	1	1	14
AST	U/L	8	1189	2137	1409	439	142	3468	13	1295	3020	1981	453	108	5039
ALT	U/L	8	10	34	36	22	0	186	13	72	220	111	31	2	291
LH	mIU/ml	7	0	0	0	0	0	0	11	0	0	0	0	0	0
FSH	mIU/ml	6	1	1	1	0	0	1	11	1	1	1	0	0	1
Progesterone	nmol/L	8	4,46^{R,S}	3	5	1	4	8	13	2	2	2	0	1	5
Oestradiol	nmol/L	8	2275^{T}	519	2155	157	1199	2596	11	533	1248	852	198	139	1785

Abbreviations: ALP: alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; Ca:P: Calcium: Phosphate ratio; FSH: Follicle stimulating Hormone; g/L: grams/litre; G: gravid females; IQR: Interquartile Range of the median; LH: Luteinising Hormone; mmol/L: millimole/litre; mIU/ml: milli-international units /millilitre; min: minimum; max: maximum; *n*: sample size; nmol/l: nanomole/litre; Na:K: Sodium: Potassium ratio; SEM: Standard error of mean; RS: reproductive stage; RS4: females with capsules; RS5: females with embryos and capsules; U/L:units/litre; umol/L: micromole/litre.

APPENDIX J: Descriptive statistics (Median ± IQR, Mean ± SEM and range) for the plasma analytes in GF *C. taurus* sub-groups (RS4 and RS5)

		RS5A					RS5C							RS5D						
	Unit	***n***	**Median**	**Me an**	**Min**	**Max**	***n***	**Median**	**IQR**	**Me an**	**SEM**	**Min**	**Max**	***n***	**Median**	**IQR**	**Me an**	**SEM**	**Min**	**Max**
Sodium	mmol/l	1	256	256	256	256	2	244	24	244	12	232	256	7	229	19	231	8	187	256
Potassium	mmol/l	1	11	11	11	11	3	22	15	20	5	11	27	7	16	12	18	4	8	39
Na: K ratio		0	NE	NE	NE	NE	1	18	0	18	0	18	18	6	14	11	14	3	5	25
Chloride	mmol/l	1	228	228	228	228	4	213	25	214	7	201	228	9	207	31	214	6	198	241
Anion Gap		1	34	34	34	34	2	42	15	42	8	34	50	6	33	23	37	5	25	57
Urea	mmol/l	1	444	444	444	444	4	398	71	400	18	359	444	8	380	38	379	10	327	415
Creatinine	mmol/l	1	24	24	24	24	4	81	59	72	17	24	102	8	69	54	73	12	28	129
Calcium	mmol/l	1	4	4	4	4	3	4	0	4	0	4	5	7	4	0	4	0	4	5
Phosphate	mmol/l	1	4	4	4	4	4	8	5	8	1	4	11	9	8	6	7	1	2	12
Ca: P ratio		1	1	1	1	1	3	1	0	1	0	1	1	8	1	1	1	0	0	2
Total Protein	g/L	1	27	27	27	27	4	28	3	27	1	24	28	9	26	6	25	1	21	30
Total Bilirubin	umol/L	1	8	8	8	8	4	2	6	3	2	1	8	7	2	4	3	1	1	9
Cholesterol	mmol/L	1	1	1	1	1	4	1	0	1	0	0	1	9	0	0	0	0	0	1
Magnesium	mmol/L	1	2	2	2	2	4	3	2	3	1	2	4	9	3	1	3	0	2	4
Triglyceride	mmol/L	1	1	1	1	1	4	1	1	1	0	0	1	9	0	0	0	0	0	1
ALP	U/L	1	8	8	8	8	4	9	8	9	2	4	14	8	7	8	6	1	1	11
AST	U/L	1	4091	4091	4091	4091	4	2793	3139	2499	828	317	4091	8	1092	898	1459	537	108	5039
ALT	U/L	1	215	215	215	215	4	144	250	146	65	5	291	8	17	202	81	38	2	249
LH	mIU/ml	1	0	0	0	0	2	0	0	0	0	0	0	8	0	0	0	0	0	0
FSH	mIU/ml	1	1	1	1	1	3	1	1	1	0	0	1	7	1	1	1	0	0	1
Progesterone	nmol/L	1	1	1	1	1	4	1,2	3	2	1	1	5	8	2,81	2	2	0	1	3
Oestradiol	nmol/L	1	1570	1570	1570	1570	3	1640	215	1665	63	1570	1785	7	376	394	401	95	139	864

