Title

Nile crocodile (*Crocodylus niloticus*) urine as sample for biochemical and hormonal analyses

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Thesis submitted to the Department of Paraclinical Sciences,
Faculty of Veterinary Science, University of Pretoria,
in fulfilment of the requirements for the degree Doctor of Philosophy (PhD)

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CO-SUPERVISOR: PROF A Duncan Cromarty (PhD)

2017

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QUOTE

God sleeps in the minerals, awakens in plants, walks in animals, and thinks in man.
Arthur Young (1741-1820)

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ACKNOWLEDGEMENTS

I specifically wish to extend my sincere appreciation and gratitude to the following persons and/or organisations:

1. Prof. Jan Myburgh, Exotic Leather Research Centre and Department of Paraclinical Sciences, Faculty of Veterinary Science, Onderstepoort; my promoter, who offered constant support and was the key person in the arrangement of all the field work and laboratory investigations. Thank you for your initiatives and leadership.
2. Prof. Christo Botha, the current Head of the Department of Paraclinical Sciences, for his valuable advice with and interest in the project.
3. Prof. Duncan Cromarty, Department of Pharmacology, Faculty of Health Sciences, my co-supervisor, for his help and guidance.
4. The Faculty of Veterinary Science of the University of Pretoria for granting me permission to do the research and to publish the results.
5. Laboratory colleagues in the Department of Paraclinical Sciences: Motjie Mulders, William Mokgojane, Reckson Ramuageli, Arina Ferreira, Annette Venter, Tharien de Winnaar, and Lyndy McGaw for their interest and encouragement. A special thank you to William for always finding publications in record time, and Arina for assistance in the GC/MS laboratory.
6. Johan Spies, Department(s) of Chemical Pathology and Chemistry, University of Pretoria, for the GC/MS analyses that set the baseline for the clinical validation of steroid metabolites in crocodilian urine.
7. Michelle Kirsten for administrational and financial arrangements; Madelyn de Wet for assistance with the printing, and Dr. Francien Botha for guidance with the editing; all from the Department of Paraclinical Sciences, Faculty of Veterinary Science, Onderstepoort.
8. Dirk Booyse from the Department of Anatomy and Physiology, Faculty of Veterinary Science, Onderstepoort, for always being available to assist with specimen sampling.
9. Prof. Alison Leslie for the urine samples she provided from Nile crocodiles studied in African countries.
10. Elsbe Myburgh from the Clinical Pathology laboratory at the Faculty of Veterinary Science, Onderstepoort, for coordinating the analyses of the plasma

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samples in the biochemistry survey.

11. Volker Schillack and colleagues from the AMPATH laboratory in Pretoria, who assisted with the urine biochemistry analyses.
12. The Norwegian Council for Higher Education's Programme for Development, Research and Education (NUFU), and the Royal Netherlands Embassy in South Africa (Prof. Nico Visser) for the grant that made the biochemistry survey possible.
13. The Crocodile Specialist Group, for their generous financial support, which enabled me to collaborate with colleagues in the USA during 2009 to do analyses on samples from American alligators.
14. The late Prof. Louis Guillette Jr. for the invitation to do analytical work in his laboratory at the University of Florida, Gainesville, FL, USA; his enthusiasm, support and guidance had a great impact on my projects.
15. Dr. Timothy Garrett for his support and assistance in the GC/MS laboratory of the Howard Hughes Medical Institute, University of Florida.
16. The Department of Zoology, University of Florida staff, for their cooperation; in particular Dr. Satomi Kohno, who provided lodging to me as a student.
17. SAVF for funding regarding chemicals, sample preparation and analyses costs during the ACTH project.
18. Dr. Mauritz Wentzel for assistance with analyses of the ACTH project samples.
19. Dr. Pete Laver from the Department of Anatomy and Physiology, Faculty of Veterinary Science, Onderstepoort, who took care of the statistical analysis of the plasma and urine biochemistry parameters.
20. Prof. Aye Aye Khine, current Head of the Department of Chemical Pathology, Sefako Makgatho Health Sciences University, for her support and encouragement during the past two years.
21. The different commercial crocodile farms, close to the Faculty of Veterinary Science, Onderstepoort, for help received with my research projects. I especially want to thank the owners, Stefan van As, Pit Suessmann, Albert Pretorius, and Trevor and Karin Dicker for their interest and assistance.
22. Adri, Ed, Eamonn, Bets, Adriaan, Marizahn, Louis, Stefanie, Hans, Marie and Francois for their encouragement.
23. All my friends and colleagues for their interest in my projects.
24. Last but not least, my parents, for their motivation and support.

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DEDICATION

This thesis is dedicated to:

God, for the ability, energy and health, to reach my goals.

My mother and father, Marie and David Bekker, for your constant interest and encouragement. I cherish the example you have set to me during your lives.

My friend, Max Gmür, for always being supportive, both with training on the water and with my studies. Thank you Max, for your never-ending motivation and dedication. You are a true mentor; I learned the value of determination, commitment and perseverance from you.

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DECLARATION

I, **Lasya Christina Bekker**, declare that this Thesis entitled: **Nile Crocodile (*Crocodylus niloticus*) urine as sample for biochemical and hormonal analyses,** which I herewith submit to the University of Pretoria in fulfilment of the requirements for the degree Doctor Philosophy (Doctor of Science) is my own original work, and has never been submitted for any academic award to any other institution of higher learning.

31 October 2016

______________________ ______________

DATE

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FORMAT OF THIS THESIS

1. Each technical chapter (Chapters 5, 6, 7 and 8) contains a more specific and dedicated introduction/literature review, hence Chapters 1 and 3 give a more general overview of the relevant published literature.
2. One article has been submitted for publication so far, namely:
   Lasya C Bekker, Peter N Laver, Maria M van Niekerk, Amelia Goddard, Jan G Myburgh, 2016. Urine and plasma concentrations of routine biochemical analytes in healthy captive pre-slaughter Nile crocodiles (*Crocodylus niloticus*). Journal of Veterinary Diagnostic Investigation (Chapter 5).
3. All the references for this thesis are listed in Chapter 10 (References).
4. All the figures for this thesis are listed under: *List of Figures*. Figures are listed per chapter.
5. All the tables for this thesis are listed under *List of Tables*. Tables are listed per chapter.
6. All the equations for this thesis are listed under *List of Equations*. Equations are listed per chapter.
7. All the appendices for this thesis are listed under *List of Appendices*.
8. Appendices can be found at the end of this thesis after Chapter 10.

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LIST OF ABBREVIATIONS

α-THF	allotetrahydrocortisol
ACTH	adrenocorticotropic hormone
An	androsterone
Ca	calcium
iCa	ionised calcium
CITES	Convention on International Trade of Endangered Species of Wild Fauna and Flora
Cl	chloride
C n-But	cholesteryl n-butyrate
CRH	corticotropin-releasing hormone
CSG	Crocodile Specialist Group
DHEA	dehydroepiandrosterone
EI	electron ionization
Et	etiocholanolone
g	gram
GC/MS	gas chromatography mass spectrometry
GFR	glomerular filtration rate
h	hour/hours
HAn	11β-hydroxy androsterone
HEt	11β-hydroxy etiocholanolone
HPDI	highest posterior density intervals
HPLC	high-performance liquid chromatography
HRMS	high resolution mass spectrometry
IS	internal standard
IUCN	International Union for Conservation of Nature
K	potassium
KAn	11-keto androsterone
KNP	Kruger National Park
LC-MS	liquid chromatography–mass spectrometry
LC/MS/MS	liquid chromatography-tandem mass spectrometry
MCMC	Markov Chain Monte Carlo
Mg	magnesium

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µg	microgram
min	minute/minutes
ml	millilitre
mm	millimeter
mmol	millimole
µmol	micromole
MO-TMS	methyloxime trimethylsilyl
MS	mass spectrometry
m/z	mass to charge ratio
n	number
N	Nitrogen
Na	sodium
NH_4^+	ammonium ion
NIST	National Institute of Standards and Technology
nmol	nanomole
NUFU	Norwegian Programme for Development, Research and Education
Osmol	osmolality
P	phosporous
PTH	parathyroid hormone
RT	retention time
SIM	Select Ion Monitoring
SVL	Snout Vent Length
THB	tetrahydrocorticosterone
THE	tetrahydrocortisone
THF	tetrahydrocortisol
THS	tetrahydro compound S
TIC	total ion chromatogram
TMSI	N-Trimethylsilylimidazole
TL	total length
TOFMS	time-of-flight mass spectrometry
TSD	temperature-dependent sex determination
TSP	thermosensitive period
U/A	uric acid
USA	United States of America

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ABSTRACT

Urine samples are routinely used in human and animal patients to diagnose health problems; often to investigate or monitor specific health-related problems that essentially may remain silent for extended periods. However, not much work has been performed on crocodilian urine for diagnostics. In general, crocodilian species lack a bladder as a separate storage organ (as found in mammals), possess metanephric kidneys (unable to concentrate urine) and have functional salt excreting glands. Collection of urine from the Nile crocodile (*Crocodylus niloticus*) is a simple and atraumatic procedure where a dog urinary catheter is used to collect relatively clean urine from the urinary chamber in the crocodile's cloaca. Unfortunately, in-depth investigations of urine variables, and establishing baseline concentrations, have not been performed on Nile crocodile urine samples before. The specific focus areas of this research project were: (1) determination of urine and plasma biochemical concentrations by means of a standard veterinary clinical pathology profile and the establishment of the ratio between urine and blood biochemical parameters; (2) the validation of a gas chromatographic–mass spectrometric (GC/MS) method for the determination of steroid metabolite concentrations in urine; and (3) using this established analytical method to determine the presence (identify) and concentrations of steroid metabolites in the urine of individual crocodilians.

Urine and plasma samples collected at Izintaba Crocodile Farm during the period November 2005 to July 2006, from captive bred, healthy young Nile crocodiles, were analysed for standard biochemistry variables. The urine samples (n = 101) were analysed for sodium, potassium, chloride, urea, creatinine, calcium, magnesium, phosphate, uric acid, osmolality, and ammonium ion, while the plasma samples (n = 101) were screened for total protein, glucose, sodium, potassium, chloride, urea, creatinine, total calcium, ionised calcium, magnesium, phosphate, uric acid and osmolality. Means, medians and standard deviations were statistically determined, as well as urine to plasma (U/P) ratios for corresponding variables. The value of this project is the establishment of reference concentrations for Nile crocodile urine samples that may become useful for interpretation of laboratory results, in future.

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The clinical validation of a GC/MS method for the analysis of urinary steroids in the Nile crocodile was achieved using urine samples from two-year-old Nile crocodiles. The main objective of this investigation was to develop, optimize and validate the laboratory analysis of urinary steroid metabolites. Steroid profiling was performed on individual and pooled Nile crocodile urine samples. Ascending concentrations of representative steroid standards: androsterone, etiocholanolone, dehydroepiandrosterone, 11-OH androsterone, pregnanediol, pregnanetriol, 11-deoxytetrahydrocortisol, tetrahydrocortisone, tetrahydrocortisol and tetra-hydrocorticosterone, were spiked into aliquots of the pooled urine samples, to obtain calibration samples ranging from 0.2 to 20 µg. Sample preparation and analysis methodology were based on a well-established, validated GC/MS method for determination of human urinary steroid metabolites. The validation of the GC/MS method for Nile crocodile urine was successfully completed, by determining lower limits of quantitation and limits of detection for each analyte, obtaining linearity up to the highest calibration level, correlations exceeding 0.90, and recoveries of 82% and more.

Steroid profiling was performed on urine samples collected from a number of mature crocodilian species, namely Nile crocodile, American alligator (*Alligator mississippiensis*), spectacled caiman (*Caiman crocodilus*) and dwarf crocodile (*Osteolaemus tetraspis*). Steroid metabolites were identified and were quantitated and reported per urinary creatinine. Qualitative reporting was conducted in cases where creatinine concentrations were not available. Results included identification and quantitation of the steroid metabolites: androsterone, etiocholanolone, 11-hydroxy androsterone, pregnanediol, pregnanetriol, and the tetrahydro-metabolites of cortisone (THE), cortisol (THF), and corticosterone (THB). In some urinary steroid profiles, several prominent peaks were observed which could not be identified. The study findings confirmed that crocodile urine could successfully be used, as it is commonly used in humans, to determine steroid metabolite profiles. A follow-up study to identify the unknown peaks by structure elucidation with more sophisticated equipment is recommended - this could lead to valuable information about liver metabolism of steroids in crocodilians.

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An adrenocorticotrophic hormone (ACTH) stimulation test was conducted on 18 captive Nile crocodiles. The experimental animals were temporarily housed in separate enclosures at Le Croc Crocodile Farm for four weeks, to ensure controlled conditions and easy and frequent access to the animals. Twenty-seven urine samples were collected both pre- and post-ACTH or saline injections. Steroid profiling was performed on 24 of the 27 urine samples to assess the corticosterone and tetrahydrocorticosterone concentrations following the ACTH treatment. Quantitation relative to urine creatinine levels was recorded following analyses with a standardised liquid chromatography/mass spectrometry (LC/MS) method, reporting the concentrations in nmol steroid/µmol creatinine. Unfortunately, a significant increase in urinary corticosterone concentrations 6 h after the injection of Synacthen® (5 µg/kg) was not observed. A possible explanation for this could be that the 6 h period was too short for a significant increase in urinary glucocorticoid metabolite excretion in the Nile crocodile.

In conclusion, this is the first in-depth study that focused, specifically, on Nile crocodile urine for analyses as diagnostic tools and for indices of health. The screening of the urine samples, collected from healthy Nile crocodiles, for a large array of biochemical variables contributed significantly to the database of “normal” concentrations. The establishment of a validated urinary steroid profiling method may significantly contribute to future validation and implementation of innovative diagnostic methods to monitor the health status and endocrine systems of wild Nile crocodiles in Africa.

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CHAPTER 1: INTRODUCTION

Crocodiles - why do humans have an interest in these reptiles? First of all, they are man-eaters! These charismatic cold-blooded animals have been called giant lizards, and described as living fossils from dinosaur-time (Grigg and Kirshner, 2015). Expanding populations of both humans and crocodilians may lead to the invasion of each others' territories, resulting in human-crocodile conflict (Caldicott et al., 2005; Wallace et al., 2011). In addition, economic interest in crocodilian skins and meat has promoted the establishment of numerous commercial crocodile farms in southern Africa, focusing on raising large numbers of crocodiles (Davis, 2001; Blessing, 2014).

The group, Archosaurs (meaning "ruling reptiles"), has traditionally been regarded as a subclass of the class Reptilia, the taxonomic status of which required revision during recent decades (Benton, 1982). Together with the extinct theocodants (includes phytosaurs), dinosaurs and pterosaurs (flying reptiles, ancestors of birds), crocodilians belong to the great group of archosaurs. The phytosaurs, believed to have preyed upon Triassic crocodilians, died out at the end of the Triassic (195 million years ago). Crocodilian fossils started appearing ±200 million years ago, in the Upper Triassic, a time when turtles, lizards and dinosaurs (another important group of reptiles) came into prominence (Bellairs, 1987).

The Triassic crocodilians were primitive reptiles and belong to the order *Protocuchia*, consisting of *Protosuchus* (Colbert and Mook, 1951), *Orthosuchus* (Nash, 1975) and *Lesothosuchus* (Whetstone and Whybrow, 1983). The *Protocuchia* were true crocodilian descendants and differed in many ways from modern forms, superficially lizard-like creatures less than one meter in length, presenting with heavy armour of bony plates, a broad head with a rather short, narrow snout and fairly long legs. The hind legs were longer than the front legs, as in modern crocodiles. The long bony palate which separates the mouth from the nose is a very characteristic crocodilian adaptation to amphibious life (Bellairs, 1987). In *Orthosuchus*, the bony palate was quite short, but Nash (1975) believes that it was functionally completed by soft tissue, indicating that the animal may have been partially aquatic.

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Some 230 to 65 million years ago, during the Mesozoic period, adaptive radiation of the crocodilians involved diversity in habits and snout form, presuming specialisation in catching fish by the slender-snouted types (Bellairs, 1987). The sub-order Mesosuchia, appearing in the Jurassic (195 to 140 million years ago), showed quite extensive bony palates, with internal nostrils "pushed" towards the posterior parts of the skull. Gharial-like teleosaurs and metriorhynchids (a truly marine group which had evolved paddle-like limbs and finned tails) were among the mesosuchians. In the late Jurassic, a generalized "mainstream" group, the goniopholids, and the little broad-snouted atoposaurs (average length of fourty centimetres; may have been terrestrial) appeared (Bellairs, 1987).

The Cretaceous period (140 to 65 million years ago) presented the sub-order Eusuchia (crocodilians of the most advanced kind). Eusuchia radiated into a variety of broad and narrow-snouted types (Langston, 1973). Although many spectacular types of reptiles perished during the holocaust (Cretaceous-Palaeogene extinction event) at the end of the Cretaceous, crocodilians from the Mesosuchian and Eusuchian lines somehow managed to survive and continue into the Tertiary (65 to 2 million years ago). The Mesosuchian and Eusuchian groups produced parallel lines of (possibly) terrestrial crocodiles having blade-like teeth with serrated edges, and both these lines may have persisted in Australia until about a million years ago. From the Eusuchia, a single family, the Crocodylia diverged about 100 million years ago (Buffetaut, 1979; Bellairs, 1987; Taplin, 1984a; Grigg and Kirshner, 2015).

From the order Crocodylia three lineages or superfamilies, recognized as clades (phylogenic taxonomy) are described: the gharial lineage (Gavialoidea), crocodile lineage (Crocodiloidea), and alligator and caiman lineage (Alligatoroidea). The surviving families or sub-clades of these clades are the Gavialidae, the Crocodylidae and the Alligatoridae (Richardson et al., 2000; Huchzermeyer, 2003; Grigg and Kirshner, 2015).

Currently there are 24 species of extant crocodilians recognised by the IUCN and CITES, of which two species are in the family Gavialidae, 14 species forming part of the Crocodylidae, and the Alligatoridae consisting of eight species. Table 1.1 is

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a summary of the extant crocodilian species as recognised by the Crocodile Specialist Group (http://www.iucncsg.org/; accessed 10 October 2016).

Table 1.1. The families and species that belong to the order Crocodilia (Source: http://www.iucncsg.org/pages/Crocodilian-Species.html).

Class Reptilia		
Order Crocodilia		
Family Alligatoridae (alligators, caimans)		
	American alligator	*Alligator mississippiensis*
	Chinese alligator	*Alligator sinensis*
	Black caiman	*Melanosuchus niger*
	Broad-snouted caiman	*Caiman latirostris*
	Spectacled caiman	*Caiman crocodilus*
	Yacare caiman	*Caiman yacare*
	Cuvier's Dwarf caiman	*Paleosuchus palpebrosus*
	Schneider's Smooth-fronted caiman	*Paleosuchus trigonatus*
Family Crocodylidae ("true" crocodiles)		
	African Slender-snouted crocodile	*Mecistops cataphractus* *
	American crocodile	*Crocodylus acutus*
	Australian Freshwater crocodile	*Crocodylus johnstoni*
	Cuban crocodile	*Crocodylus rhombifer*
	Dwarf crocodile	*Osteolaemus tetraspis*
	Morelet's crocodile	*Crocodylus moreletii*
	Mugger crocodile	*Crocodylus palustris*
	New Guinea Freshwater crocodile	*Crocodylus novaeguineae*
	Nile crocodile	*Crocodylus niloticus*
	West African crocodile	*Crocodylus suchus*
	Orinoco crocodile	*Crocodylus intermedius*
	Philippine crocodile	*Crocodylus mindorensis*
	Saltwater/estuarine crocodile	*Crocodylus porosus*
	Siamese crocodile	*Crocodylus siamensis*
Family Gavialidae (gharial and tomistoma)		
	Gharial	*Gavialis gangeticus*
	Tomistoma	*Tomistoma schlegelii*

* previously *Crocodylus cataphractus*

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Numerous genetic studies that were recently conducted provide more clarity on the phylogenic taxonomy of the extant (and even extirpated) crocodilians. Eaton et al. (2009), sequenced three mitochondrial and two nuclear genes from a large number of African dwarf crocodiles, confirming species diversity in the genus *Osteolamus* and, Meredith et al. (2011) reported on the revelation of a New World clade nested within *Crocodylus niloticus*. A study by Hekkala et al. (2011) revealed a cryptic evolutionary lineage within the Nile crocodile by examining DNA from samples throughout Africa, including from mummified crocodiles found in temples in Egypt. This study resulted in the West African crocodile, *Crocodylus suchus* (formerly part of *C. niloticus*) being recognised as a separate species, and the most recent addition to the Family of Crocodylidae. Eaton (2010) and Shirley et al. (2013) suggested an additional split in the two species from West Africa into five species, and the slender snouted crocodile, *Crocodylus cataphractus* to change to *Mecistops cataphractus*. However, none of this has been formalized to date. Shirley et al. (2013) also supported at least two species of the slender snouted crocodile (*Mecitops cataphractus*). In more recent studies, Milián-Garcia et al. (2015) provided evidence of hybridization between the Cuban crocodile (*Crocodylus rhombifer*) and the American crocodile (*Crocodylus acutus*), and Pacheco-Sierra et al. (2016) evaluated the hybridization between *Crocodylus acutus* and Morelet's crocodile (*Crocodylus moreletii*) using genetic and morphological data.

Crocodilians usually live in tropical and subtropical areas in various aquatic-type habitats. These carnivourous and opportunistic predators can be considered the largest freshwater dwellers (Martin, 2008), but may occasionally also prefer more saline waters, e.g the estuarine crocodile (*Crocodylus. porosus*) (Dunson, 1982; Mazzotti and Dunson, 1984).