Abbreviations: ALP: alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; Ca:P: Calcium: Phosphate ratio; FSH: Follicle stimulating Hormone; G: gravid females; IQR: Interquartile Range of the median; LH: Luteinising Hormone; min: minimum; max: maximum; *n*: sample size; Na:K: Sodium: Potassium ratio; SEM: Standard error of mean; RS: Reproductive Stage; RS5A: pre-hatch females; RS5C: females with intrauterine cannibalistic embryos;RS5D: females with oophagous embryos ; NE: Not Established. Units: mmol/L: millimole/litre g/L: grams/litre; U/L: units/litre; mIU/ml: milli- international units /millilitre umol/L: micromole/litre.

APPENDIX K: Descriptive statistics (Median ± IQR, Mean ± SEM and range) for the UF analytes in *C. taurus* GF sub-groups (RS4 and RS5)

		RS4							RS5						
	units	***n***	**Median**	**IQR**	**Mean**	**SEM**	**Min**	**Max**	***n***	**Median**	**IQR**	**Mean**	**SEM**	**Min**	**Max**
Sodium	mmol/l	4	386	150	369	40	265	440	14	340	46	330	7	276	360
Potassium	mmol/l	2	10	3	10	2	8	11	10	10	7	10	1	6	15
Chloride		3	359	179	351	52	257	436	16	354	46	342	12	184	385
Na:K ratio	mmol/l	2	46	16	46	8	38	54	10	27	16	30	4	7	50
Anion Gap		4	-5	14	-6	4	-16	3	16	-25	27	-23	5	-69	5
Urea	mmol/l	4	200	145	215	39	149	312	16	229	46	230	9	160	315
Creatinine	mmol/l	4	13	25	18	7	8	39	16	16	17	19	2	6	39
Calcium	mmol/l	4	5,1	1	5	0	4	6	17	9,9	3	9	1	1	17
Phosphate	mmol/l	2	1	0	1	0	1	1	11	1	2	1	0	0	4
Ca:P ratio		2	8	7	8	3	5	12	11	13	22	17	4	4	43
Total Protein	g/L	4	4	5	5	2	3	9	16	5	3	5	0	2	7
Total Bilirubin	umol/L	3	1	1	1	0	1	2	12	4,5	7	5	1	1	11
Cholesterol	mmol/L	2	0	0	0	0	0	0	14	0	0	0	0	0	0
Magnesium	mmol/L	4	3,9	1	4	0	3	4	17	6,16[y]	2	6	0	2	10
Triglyceride	mmol/L	4	0	0	0	0	0	1	14	0	1	0	0	0	1
ALP	U/L	2	260	167	260	84	176	343	17	194	242	169	33	3	414
ALT	U/L	2	4	0	4	0	4	4	15	14	19	15	4	2	47
LH	mIU/ml	2	0	0	0	0	0	0	13	0	0	0	0	0	0
FSH	mIU/ml	2	1	0	1	0	1	2	13	4	3	4	1	1	9
Progesterone	nmol/L	1	1	0	1	0	1	1	12	1	1	1	0	0	2
Oestradiol	nmol/L	0	NE	NE	NE	NE	NE	NE	7	139	0	124	15	37	139