Goombridge (1987) produced an outline of the distribution of world crocodilians by country. This was used, together with data by Grigg and Kirshner (2015), Hekkala et al. (2011) and Young (2011) to compile Table 1.2, to provide information on the distribution and habitat of the different crocodilian species currently recognised.

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Table 1.2. Distribution and habitat of world crocodilians (Goombridge, 1987; Grigg and Kirshner, 2015; Hekkala et al., 2011; Young, 2011).

Sub-family/species	Distribution	Habitat
Alligatoridae		
Alligator missisipiensis	South-eastern United States	Freshwater, marshes, streams, lakes, sometimes brackish coastal areas, temporary foraging in ocean
Alligator sinensis	South-east China	Freshwater rivers, streams, ponds and swamps
Caiman crocodilus	Northern two thirds of South America and north to the southern tip of Mexico	Freshwater wetland habitats, prefers slow moving water
Caiman latirostris	Argentina, Paraguay, Bolivia, Uruguay and drainage of Brazil's SE coast	Freshwater and brackish wetlands, and mangrove-lined estuaries on Brazilian coast
Caiman yacare	Lowlands of Argentina, Bolivia and SW Brazil, and Paraguay	Freshwater wetland habitats, eg. Pantanal
Melanosuchus niger	Northern South America	Freshwater rivers, streams, lakes, swamps, prefer slow moving waters
Paleosuchus palpebrosus	South America east of the Andes as far south as Paraguay	Forest streams, rivers, flooded forest
Paleosuchus trigonatus	South America east of the Andes as far south as Bolivia. Orinoco and Amazon drainage basins	Forest streams
Crocodylidae		
Crocodylus acutus	Central America from Mexico to Colombia, Cuba, Jamaica, Dominican Republic, Southern Florida	Fresh, brackish and coastal waters, coastal lagoons and mangrove swamps. Burrows
Crocodylus cataphractus	The West African countries, fronting the Atlantic ocean from Congo DR to Gambia	Riverine habitat with dense vegetation cover and large lakes. Also in brackish water and an off-shore island
Crocodylus intermedius	Orinoco River basin, Colombia, Venezuala	Freshwater rivers and waterlogged llanos in wet season. In dry season may travel overland to find pools and also dig burrows
Crocodylus johns(t)oni	Northern Australia	Freshwater river systems, lakes, billabongs and swamps. Some populations in brackish and even hyperosmotic estuaries
Crocodylus mindorensis	A Philippines endemic (suitable habitat on islands of Busuanga, Luzon, Masbate, Mindanao, Mindoro, Negros and Samar)	Freshwater lakes and ponds, marshes
Crocodylus moreletii	Caribbean-facing watersheds of Belize, Guatemala, Mexico	Freshwater swamps and marshes in forested areas, also brackish water
Crocodylus niloticus	Some African countries and Madagascar	Freshwater and brackish water, common in estuaries
Crocodylus novaeguinae	Iran, Jaya, Papua New Guinea	Freshwater swamps, marshes and lakes, rarely in coastal areas

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Table 1.2. (continued)

Sub-family/species	Distribution	Habitat
Crocodylus palustris	India, Sri Lanka, South Pakistan and South-East Iran, South Nepal	Freshwater rivers, lakes and marshes, preferring slow-moving, shallower areas. Also in reservoirs, irrigation canals, and other man-made bodies of freshwater
Crocodylus porosus	East coast of India through tropical and subtropical coastlines and seas to Western Pacific	Freshwater and brackish water, common in estuaries, capable of sea journeys
Crocodylus rhombifer	In one swamp on Cuba and another on a nearby island	Freshwater swamps, though tolerant or brackish water also
Crocodylus siamensis	Historically in Cambodia, Indonesia (incl. Borneo and possibly Java), Laos, Malaysia, Thailand, Vietnam	Thought to prefer slow-moving, sheltered parts of lakes, rivers, streams and swamps
Crocodylus suchus	West Africa	Dry interior of West Africa
Osteolaemus tetraspis	Congo, Gabon, Cameroon, and Central African Republic. Situation westwards, from Nigeria to Gambia uncertain	Mostly rainforest, but also a variety of freshwater habitats, including cool streams under closed canopy rainforest, savannah pools and even mangrove swamps
Tomistoma schlegeli	Peninsular Malaysia, Borneo, Sumatra, possibly Java	Forested freshwater lakes, slow-moving rivers and swamps, often with floating matsof vegetation. Reported to use burrows
Gavialidae		
Gavialis gangeticus	Northern India, Nepal, Ganges river drainage	Slower pools of fast flowing rivers. Fish-eaters, nest on samdbanks

In non-crocodilian reptiles (snakes, lizards and turtles), the heart is subdivided into three intraventricular compartments (this allows intracardiac mixing of oxygenated and deoxygenated blood). The crocodilian heart, however, as in birds and mammals, possesses a complete intervertricular septum, with separate and complete left and right ventricles (Grigg and Gans, 1993). This allows a full intracardiac separation of blood and flows in the systemic and pulmonary circulations. As in most reptiles, both left and right systemic aortas are present, but, particularly in larger specimens, asymmetry is observed with a larger right aorta. The left aorta comes from the right ventricle, adjacent to the pulmonary artery. Another unique feature of the crocodilian heart is the possibility of shunting of blood, away from the pulmonary circulation (the right ventricle gives rise to the pulmonary arteries as well as to the left aorta). Deoxygenated blood from the right ventricle is allowed to bypass the lungs and is recirculated into the systemic circulation (pulmonary-to-systemic shunt). At the base of each (right and left)

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aorta, a bicuspid valve is found; the two valves share a common wall in which the foramen of Panizza is located. (Axelsson, 2001) A unique valve mechanism is supported by connective tissue nodules (protruding into the outflow tract of the right ventricle), situated in the subpulmonary conus. The two aortic arches, connected just posterior to the heart by the aortic anastomosis, is another feature unique to the crocodilian circulation, and probably in close regulation with the Foramen of Panizza and subpulmonary conus, in the normal function of the cardiovascular system (Grigg, 1991).

Unlike in humans, all crocodilian blood cells possess nuclei, and the erythrocytes (as in other reptiles and birds) are oval, not round (Huchzermeyer, 2003; Grigg and Kirshner, 2015).

The mechanism of crocodilian ventilation is complex; involving the viscera (forward and aft movement), rib cage (costosternal movement), ribs in three joined sections (allow extended expansion of thorax), pubes (rotate to increase abdominal volume), gastralia (movement), and vertebral flexion and extension (Grigg and Kirshner, 2015).

Reproduction in sexually mature crocodilians starts with internal fertilization, followed by the female making a nest, laying the eggs, and often defending them (Grigg and Kirshner, 2015). After collection of eggs on the farm, females have been observed to continue guarding their nests (Huchzermeyer, 2003). The clutch size of a Nile crocodile varies between 35 and 50 eggs, but up to 95 have been reported (Guggisberg, 1972). Lance (1987) reported on the influence of diet and stress on clutch size. The size of crocodilian eggs varies with the species and size of the female that laid the eggs. The eggs have hard shells consisting of different calcified layers and are of oblong ovoid shape. Between the shell and the embryo, two membranes are found: a fibrous membrane facing the shell, and a limiting membrane (containing numerous tiny pores and fewer large pores) facing the embryo. Under optimal conditions, the production of one clutch per year is expected in a fertile female Nile crocodile, and the percentage of fertile eggs laid, is strongly influenced by weather conditions, nutrition and various stressing incidents (Huchzermeyer, 2003).

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Sex chromosomes are absent in crocodiles and the sex of the embryo is determined by the incubation temperature of the egg at a temperature-sensitive stage, early in embryonic development (Huchzermeyer, 2003). Considering the crocodile farmer's economical interest, production of faster growing male animals is desirable, for skin and meat harvesting. Lang and Andrews (1994) described evidence of temperature-dependent sex determination (TSD) in eleven crocodilian species (reviewing reports on five and presenting new data for six) which included *Crocodylus niloticus* showing a female-male-female (FMF) pattern; males at intermediate temperature, females at low and high temperatures. They also stated that the duration (cumulative effect) and/or magnitude (potency effect) of incubation temperatures during the thermosensitive period (TSP) could predictably alter sex ratios (Webb et al., 1987; Lang and Andrews, 1994).

Topical treatment of eggs at the beginning of the TSP, with exogenous steroid hormones, was successfully employed in attempts to achieve altered ratios of the expected sex of the hatchlings produced at a defined incubation temperature. Some differences in the oviducts and genitalia of treated animals compared to control animals were however, observed (Lang and Andrews, 1994).

Maintenance of a constant body temperature, independent of the environment, is not possible in exothermic reptiles like crocodiles. Environmental thermogradients are employed to cool down or warm up these animals. Rates are controlled by restriction or an increase of blood circulation to the body surface. Optimal temperature conditions can ensure high growth rates in juvenile crocodiles (Huchzermeyer, 2003; Grigg and Kirshner, 2015).

Exposure to hyperosmotic environments (like seawater) requires strategies (behavioural and physiological) to maintain salt homeostasis in crocodilians. (Leslie and Spotila, 2000). Although estuarine populations of four crocodile species; *C. porosus*, *C. johnstoni*, *C. niloticus* and *C. acutus* have been documented, all alligateroids and most crocodiles have poor salt tolerance and are mainly regarded as freshwater species (Huchzermeyer, 2003). Evidence of the

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functionality of lingual salt glands was found in the Nile crocodile showing osmoregulatory responses similar to the estuarine crocodile (Taplin and Loveridge, 1988). Grigg and Kirshner, (2015) reported that after slow acclimation, the Nile crocodile can osmoregulate effectively in seawater.

Reptilian kidneys lack loops of Henle (Moore et al., 2009), so reptiles consistently produce isosthenuric urine with specific gravity of 1.005 to 1.010, regardless of their hydration status (Moore et al., 2009; De La Navarre, 2006). Crocodilians lack a proper bladder, and the urine accumulates in the urinary chamber (in the cloaca) formed by the coprodeum and urodeum (very similar to the ostrich) (Myburgh et al., 2012). They have metanephric kidneys (unable to concentrate urine) and crocodilinae have lingual glands to excrete salt when taking in brackish water (Moore et al., 2009; Kuchel and Franklin, 1998).

Although crocodilians may be considered the oldest survivors, human modernisation is slowly taking its toll contributing to the decline in numbers of crocodilian worldwide due to habitat destruction and human-crocodile conflict. For more than a third of the crocodilian species, concern is raised regarding their conservation status. The International Union for Conservation of Nature (IUCN) Red List of Threatened Species (IUCN, 2016-2) is compiled by the Species Survival Comission (SSC) with assistance from the IUCN Species Program and is a scientifically based call to action for preventing the extinction of wild species around the world. Deficiencies in the Red List criteria have been described (Webb, 2008; Godfrey and Godley, 2008; Seminoff and Shanker, 2008), but it is still believed to give important guidance and is more or less in line with CITES listings.

Table 1.3 reflects data on the conservation status of crocodilians, published by the International Union for Conservation of Nature (IUCN) (Red List accessed through http://www.iucncsg.org, 10 October 2016). Of the 24 extant species, six are currently listed as Critically Endangered, one as Endangered and three are listed as Vulnerable.

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Table 1.3. Current IUCN Red List categories for crocodilians (Source: http://www.iucncsg.org/pages/Conservation-Status.html)

Common Name	Species Name	IUCN Red List Category
Chinese alligator	*Alligator sinensis*	CR
Philippine crocodile	*Crocodylus mindorensis*	CR
Orinoco crocodile	*Crocodylus intermedius*	CR
Siamese crocodile	*Crocodylus siamensis*	CR
Gharial	*Gavialis gangeticus*	CR
Cuban crocodile	*Crocodylus rhombifer*	CR
Tomistoma	*Tomistoma schlegelii*	EN
American crocodile	*Crocodylus acutus*	VU
Mugger crocodile	*Crocodylus palustris*	VU
Dwarf crocodile	*Osteolaemus tetraspis*	VU
American alligator	*Alligator mississippiensis*	LR (lc)
Australian freshwater crocodile	*Crocodylus johnstoni*	LR (lc)
Nile crocodile	*Crocodylus niloticus*	LR (lc)
New Guinea freshwater crocodile	*Crocodylus novaeguineae*	LR (lc)
Saltwater/estuarine crocodile	*Crocodylus porosus*	LR (lc)
Spectacled caiman	*Caiman crocodilus*	LR (lc)
Broad-snouted caiman	*Caiman latirostris*	LR (lc)
Yacare caiman	*Caiman yacare*	LR (lc)
Cuvier's smooth-fronted caiman	*Paleosuchus palpebrosus*	LR (lc)
Schneider's smooth-fronted caiman	*Paleosuchus trigonatus*	LR (lc)
Morelet's crocodile	*Crocodylus moreletii*	LR (cd)
Black caiman	*Melanosuchus niger*	LR (cd)
African slender-snouted crocodile	*Crocodylus cataphractus*	DD
West African crocodile	*Crocodylus suchus*	not assessed

- CR: Critically Endangered – considered to be facing an extremely high risk of extinction in the wild.
- EN: Endangered – considered to be facing a very high risk of extinction in the wild.
- VU: Vulnerable – considered to be facing a high risk of extinction in the wild.
- LR: Least Risk – widespread and abundant taxa, which can be further subdivided into one of three sub-categories: Conservation Dependent (cd), Near Threatened (nt) and Least Concern (lc)
- DD: Data Deficient – inadequate information to make a direct, or indirect, assessment of its risk of extinction based on distribution and/or population status. Data Deficient is not a category of threat.

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Crocodilians are apex predators and many anatomical and physiological features contribute to their evolutionary success. However, numerous reports have documented that reptilians have a great sensibility to pollutants (Crain et al., 1997; Guillette et al., 2000; Rey et al., 2009). Guillette and Iguchi (2003) documented contaminant-induced alterations in endocrine and reproductive functions in the American alligator. Studies on exposure to environmental pollutants and disruption of steroidogenesis and steroid signalling were reported (Guillette et al., 1996; Gunderson et al., 2001; Hamlin and Guillette, 2011; Arukwe et al., 2016).

Aquatic animals are recognized as valuable sentinels of ecosystem health (Bornman et al., 2007; Colborn and Thayer, 2000; Guillette and Edwards, 2008; Milnes and Guillette, 2008). Pollutants can accumulate in the body both through diet and from the aquatic environment, and because crocodilians are quite widespread through various habitats, it is plausible to assume that Nile crocodiles may be good sentinels to monitor aquatic ecosystem changes and its endocrine effects (Godal, 2015). A large demise of Nile crocodiles in the Kruger National Park, South Africa, was reported during 2008 (Myburgh, 2014; Ferreira and Pienaar, 2011). The most likely explanation was the consumption of the non-endemic fish, *Hypophthalmichthys molitrix*, during April/May of that year (Huchzermeyer, 2012; Myburgh, 2014; Ferreira and Pienaar, 2011). In addition, the health of wild Nile crocodiles may give an indication of the environmental status, which could also be a health indicator for the people living in the area (Warner et al., 2016).

The health-status of crocodilians is of great importance to crocodile farmers. Apart from the fact that captive breeding has an important conservation value, the crocodile farmers persue commercial value. Thorbjarnarson and Velasco (1999) reported that the export value of spectacled caiman (*Caiman crocodilus*), harvested (>1 million caiman) between 1983 and 1995 in Venezuela, was more than $US 115 million. Focusing on increased production of first grade skins in farmed estuarine crocodiles, Davis (2001) reported on nutrition and management aimed at improved growth rates and reduced disease and mortality rates. Contributing to the global luxury market for leather products, crocodile farming in Africa shows a yearly trade of over one million skins. These are from about 30

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African countries with Zimbabwe, Zambia and South Africa the leaders (Jan Myburgh pers. comm.).

Biomonitoring relies strongly on collected samples, so that chemical concentrations (Hays et al., 2007) or clinical effects (Barr et al., 2005a; Esteban and Castaño, 2008; Paustenbach and Galbraith, 2006) can be determined. Blood is the sample most often collected from crocodilians (Myburgh et al., 2014) due to its contact with the body and equilibrium with the organs and tissues (Esteban and Castaño, 2008), but blood sample collection has a disadvantage of being an invasive procedure (Barr et al., 2005b; Esteban and Castaño, 2008). The development of new methodology and modern analytical techniques allow scientists to use other samples that are less invasive, such as urine, hair and milk (Esteban and Castaño, 2008; Paustenbach and Galbraith, 2006).

Urine is a valuable diagnostic sample that is used daily to evaluate the health of animal and human patients (Bekker et al., 2001; Dufel, 1986). However, clinicians are very often guided by the specific clinical condition of the patient, in the choice of sample parameters to be evaluated. Most organic contaminants and pharmaceutical agents are biotransformed to water-soluble metabolites by the liver and excreted in the urine, making urine a very important diagnostic sample (Larsen, 1995; McClellan-Green et al., 2006). Applying this knowledge, measurement of pesticide metabolites in urine is commonly used to monitor exposure to these chemicals (Panuwet et al., 2008). Esteban and Castaňo (2008) reported that urine is the second most common sample used for human examination, and the preferred non-invasive sample for the diagnosis of heavy metal exposure.

It is relatively easy to collect clean urine samples from crocodilians (Myburgh et al., 2012), as shown in Figures 1.1 and 1.2, inserting a urinary dog catheter into the cloaca of a captive juvenile Nile crocodile. Furthermore, the biochemical evaluation of the urine samples might be, diagnostically, just as helpful as in humans and other animals (Esteban and Castaño, 2008; De La Navarre, 2006; Dufel, 1986; Huchzermeyer, 2003).

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This project addressed Nile crocodile urine as diagnostic tool, evaluating biochemical parameters measured in urine in relation to corresponding plasma concentrations. Steroid metabolites in crocodilian urine were also investigated using a well established method for urinary steroid profiling in humans (Bekker, 2004). These results may provide baseline concentrations as guidelines for the investigation of the health status of farm and wild Nile crocodiles.

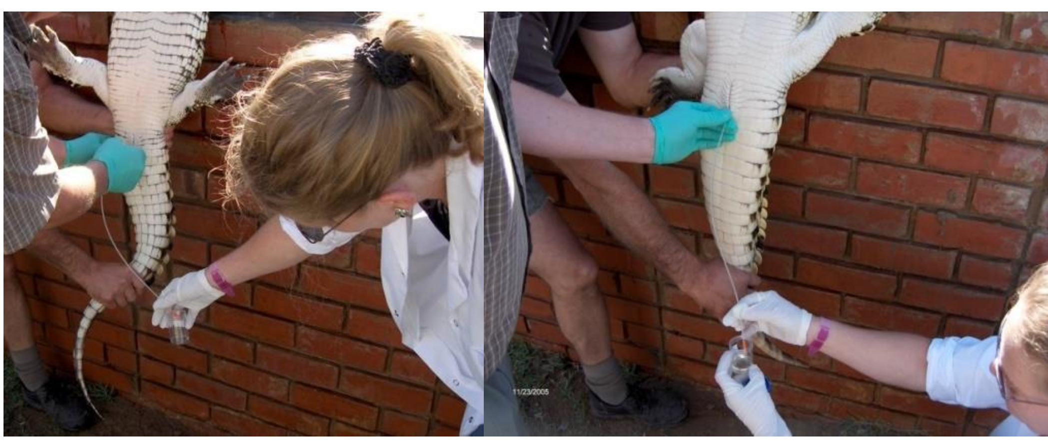

Figure 1.1. Urine being collected from a captive juvenile Nile crocodile by inserting a dog catheter into the cloaca.

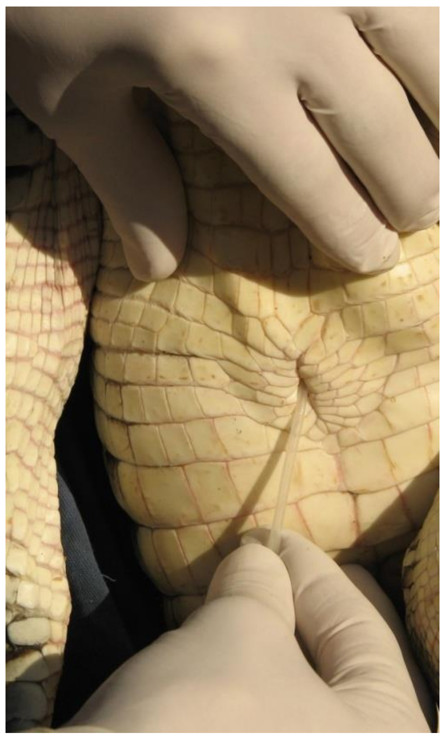

Figure 1.2. A close-up of the urine collection procedure.

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CHAPTER 2: AIM AND OBJECTIVES

2.1. Aim

The aim of this project was to evaluate urine from the Nile crocodile (*Crocodylus niloticus*) as sample for biochemical and hormonal analyses.

2.2. Objectives

To be able to achieve the aim of this specific research project the following objectives were undertaken:

- Determination of urine and plasma biochemical variables in juvenile Nile crocodiles by means of a standard veterinary clinical pathology profile, and establishment of the relationship between the urine- and the plasma biochemical variables (Chapter 5),
- Validation of the sample preparation and gas chromatographic mass spectrometric (GC/MS) technique by optimizing the sample preparation procedure and the instrument parameters to optimally detect steroid metabolites in crocodilian urine (Chapter 6),
- Application of this validated analytical method to perform qualitative and quantitative measurements of steroids and steroid metabolites in the urine of a cohort of individual crocodilians (Chapter 7),
- Investigation if a 6 h period was adequate to detect a significant increase of adrenal steroid metabolites in the urine of Nile crocodiles after an ACTH stimulation test (Chapter 8).