Abbreviations: ALP: alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; Ca:P: Calcium: Phosphate ratio; g/L: grams/litre; FSH: Follicle stimulating Hormone; G: gravid females; IQR: Interquartile Range of the median; LH: Luteinising Hormone; mmol/L: millimole/litre; mIU/ml: milli-international units /millilitre; min: minimum; max: maximum; *n*: sample no; nmol/L: nanomole/litre; Na: K: Sodium: Potassium ratio; SEM: Standard error of mean; RS: reproductive stage; RS4: females with capsules; RS5: females with embryos and capsules; NE: Not Established; U/L:units/litre; umol/L: micromole/litre

APPENDIX L: Descriptive statistics (Median ± IQR, Mean ± SEM and range) for the UF analytes in *C. taurus* GF sub-groups (RS5A and RS5B)

		RS5A							RS5B						
	Units	*n*	Mean	SEM	Median	IQR	Min	Max	*n*	Mean	SEM	Median	IQR	Min	Max
Sodium	mmol/l	2	346,5	8,5	346,5	17	338	355	2	357,5	2,5	357,5	5	355	360
Potassium	mmol/l	0	NE	NE	NE	NE	NE	NE	1	14,1	0	14,1	0	14,1	14,1
Chloride		2	358	17	358	34	341	375	2	374	1	374	2	373	375
Na: K ratio	mmol/l	0	NE	NE	NE	NE	NE	NE	1	25,53	0	25,53	0	25,53	25,53
Anion Gap		2	-16,5	8,5	-16,5	17	-25	-8	2	14,45	10,55	-14,45	21,1	-25	-3,9
Urea	mmol/l	2	219,2	12,35	219,2	24,7	206,8	231,5	2	208,4	1,55	208,4	3,1	206,8	209,9
Creatinine	mmol/l	2	7	1	7	2	6	8	2	15	7	15	14	8	22
Calcium	mmol/l	2	8,59	1,06	8,59	2,12	7,53	9,65	2	9,82	0,17	9,82	0,34	9,65	9,99
Phosphate	mmol/l	0	NE	NE	NE	NE	NE	NE	1	2,09	0	2,09	0	2,09	2,09
Ca: P ratio		0	NE	NE	NE	NE	NE	NE	1	4,78	0	4,78	0	4,78	4,78
Total Protein	g/L	2	6	0	6	0	6	6	2	6,5	0,5	6,5	1	6	7
Total Bilirubin	umol/L	1	1	0	1	0	1	1	1	2	0	2	0	2	2
Cholesterol	mmol/L	2	0,1	0	0,1	0	0,1	0,1	1	0,1	0	0,1	0	0,1	0,1
Magnesium	mmol/L	2	6,515	0,355	6,515	0,71	6,16	6,87	2	7,205	0,335	7,205	0,67	6,87	7,54
Triglyceride	mmol/L	2	0,135	0,075	0,135	0,15	0,06	0,21	1	0,21	0	0,21	0	0,21	0,21
ALP	U/L	2	257	46	257	92	211	303	2	275	28	275	56	247	303
ALT	U/L	2	18,5	4,5	18,5	9	14	23	2	35	12	35	24	23	47
LH	mIU/ml	2	0,35	0,05	0,35	0,1	0,3	0,4	2	0,3	0,1	0,3	0,2	0,2	0,4
FSH	mIU/ml	2	5,85	1,45	5,85	2,9	4,4	7,3	2	4,05	0,35	4,05	0,7	3,7	4,4
Progesterone	nmol/L	2	0,25	0,05	0,25	0,1	0,2	0,3	2	0,65	0,35	0,65	0,7	0,3	1
Oestradiol	nmol/L	2	139	0	139	0	139	139	2	139	0	139	0	139	139

Abbreviations: ALP: alkaline phosphatase; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; Ca:P: Calcium: Phosphate ratio; FSH: Follicle stimulating Hormone; g/L: grams/litre; G: Gravid females; IQR: Interquartile Range of the median; LH: Luteinising Hormone; *n*: sample no; nmol/L: nanomole/litre; Na:K: Sodium: Potassium ratio; mIU/ml: milli- international units /millilitre; mmol/L: millimole/litre; min: minimum; max: maximum; SEM: Standard error of mean; RS: reproductive stage; RS5A: pre-hatch females; RS5B: post-hatch females; NE: Not established; Units: U/L:units/litre; umol/L: micromole/litre.