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CHAPTER 3: LITERATURE REVIEW

3.1. Urine sample analysis for diagnostic tools

3.1.1. Introduction

Testing of human urine to diagnose medical conditions originated over 6000 years ago, with evidence available of visual testing of the urine in the earliest civilizations (Echeverry et al. 2010). Until the 17th century, this was termed uroscopy, derived from the Greek '*ouron*' meaning '*urine*' and '*skopeo*', meaning to '*behold, contemplate, examine or inspect*'. Uroscopy is today known as urinalysis (Armstrong, 2007). Urinalysis was first introduced as part of the routine physical examination by Richard Bright in 1827, linking albuminuria to kidney pathology (Bright, 1827; Weller and Nester, 1972; Bright P, 1983; Fine, 1986; Berry et al., 1992). Advances in laboratory test equipment led to the development of dip-sticks (strip tests), which today form a very important part of a clinical urine sample investigation. The advantages are that it provides a quick and in-depth assessment and as a diagnostic tool of the urinary system (Lynch and Wu, 2013).

Abnormal urinalysis results may disclose evidence of diseases, even some that do not yet show significant visual signs or symptoms. Urinalysis remains a valuable method of diagnosis; commonly part of a routine health screening (Wu, 2010), but additional tests and clinical assessment are often needed to further investigate and ultimately diagnose the causes of underlying health problems (Simmerville et al., 2005). Non-routine urinalyses include screening that may reveal chemical exposure (occupational health) (Ahmed et al., 2008; Yin et al., 2016), intake of herbal compounds (monitoring), and a pregnancy test (Simmerville et al., 2005). Chemical testing of urine samples has the added advantage that large numbers of samples can be screened in short periods by automated laboratory equipment. Furthermore, the effectiveness of urine testing has been improved by recent advances in chemical and microscopic techniques (Roxe, 1990; Wu, 2010).

Urine collection is a non-invasive sampling technique, even in newborns (Herreros Fernández et al., 2013) and mammalian species (Kersey and Dehnhard, 2014) which renders urine a popular sample in diagnostic medicine. Random urine

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samples, also called spot samples are preferred for routine urinalysis and other tests e.g. pregnancy diagnosis and drug screening. For the pregnancy test, the human urine sample should be a first morning void, or a random specimen with a specific gravity of at least 1.010. Most other tests on random samples should preferably be performed on a midstream specimen (Schmiemann et al., 2010). A 24 h urine collection is the usual standard for urine chemistry tests and the volume of the collection should be recorded to aid in the reporting of the result, e.g. mmol or mg analyte per day (Guyton and Hall, 2006a). When these tests are performed on random samples, they can be normalised against creatinine (Arndt, 2009).

Changes in urine composition begin to take place as soon as it is voided, therefore great care should be taken with collection (avoiding contamination), transportation, storage and handling to maintain the integrity of the specimen (Simmerville et al., 2005). Urinalysis should preferably, be performed within 15 min of collection of the sample. A maximum storage time of 2 h per sample at room temperature and exposed to minimal light (or stored in darkness) is preferable. Longer storage at 4°C up to 24 h will slow down, but not prevent the decomposition process (Echeverry et al., 2010). Some tests require the addition of preservatives (e.g. 10 ml concentrated HCl/24 h urine collection for Ca measurement), whereas others (e.g. samples for drug testing) should be collected with no preservatives. For some urine tests, dietary restrictions may be required, e.g. a normal sodium diet for urinary aldosterone determination is required before and during the collection. If the patient could not refrain from medications that may interfere with test values (e.g. thiazide diuretics during Ca determination) a thorough medication history should be recorded (Wu, 2010).

3.1.2. Laboratory analysis of urine

Simple human urinalysis can be performed in a doctor's consulting room, or more complex analysis can be carried out in the laboratory when requested by a physician. The urine is physically examined regarding colour, odour, clarity, volume, crystal formation and specific gravity. Chemical examination of urine is performed using a reagent strip (dip-stick) test, which includes the identification of

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protein, blood cells, glucose, pH, bilirubin, urobilinogen, ketone bodies, nitrites and leukocyte esterase, and the tablet test (copper reduction test), for quantitative confirmation of reducing substances. Finally, microscopic examination entails the detection of crystals, cells, casts and microorganisms (Echeverry et al., 2010; Strasinger and Di Lorenzo, 2014).

Gross examination of urine involves checking the appearance and colour, odour and volume (Wu, 2010). The appearance may be noted as cloudy (turbid), clear or layered. Precipitation of phosphate crystals in an alkaline urine specimen, or precipitation of calcium and urate salts due to refrigeration, may also affect clarity (Simmerville et al., 2005). A turbid appearance may be due to the presence of white cells and casts, vaginal secretions, sperm, bacteria, blood clots, small calculi, fecal material, and less often chylomicrons (Echeverry et al., 2005). Colour variations observed are from pale (almost colourless) yellow to dark yellow, even red, green or blue. Urine may be abnormal in colour and cloudy due to infection or other non-pathological causes (Simmerville et al., 2005; Echeverry et al., 2010). The concentration of the urine usually determines the colour, but certain foods (e.g. beets, asparagus, blackberries), ingested dyes (e.g. food colouring) and a number of medications can affect urine colour (Lynch and Wu, 2013). Urine appears dark (even olive green) in the presence of bilirubin, which may be indicative of liver disease. Injury to the urinary tract or menstrual contamination may produce hemoglobin and urine will present with a pink or red colour. Brownish black urine colour is observed in cases of alkaptonuria and malignant melanoma. Dyes (e.g. in medications) or the presence of riboflavin (B complex vitamin), may cause urine to be coloured green or blue. Medications like pyridium (used in the treatment of urinary tract infections), warfarin, and laxatives, as well as B complex vitamins and carotene, can cause urine to have an orange colour (Strasinger and Di Lorenzo, 2014). Fresh urine normally has a faint aromatic odour, whereas in urinary tract infections, a noxious faecal smell may be present; diabetic urine can have a fruity smell, and certain inborn errors of metabolism can cause characteristic odours in urine (Simmerville et al., 2005; Echeverry et al., 2010; Strasinger and Di Lorenzo, 2014).

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The balance between fluid ingestion and water lost from the body (lungs, sweat, intestine) could be indicated by urine volume; excessive excretion (polyuria) can be indicative of diseases susch as diabetes mellitis and insipidus, chronic renal disease, acromegaly and myxedema; reduced excretion (volumes <200 mL/day) is observed in nephritis, urinary tract obstruction, acute kidney injury and kidney failure. To evaluate the ability of the kidneys to concentrate or dilute urine, specific gravity and osmolality tests are performed. The correlation of urine specific gravity (USG) with urine osmolality gives an indication of the hydration status of the patient. An increased USG is associated with glycosuria and the syndrome of inappropriate antidiuretic hormone. With diuretic use, or in diabetes insipidus, adrenal insufficiency, aldosteronism, and impaired renal function, the USG may be decreased (Simmerville et al., 2005; Echeverry et al., 2010; Wu, 2010).

Urinary pH values can reflect the acid-base status of a patient, but may be influenced by diet and medications. In normal healthy humans, the urine pH is mostly slightly acidic due to predominant acid formation, but can rise after meals during the “alkaline tide”. Alkaline urine is usually associated with urinary tract infections and Fanconi syndrome (Simmerville et al., 2005; Echeverry et al., 2010; Strasinger and Di Lorenzo, 2014).

Reagent strip tests are used to examine the presence of substances which are not normally found in urine and may indicate disease or pathology, namely: bilirubin (a pigment in bile; may indicate liver disease); glucose (may indicate diabetes); haemoglobin (iron-containing pigment in red blood cells may indicate injury of the urinary tract, anaemia or infection); ketones (e.g. acetone may indicate diabetes or metabolic disease); protein (may indicate kidney disease); and urobilinogen (a by-product of bilirubin; may be found in trace amounts in normal urine, but elevated in patients with severe liver disease or hemolytic disorders) (Echeverry et al., 2010).

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The copper reduction test or Benedict's test is a non-specific test for reducing substances, and employs a tablet which, upon reaction with the urine sample, will cause a colour reation, indicating the presence of reducing sugars. Apart from glucose, fructose, galactose (clinically most significant, indicating galactosemia), lactose, maltose and pentose can cause a positive reaction, but the tablet test is subject to interferences from other reducing substances like ascorbic acid, certain drug metabolites, and antibiotics such as cephalosporins (Strasinger and Di Lorenzo, 2014).

Sediment in human urine may be microscopically analysed to confirm the presence of: bacteria and other microorganisms (indicating infection); blood cells (red blood cells indicate hematuria, white blood cells indicate infection); casts (e.g. hyaline, granular; may indicate kidney disease); epithelial cells from the lining of the urinary tract, vagina, or from the kidney (abnormal quantities may indicate injury, inflammation or neoplasm); crystals (may indicate metabolic disease); fat (may indicate nephrotic syndrome of diabetic nephropathy); or renal tubular cells (may indicate acute tubular necrosis) (Simmerville et al., 2005; Lynch and Wu, 2013; Strasinger and Di Lorenzo, 2014).

3.1.3. Specific laboratory analyses

Biochemical tests that are available to be performed on urine samples include tests on the routine biochemistry auto-analyzer, as well as analyses on more sophisticated equipment in the modern laboratory. Routine tests include the normal urinary test panels: sodium (Na), potassium (K), chloride (Cl), urea, creatinine, ionised calcium (iCa), magnesium (Mg), phosphorous (P), and uric acid (U/A) (Frank, 2013; Harwell, 2013). Additionally, concentrations of total protein, microalbumin, Beta-2 macroglobulin, ammonium ion (NH_4^+), amylase, myoglobin, hormones (incl. PTH, cortisol and aldosterone), certain metals and pharmaceuticals (acetaminophen, salicylate and digoxin) may be determined (Schütz et al., 2006; Keil, 2013) .Urine protein electrophoresis (UPEP) and immunofixation electrophoresis (IFE) tests are performed on separate instruments, and require somewhat more manual handling regarding sample preparation and

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application (Keil, 2013). Atomic absorption spectroscopy (AAS), optical emission spectroscopy (OES) or inductively coupled plasma spectroscopy (ICP) could be applied for the analysis of some metals (Goullé et al., 2005; Keil, 2013). Analyses of vanillylmandelic acid (VMA), catecholamines, metanephrines, oxalic acid, toxins, anabolic steroids and steroid profiling need manual sample preparation and handling and are performed on sophisticated chromatography equipment (HPLC, LC-MS/MS, GC or GC-MS) (Yap et al., 1992; Ojanpera et al.; 2005; Krone et al., 2010; Drees et al., 2013).

3.1.4. Routine testing of urine samples in veterinary medicine

In veterinary medicine, the examination of urine samples (urinalysis) is not only useful to evaluate renal function, but may also confirm various systemic diseases. Clinical analyses of urine samples include examining the physical characteristics (colour, transparency, odour, volume, solute concentrations) chemistry (urinary test strips), and sediment (microscopic examination). Qualitative assessment of substances that are not usually present in the urine is common practice on midstream (“clean catch”) samples. Most normal urine constituents are excreted in a diurnal pattern, and influenced by the the rate of excretion by the kidney. However, collecting 24 h urine samples from animals is not only impractical but lacks baseline normal concentrations. Quantitation of these urinary parameters on midstream spot samples can be achieved by normalization with urinary creatinine, which has a relatively constant excretion rate (Kerr, 2002a; Tripathi et al., 2011).

Normal animal urine is typically yellow or amber in colour (depending on the concentration of the urine) and freshly voided urine is usually transparent or clear (Kerr, 2002a). Changes in the colour or clarity of the urine should be further investigated, to confirm systemic abnormalities or infections (Tripathi et al., 2011). For most animal species, normal urine has a slight odour of ammonia, which could become quite prominent in retained urine or old urine specimens, or in the case of a bacterial infection in the urinary tract. The prescence of ketones can cause an acetone odour, and certain drugs can give a characteristic odour to the urine. Abnormal urine volume may be caused by a variety of conditions, including: both polyuria and oliguria (acute renal disease); polyuria (hepatic failure, pituitary

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diabetes insipidus, pyelonephritis, stress); and oliguria (dehydration, shock, urinary tract obstruction). Exact determination of urine output is difficult unless making use of a metabolism cage, measuring 24 h urine volume. Alternatively, urine volume could be inferred from urine specific gravity or osmolality, which is also measured to determine solute concentration in the urine (Tripathi et al., 2011).

In the veterinary consulting room, chemical analysis of spot urine specimens is also achieved with urine test strips (dip-stick tests), available from a variety of manufacturers. This allows qualitative or semi-quantitative determination of nitrites, pH level, protein, blood (red blood cells or free haemoglobin), glucose, ketones, bilirubin and urobilinogen, all of which can indicate disease, injury, or defects (Kerr, 2002a). Microscopic examination of urine sediment (the solid part of urine) should be performed to complete a routine urinalysis. A drop of resuspended urine sediment is placed on a glass slide and inspected for the presence of epithelial cells, erythrocytes, leukocytes, casts, mucous, fat, bacteria, sperm, parasite ova, fungi, algae and crystals (Tripathi et al., 2011).

Inter-species variability complicates the evaluation of animal urine biochemistry. A basic test panel is offered by most veterinary clinical pathology laboratories. Tests usually included in this panel are: Na, K, Cl, iCa, Mg, P, urea, creatinine, U/A, and osmolality. Evaluation of fractional excretion and/or clearance of electrolytes and other substances provide information regarding glomerular function (Braun and Lefebvre, 2008; Tripathi et al., 2011).

3.2. Urine from crocodilians

All crocodilians lack a distinct bladder, and urine is stored in the cloaca (Huchzermeyer, 2003; Myburgh et al., 2012; Grigg and Kirshner, 2015). To date, examinations of cloacal urine mostly focused on osmoregulation and nitrogenous excretion. Laboratory invesigations focusing on osmolality, and concentrations of Na, K, Cl, urea, U/A and ammonia were done on urine samples collected from the estuarine crocodile (*Crocodylus porosus)* (Dantzler and Braun, 1980; Grigg et al., 1980; Pidcock et al., 1997; Taplin and Loveridge, 1988; Kuchel and Franklin, 1998); American alligator (*Alligator. mississipiensis*) (Herbert, 1981; Lauren, 1985;

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Pidcock et al., 1997); Australian freshwater crocodile (*Crocodylus. johnstoni*) (Dantzler and Braun, 1980; Taplin et al., 1999), American crocodile (*Crocodylus acutus*) (Dantzler and Braun, 1980; Ellis, 1981); and Nile crocodile (*Crocodylus niloticus*) (Taplin and Loveridge,1998; Leslie and Spotila, 2000). In these studies, the urine was found to be hypo-osmotic to blood; the liquid fraction of the cloacal urine was extremely low in Na, with Cl usually much higher; and urea absent or detected in only small quantities. If the animals were in fresh water, K, similar to N, would be very low in the urine, but the concentrations of Cl and K would significantly rise in hyper-osmotic conditions.

In crocodiles, urine may be affected by post-renal water reabsorption, and the low levels of urinary Na may be due to Na excretion via the salt glands (Grigg and Kirshner, 2015). According to Grigg and Gans (1993), the mesonephric kidneys, supported by the cloaca and lingual salt glands, take care of ionic and osmotic regulation. Kuchel and Franklin (1998), who investigated kidney and cloaca function in the estuarine crocodile, reported that crocodilians possess metanephric kidneys (unable to concentrate urine), and have functional salt glands.

It is known that the true crocodiles (Crocodylidae) differ from alligators and caimans (Alligatoridae) in having lingual salt excreting organs (Pidcock et al., 1997; Kuchel and Franklin, 1998). In the estuarine crocodile and the Nile crocodile, the lingual salt glands are truely functional (Dunson, 1970; Taplin, 1985; Grigg et al., 1980; Taplin and Loveridge, 1998; Huchzermeyer, 2003). This ability of crocodylids to excrete excess salt through lingual salt glands is an indication of their saltwater origin (Pidcock et al., 1997; Taplin and Grigg, 1981; Taplin et al., 1982; Cramp et al., 2008).

In the Nile crocodile it was also confirmed by Leslie and Spotila (2000), that physiological osmoregulation involves lingual salt glands in saline habitats. The study was conducted on Nile crocodiles of three age classes, exposed to water of extremely high salinity (17 and 35 ppt NaCl) after being reared in fresh water, and noted dehydration, lethargy, ceasing to feed and loss of mass in the animals.

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Exposure to gradually increasing salinities (3 to 35 ppt), allowing short acclimation periods at each salinity, saw the crocodiles survive, kept on feeding and had an increase in mass and size. Plasma osmolality was observed to be relatively constant across the salinity spectrum, whereas variability in the cloacal urine osmolality, not related to increasing salinity, was monitored. Cloacal urine Na and Cl concentrations tended to decrease, whereas the K concentration tended to increase. It was concluded that the Nile crocodile possesses the physiological ability to survive and thrive in periodically hyper-osmotic environments, but need an acclimation period at lower salinities (Leslie and Spotila, 2000).

In crocodilians, ammonia (excreted as ammonium bicarbonate) and U/A are the principal nitrogenous end-products (Huchzermeyer, 2003; Singer, 2003). The urine of crocodilians (*C. porosus*) that spent time in fresh water is copious (abundant), with a clear and dilute appearance. Cloacal urine from crocodilians that were exposed to hyperosmotic conditions, appears opaque (white and cream-like) and is often decreased in volume (Grigg and Gans, 1993). Khalil and Haggag (1958) described excretions in the Nile crocodile: "*partly as liquid urine and partly as amorphous white thread-like deposits*". Cragg et al. (1961) reported that the urine samples of Nile crocodiles and spectacled caiman (*Caiman crocodilus*) were: "*a colourless liquid with a solid white deposit*". Hopping (1923) reported on nitrogen excretion in the American alligator; in summary his findings were: urea up to 17%, ammonia 50-85% and U/A 7-20%. Coulson et al. (1950) claimed that American alligators are ammonoteles. However, they may have overlooked the presence of solid deposits that were found to contain large amounts of U/A (Herbert, 1981; Pidcock et al., 1997). Khalil and Haggag (1958) found that the deposits in the urine of Nile crocodiles contained 88.61% U/A, concluding that whole urine consists of 25.4% ammonia, 4.5% urea and 68.5% U/A. Overwhelming evidence was published that crocodiles and most probably alligators too, are in fact ammono-uricoteles (Schmidt-Nielsen and Skadhauge, 1966; Herbert, 1981), and not ammonoteles as believed earlier. Leslie and Spotila (2000) observed a marked increase in the U/A concentration of Nile crocodile urine with increasing salinity.

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3.3. Crocodilian blood parameters

Morpurgo et al. (1992) measured Ca concentrations, while Leslie and Spotila (2000) reported on Na, Cl, K and osmolality in Nile crocodiles. In the estuarine crocodile, Taplin (1984b) investigated electrolytes and osmolality, and Kuchel and Franklin (1998) measured N, Cl, K and osmolality. Taplin et al. (1999) reported on Na, K, Cl and osmolality in *C. johnstoni*. In the American alligator, Hopping (1923) reported on blood sugar, urea, ammonia and chloride; Guillette et al. (1997) measured total protein, phosphorus and Ca; Lance et al. (2004) studied glucose and protein. Huchzermeyer (2003) listed blood biochemistry parameters in six crocodilian species, including the Nile crocodile. Concentrations of total protein, albumin, globulin, glucose, Ca, P, Na, K, Mg, Cl, total serum iron, U/A, urea, bilirubin, cholesterol, triglyceride, creatinine, GOT, GPT, ALP, ALT, AST, BUN, T_4, T_3, rT_3 (reverse T_3, the inactive form of T_3, secreted instead of normal T_3 in the presence of acute illness, eg. with inflammation and under stressfull conditions) and LDH were reported.

For all the above variables, individual concentrations were published and therefore no suitable reference ranges are available. With the exception of Lauren (1985), who investigated urine osmolality, Na, K, Cl, U/A, and urine:plasma (U/P) ratios in American alligators, no other crocodilian species has been investigated regarding the U/P ratios of biochemical parameters. Moreover, no Nile crocodile blood and urine hormonal (steroids) studies have been published.

Extensive investigations on serum and plasma parameters have been reported, reflecting the reproductive and adrenal endocrine systems of other crocodilians: Lauren (1985) measured corticosterone in the plasma of American alligators exposed to salt water; plasma samples were analysed for oestradiol-17β, testosterone and corticosterone in a study of the reproductive cycle of female American alligators (Guillette et al., 1997); Lance et al. (2003) analysed plasma from juvenile and adult male and female American alligators in a study investigating testicular production of oestradiol-17β; Lance et al. (2004) monitored the effect of capture on the testosterone secretion in male American alligators.

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Furthermore, contaminant-induced endocrine disruption was investigated: Guillette and Iguchi (2003) reported on contaminant-induced endocrine and reproductive alterations in the American alligator. Plasma testosterone concentrations were monitored in juvenile alligators living in a lake contaminated with DDT (Guillette et al., 1996). Plasma oestradiol-17β and testosterone concentrations were evaluated in juvenile alligators from contaminated and control lakes in Florida, USA (Guillette et al., 1994; Guillette et al., 1996; Guillette et al., 1999; Moore et al., 2012); and Milnes et al (2005) measured testosterone in the plasma of Amerian alligator hatchlings to determine the effect of ovo-exposure to *p,p'* DDE. Serum testosterone concentrations in male broad-snouted caiman (*Caiman latirostris)*, prenatally exposed to pesticides, were reported by Rey et al. (2009); and plasma testosterone and DHEA concentrations were measured in male American alligators in a Barrior Island population near Kennedy Space Centre, Florida, USA (Hamlin et al., 2011).