APPENDIX M: Descriptive statistics (Median ± IQR, Mean ± SEM and range) for the UF analytes in GF *C. taurus* sub-groups

		RS5C							RS5D						
	Units	***n***	**Mean**	**SEM**	**Median**	**IQR**	**Min**	**Max**	***n***	**Mean**	**SEM**	**Median**	**IQR**	**Min**	**Max**
Sodium	mmol/l	2	328	32	328	64	296	360	8	319	9	319	49	276	349
Potassium	mmol/l	2	12,2	1,9	12,2	3,8	10,3	14,1	7	9	1	9	5	6	15
Chloride		2	348,5	24,5	348,5	49	324	373	10	331	18	349	60	184	385
Na: K ratio	mmol/l	2	27,14	1,605	27,14	3,21	25,5	28,7	7	32	5	35	21	7	50
Anion Gap		2	-13,3	9,4	-13,3	18,8	-22,7	-3,9	10	-27	6	-32	25	-69	5
Urea	mmol/l	2	193,9	16,05	193,9	32,1	178	210	10	243	13	238	45	160	315
Creatinine	mmol/l	2	25	3	25	6	22	28	10	21	3	16	18	9	39
Calcium	mmol/l	2	9,84	0,15	9,84	0,3	9,69	9,99	11	9	1	10	6	1	17
Phosphate	mmol/l	2	1,875	0,215	1,875	0,43	1,66	2,09	8	1	0	0	1	0	4
Ca: P ratio		2	5,31	0,53	5,31	1,06	4,78	5,84	8	21	5	22	25	4	43
Total Protein	g/L	2	5,8	1,2	5,8	2,4	4,6	7	10	4	1	3	3	2	7
Total Bilirubin	umol/L	2	3	1	3	2	2	4	8	7	1	7*W*	6	2	11
Cholesterol	mmol/L	1	0,1	0	0,1	0	0,1	0,1	10	0	0	0	0	0	0
Magnesium	mmol/L	2	6,445	1,095	6,445	2,19	5,35	7,54	11	5	1	5	3	2	10
Triglyceride	mmol/L	1	0,65	0	0,65	0	0,65	0,65	10	0	0	0	1	0	1
ALP	U/L	2	142	105	142	210	37	247	11	139	44	58	247	3	414
ALT	U/L	2	24,5	22,5	24,5	45	2	47	9	8	2	6	12	2	16
LH	mIU/ml	2	0,3	0,1	0,3	0,2	0,2	0,4	7	0	0	0	0	0	0
FSH	mIU/ml	2	4,55	0,85	4,55	1,7	3,7	5,4	7	3	1	2	3	1	9
Progesterone	nmol/L	2	0,9	0,1	0,9	0,2	0,8	1	6	1	0	1	1	0	2
Oestradiol	nmol/L	1	139	0	139	0	139	139	2	88	51	88	102	37	139

Abbreviations: ALP: alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; Ca:P: Calcium Phosphate ratio; FSH: Follicle stimulating Hormone; g/L: grams/litre; G: gravid females; IQR: Interquartile Range of the median; LH: Luteinising Hormone; mmol/L: millimole/litre; mIU/ml: milli-international units /millilitre; min: minimum; max: maximum; *n*: sample no; nmol/L: nanomole/litre; Na: K: Sodium: Potassium ratio; SEM: Standard error of mean; RS: reproductive Stage; RS5C: females with intrauterine cannibalistic embryos; RS5D: females with oophagous embryos; U/L: units/litre; umol/L: micromole/litre. nmol/L: nanomole/litre.