Apart from biochemical and hormonal studies, reports on crocodilian blood pharmacokinetic studies and systemic metal concentrations have also been published. Helmick et al. (2004a) reported on oxytetracycline-, and Helmick et al. (2004b) studied enrofloxacin kinetics in the plasma of American alligators. Warner et al. (2016) determined blood lead concentrations in 34 sub-adult free-ranging Nile crocodiles, to monitor exposure (primarily through oral ingestion) to this inert heavy metal. The subjects were selected from three separate populations, namely Lake St. Lucia Estuary, Ndumo Game Reserve and Kosi Bay, South Africa.

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CHAPTER 4: JUSTIFICATION

Nile crocodile (*Crocodylus niloticus*) farming in South Africa and Africa has expanded substantially in recent years (Van As, 2016). There are currently more than 80 commercial Nile crocodile farms in South Africa, with an estimated million crocodiles in captivity (Gerry Swan pers. comm.). Income is primarily from the sale of good quality skins, while some of the South African farmers also export crocodile meat. In order to achieve their economic goals, commercial crocodile farmers must apply scientific principles to optimize Nile crocodile health, production, nutrition and welfare. Due to Nile crocodile's susceptibility to stress (Ganswindt et al., 2014), farmers are constantly trying to create a suitable environment that supports the health and welfare of the animals, improves productivity and so economic viability. However, monitoring of the Nile crocodile's health and production is only achievable by using validated laboratory tests.

While Nile crocodile numbers on commercial farms are increasing, most populations of wild Nile crocodiles in southern Africa are declining and becoming threatened (Combrink et al., 2011; Ferreira and Pienaar, 2011; Botha et al., 2011). The threats affecting wild crocodiles are mostly due to human-crocodile conflict, habitat destruction and anthropogenic changes to aquatic ecosystems (Combrink et al., 2013; Arukwe et al, 2016). Similarly, their health also needs to be monitored using diagnostic samples that are relatively easy to collect and employing laboratory tests that were validated for the Nile crocodile (Myburgh, 2014).

Urine as a diagnostic tool may become a critically important role-player in future. It is easy to collect through a non-invasive and atraumatic process and can play a very important role as a diagnostic tool. This study was therefore planned and conducted to investigate if urine from the Nile crocodile is a suitable sample for biochemical and hormonal analyses.

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The potential benefits of this PhD project are:

1. The baseline concentrations for plasma and urine biochemistry parameters (using standard clinical pathology tests) will be established for healthy Nile crocodiles. These results may be employed as guidelines when evaluating laboratory results, in future.
2. The determination of urine to plasma (U/P) ratios for the Nile crocodile has not been investigated before. This may be employed in the evaluation of individual crocodile's urine biochemistry results.
3. The clinically validated GC/MS method will be employed to quantitate steroids and their metabolites in Nile crocodile urine. This will contribute significantly to future investigations of endocrine function in the Nile crocodile by means of steroid profiling.
4. Future application of this validated analytical method to determine the presence (identify) and concentrations of steroid metabolites in the urine of individual crocodilians (different species), may lead to collaboration with international research institutions and individual scientists.
5. Guidelines will be determined for the assessment of adrenocortical activity during an ACTH stimulation test by monitoring glucocorticoid concentrations in urine using the GC/MS method.

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CHAPTER 5: Nile crocodile (*Crocodylus niloticus*) urine and plasma biochemistry investigation

5.1. Introduction

The number of Nile crocodiles (*Crocodylus niloticus*) in captivity is increasing in southern Africa due to the positive demand from the luxury leather industry (Van As, 2016). Contrary to the increase in crocodiles on commercial farms, wild crocodile numbers are declining (Combrink et al., 2011; Botha et al., 2011; Ferreira and Pienaar, 2011; Zisadza-Gandiwa et al., 2013; Dabrowski et al., 2013). The ability to successfully diagnose the causes of ill health or poisonings in Nile crocodiles without harming or sacrificing animals would be a significant advantage. Myburgh et al. (2012) reported that urine sample collection in the Nile crocodile is a relatively simple and an atraumatic procedure. In humans urine samples are routinely used for diagnostic purposes (Armstrong, 2007; Echeverry et al., 2010). Similarly, in veterinary medicine, urine is routinely used to monitor the health of animals (Tripathi, 2011). Urine as a potential diagnostic sample has, however, not received a lot of attention in crocodilians (Myburgh et al., 2012).

In some human diseases, changes in the composition of urine may be detected before any clinical symptoms are observed (Simerville et al., 2005; NIDDK, 2013). As urine samples are generally easily obtained from human patients (Herreros Fernández et al., 2013; Esteban and Castaňo, 2008), urine was one of the first diagnostic samples to be investigated (Eknoyan, 2007; Tuuminen, 2012). In humans and animals, urine samples may be tested to investigate or confirm: urinary tract infections (Wilson and Gaido, 2004; Schmiemann et al., 2010); kidney function, metabolic status (Dufel, 1986; Kamel et al., 1990; NIDDK, 2013); renal disease (Johnson et al., 2004; Giles et al., 2008); pregnancy (Johnson et al., 2009); and endocrine function (Vestergaard, 1978; Venturelli et al., 1995; Honour et al., 1991). In occupational health monitoring, urine samples are commonly used to screen for exposure to toxic chemicals (Ahmed et al, 2008; Colín-Torres et al., 2014). Urine samples may also be tested for: metals and metalloids (Goullé et al., 2005; Minnich et al., 2008; Esteban and Castaňo, 2009); biomarkers of hormonally

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active environmental agents (Wolff et al., 2007; Yin et al., 2016); pharmaceuticals and drugs of abuse (Theodoridis et al., 2004; Ojanpera et al., 2005; Schutz et al., 2006); cotinine (Hou et al, 2012), metabolites of pesticides (Esteban and Castaňo, 2009); endogenous (Shackleton, 1986; Yap et al, 1992; Honour and Brook, 1997; Turpeinen, 1997; Honour, 2001) and anabolic (Dumasia et al., 1985; Houghton et al., 1988; Haber et al., 2001; Guan et al., 2005; Buiarelli et al., 2010) steroids.

Even though some variables have been investigated, urine has not been used as a routine diagnostic sample in crocodilians. The focus on crocodilian urine in the past was mostly on osmoregulation (Dunson, 1982; Taplin and Loveridge, 1988; Leslie and Spotila, 2000) and nitrogenous excretion (Khalil and Haggag, 1958; Cragg et al., 1961; Dunson, 1970; Herbert, 1981). If compared with other crocodilian species, especially the estuarine crocodile (*Crocodylus porosus*) (Taplin, 1984b; Taplin, 1985; Kuchel and Franklin, 1998), the Australian freshwater crocodile (*Crocodylus johnstoni*) (Taplin et al., 1999), the American crocodile) (*Crocodylus acutus*) (Schmidt-Nielsen and Skadhauge, 1967; Ellis, 1981) and the American alligator (*A. mississippiensis*) (Lewis, 1918; Hopping, 1923; Herbert, 1981; Lauren, 1985), it is obvious that not much was done on Nile crocodile urine. Khalil and Hagag (1958) monitored total nitrogen (N), ammonia N, urea N, U/A N and undetermined N in urine, as well as total N, water soluble N, ammonia N, urea N, U/A N and water insoluble N, in urinary deposits of Nile crocodiles. Leslie and Spotila (2000) evaluated the exposure to water with increased salinity, by measuring electrolytes (Na, Cl, K) and osmolality in Nile crocodile urine and plasma. Taplin and Loveridge (1988) investigated urinary electrolytes (Na and K) and osmolality in Nile crocodiles and estuarine crocodiles.

Although crocodilians lack a distinct bladder it is possible to collect clean urine from the urinary chamber (Myburgh et al., 2012). The Nile crocodile cloaca consists of three obvious chambers: a caudal chamber (proctoceum), a smooth-walled middle chamber (urodeum), and a cranial chamber (coprodeum). The

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urinary chamber is a combination of the urodeum and coprodeum, in which the urine accumulates (Myburgh et al., 2012).

A potential complicating factor in crocodilian urine biochemistry is that the urine composition may be changed in the cloaca during storage (Skadhauge and Duvdevant, 1977; Skadhauge, 1977; Laverty and Skadhauge, 2008). Post-renal modification of the urine has been reported in the estuarine (saltwater) crocodile exposed to three environmental salinities, where osmotic pressure, Cl, K and Na were tested in the urine (Kuchel and Franklin, 1998).

Urinary biochemical parameters routinely used in mammalian veterinary diagnostics are: Na; K; Cl, urea, creatinine, iCa, Mg, P, U/A and osmolality (Wright George and Zabolotzky, 2011; Cottam et al., 2002). The urine protein to creatinine ratio (UPC) is often determined to aid in/support early diagnosis of chronic kidney disease (Waldrop, 2008). Due to urinary parameters showing a wide range when concentrations are measured in randomly collected samples, measurements are more often given as a ratio to urinary creatinine concentrations (Stechman et al., 2010). Electrolyte clearance may be quantified by comparison to the clearance of endogenous creatinine (Tripathi et al., 2011). Measurements of urinary parameters are most valuable in conjunction with plasma/serum measurements due to the balance between body fluid compartments (Carlson, 2008).

In mammals, Na concentrations in urine may be low in prerenal azotaemia, including severe hypovolemia (Guyton and Hall, 2006a). Elevated urinary Na concentrations may be seen in acute tubular necrosis, as well as in several conditions including post-renal causes of azotaemia, hypothyroidism, hypoadrenocorticism, and diuretic use (Waldrop, 2008). Secretion of K by the kidneys may be decreased in hypoaldosteronism (Lathan and Tyler, 2005) and excessive urinary K loss is associated with chronic renal disease, and renal tubular acidosis (Borgoignie et al., 1981; Autran de Morais, 2009). Urinary Cl excretion,

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similar to Na excretion, is decreased with hypovolemia, and elevated with acute renal tubular dysfunction, due to renal ischemia, prolonged prerenal azotaemia, endogenous or exogenous toxins (Waldrop, 2008).

Urinary urea concentrations in mammals may be low in the presence of renal azotaemia, with a significant decrease in glomerular filtration rate (GFR) (Tripathi et al., 2011); or decreased renal blood flow due to pre-renal causes (dehydration or decreased cardiac output); or post-renal causes (urethral obstruction or ruptured bladder); or primary renal dysfunction (Guyton and Hall 2006b). Increased urinary urea concentrations may indicate increased protein intake (Erikkson and Valtonen, 1982); increased protein breakdown in the body (Kerr, 2002b); or chronic renal failure (Deguchi and Akuzawa, 1997). The tubular resorption of urea is decreased and excretion increased in the presence of diuresis (Grauer, 2009). Creatinine concentrations in urine may be used in conjunction with serum levels to determine creatinine clearance (Watson et al., 2002) and evaluate GFR (Von Hendy-Willson and Pressler, 2011). Low creatinine clearances are seen in chronic renal failure (Deguchi and Akuzawa, 1997). Although reference ranges of urine creatinine are dependent on sex and/or muscle-mass, creatinine shows a constant rate of excretion through glomerular filtration, hence urinary creatinine concentrations can be employed in establishment of the integrity and completeness of a urine sample. Currently, in clinical biochemistry, analyte/creatinine ratios are employed in the normalization of other mammalian urinary parameters (Arndt, 2009; Hou et al., 2012).

High iCa concentrations in mammalian urine may indicate impaired parathyroid hormone (PTH) function and consequent decreased vitamin D activation (Ferguson and Hoenig, 2011), and also predict urolithiasis (Stevenson and Markwell, 2001). Increased urinary iCa excretion was detected in Nile crocodiles exposed to stressful conditions (Huchzermeyer F, unpublished data). In mammals, low iCa concentrations in urine may be due to nutritional deficiency, malabsorption (decreased Ca and/or vitamin D intake or malabsorption) or secondary

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hyperparathyroidism (Ferguson and Hoenig, 2011). Decreased Mg excretion can be indicative of inadequate Mg intake, impaired intestinal Mg absorption, or chronic Mg depletion (Jahnen-Deschent and Ketteler, 2012; Mellema and Hoareau, 2014), or dietary induced Mg deficiencies (Norris et al., 1999). Increased Mg excretion may suggest renal wasting of Mg, owing to medication or the physiological status of the patient (Jahnen-Deschent and Ketteler, 2012). Phosphorous concentrations in mammalian urine may be influenced by dietary intake (Al-Jurf and Chapmann-Furr, 1986), extracellular volume (Gradowska et al., 1973), proximal tubular defects (Riordan and Schaer, 2005), abnormal intestinal absorption of P (Schroder et al., 1996), PTH abnormalities, vitamin D status (Clark, 1991) and intestinal infarction (Jamieson, 1979).

U/A is the final compound of purine catabolism in humans. In most mammals, U/A is converted by the enzyme uricase, to allantoin, which is easily eliminated through urine. Overproduction of U/A is associated with disease severity, especially with cardiovascular disease states and urolithiasis (Foschi, 2008). The urinary NH_4^+ concentration can be assessed by using the urine osmolality to determine the osmolal gap (Kamel et al., 1990). The synthesis and excretion of ammonia play a major role in chronic acidosis.

Urinary osmolality measurements are useful in differentiating prerenal and renal causes of azotemia (Waldrop, 2008), assessing hydration status; and in diagnosis of disorders such as diabetes insipidus, or assessment of disorders involving antidiuretic hormone (ADH) (Van Vonderen et al., 1997; Waldrop, 2008). In mammalians, balances between the different body compartments are controlled by feedback mechanisms at a high level, referred to as homeostasis. Plasma osmolality and solute concentrations are usually kept within narrow ranges, while a wide variation is seen in the urine (Guyton and Hall, 2006a; Waldrop, 2008).

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Testing blood plasma or serum in animals can confirm circulating levels of Na, K, Cl, total Ca (protein bound and free Ca), iCa, Mg, P, urea, creatinine, U/A, total protein and glucose (Rose et al., 1980). Similarly, in crocodilian plasma, concentrations have been reported on the following biochemical variables: Na, Cl, K and osmolality (Taplin, 1984b; Kuchel and Franklin, 1998; Taplin et al., 1999; Leslie and Spotila, 2000); blood sugar, urea, NH_4^+ and Cl (Hopping, 1923); total protein, phosphorus and Ca (Morpurgo et al., 1992; Guillette et al., 1997; Lance et al., 2004); total protein, albumin, globulin, glucose, Ca, P, Na, K, Mg, Cl, total serum iron, U/A, urea, bilirubin, cholesterol, triglyceride, creatinine, GOT, GPT, ALP, ALT, AST, BUN, T_4, T_3, rT_3 and LDH (Huchzermeyer, 2003).

The objectives of this study were to do an in-depth investigation of urinary and plasma biochemical variables in the Nile crocodile. The following variables were determined in the urine: Na, K, Cl, iCa, Mg, P, urea, creatinine, U/A and In the plasma, Na, K, Cl, total Ca (protein bound and free Ca), iCa, Mg, P, urea, creatinine, U/A, total protein and glucose were quantitated. Baseline concentrations would be established. In addition, the ratio between urine and blood biochemical variable concentrations would be investigated to determine whether a positive correlation exists for specific variables.

5.2. Materials and methods

5.2.1. Collection of samples and subjects

Urine and blood samples were collected from healthy captive bred juvenile Nile crocodiles (approximately two years old, total length: 1.8 to 2m) at Izintaba crocodile farm close to the University of Pretoria Faculty of Veterinary Science, Onderstepoort, South Africa.

Blood samples were collected from the post-occipital spinal venous sinus according to the technique described by Myburgh et al. (2014). Each blood sample was collected with new 5 ml syringe (Omnifix®, B Braun, Germany) and 20G needle (Terumo Corporation, Japan), and transferred to a 4 ml lithium heparin

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plastic blood collection tube (SGVac, The Scientific Group, South Africa) immediately after collection. The tubes were transferred on ice and centrifuged in the Pharmacology and Toxicology laboratory, Faculty of Veterinary Science, Onderstepoort (within 60 min), after which the plasma was aliquotted into eppendorf tubes. A plasma aliquot of each subject was sent to the Onderstepoort Veterinary Clinical Pathology laboratory to be analysed, or stored at -20°C if immediate transfer to the laboratory was not possible.

Urine was collected with a urinary catheter as described by Myburgh et al. (2012). The urine samples were collected in 30 mL screw-top containers. All the urine samples transferred on ice and kept at 2 to 8°C until it was centrifuged in the Pharmacology and Toxicology laboratory, Faculty of Veterinary Science, Onderstepoort (within 8 h). The clean urine was transferred to sterile tubes and marked accordingly, and stored at -20°C until transfer to AMPATH laboratories to be analysed.

Samples were only selected for laboratory analysis where sufficient plasma and urine were collected from the same animal. In cases where urine or plasma was insufficient or absent, the samples from that specific animal were removed from this investigation. Hence, matching sets of urine and plasma results from the same subject were used to determine the correlation of variables measured between urine and plasma.

5.2.2. Laboratory examination of the urine samples

The following biochemical parameters were evaluated in the Nile crocodile urine samples (n = 101): Na, K, Cl, iCa, Mg, P, urea, creatinine, U/A, NH_4^+ and osmolality. Na, K, Cl, iCa, Mg, P, urea, creatinine and U/A were analyzed with the Beckman Coulter LX20 (Beckman Coulter, Inc., Brea, CA, USA) up to April 2006, and the Integra Cobas Roche/ Hitachi Modular P800 (Roche Diagnostics, Mannheim Germany) from 6 April 2006 onwards. (During the period that the samples were collected and sent for analyses to AMPATH laboratories, Pretoria, which is a SANAS accredited laboratory, the Beckman instrument was replaced by

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the Roche instrument. The methodology principles for the tests requested, are similar for both instrumens. These automated platforms were both utilised for daily routine analysis of patient samples and therefore fully validated to provide consistent results. This was achieved with calibration procedures as well as internal and external quality control.) NH_4^+ was determined separately on x10 to x20 dilutions in deionised water of each urine sample with Macherey-Nagel Quantofix® ammonium test strips (Macherey Nagel, Düren, Germany). Osmolality was determined with the Advanced® Model *3320 Micro-Osmometer* (Advanced Instruments, Inc., Norwood, MA, USA).

5.2.3. Laboratory examination of the plasma samples

The corresponding Nile crocodile plasma samples (n = 101) were tested for the following biochemical variables: Na, K, Cl, total Ca, iCa, Mg, P, urea, creatinine, U/A, total protein, osmolality and glucose. Na, K, Cl and iCa was analyzed with the RapidLab (Bayer, Leverkusen, Germany), and total Ca, Mg, P, urea, creatinine, U/A, total protein and glucose, with the NexCT analyzer (Alfa Wassermann, West Caldwell, NJ, USA). Osmolality was determined with the Osmomat (Gonotech GmbH, Berlin, Germany).

5.2.4. Statistical assessment

5.2.4.1. Bayesian estimates of biochemical parameters measured in urine and plasma in Nile crocodiles

The statistical distribution of the measured variables were assessed. For all Bayesian variable estimation (estimates of the mean measured concentrations), parameters that approximately followed a lognormal distribution were log-transformed, and parameters that approximately followed a normal distribution, were retained, unaltered. Of the 12 variables measured in the urine, ten needed to be log-transformed (iCa, Cl, creatinine, K, Mg, Na, P, NH_4^+, NH_4^+:U/A, ratio), and of the 13 variables measured in plasma, five required transformation (Ca, glucose, P, urea, U/A). The mean concentrations of the variables measured in urine and plasma were estimated using Bayesian methods. For each analysis, the posterior distribution for the sample mean (μ) was estimated using Markov Chain Monte

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Carlo (MCMC) methods using r2jags (Su and Yajima, 2015), JAGS (Plummer, 2003), and R (R Core Team, 2015). For each analysis three chains, 60 000 iterations, a burn in of 1000 iterations per chain, and a thinning rate of 10 was used, to give a total of 17 700 simulations per model from which posterior inference was drawn. Diffuse priors was used in all cases for group means (Normal [μ = 0, σ2 = 1 × 106]) and diffuse uniform priors was used for the variance of residual error (Uniform[0, 100]).

MCMC chain convergence was assessed using traceplot mixing, autocorrelation plots, and potential scale reduction factors. For Bayesian credible intervals on estimates of μ, 95% highest posterior density intervals (HPDI) was used to report the median, upper HPDI, and lower HPDI of the posterior distribution on μ. There is thus a 95% probability that the true mean for the measured variable lies within the given interval. The median, upper HPDI, and lower HPDI for back-transformed posterior distributions is reported for variables that were log-transformed.

5.2.4.2. Correlation of parameters measured between urine and plasma

The correlations between variables measured in urine and plasma were assessed using Bayesian methods. For each analysis, the posterior distribution for Pearson's product moment correlation (*ρ* or rho) was estimated using Markov Chain Monte Carlo (MCMC) methods employing rjags (Plummer, 2015), JAGS (Plummer, 2003), and R (R Core Team, 2015). For each analysis three chains, 18 0000 iterations, a burn in of 1000 iterations per chain, and a thinning rate of 10, were used to give a total of 15 000 simulations per model from which posterior interference was drawn. Diffuse priors were used in all cases for group means (*Normal*[$\mu = 0, \sigma^2 = 1 \times 10^6$]) and diffuse uniform priors for the variance of residual error (*Uniform*[0,1000]) and for our estimate of *ρ* (*Uniform*[-1,1)].

MCMC chain convergence was assessed using traceplot mixing, autocorrelation plots, and potential scale reduction factors. For credible intervals on estimates of *ρ*, 95% highest posterior density intervals were used, and the posterior distribution of *ρ* for each comparison is graphically reported in Figure 5.1 using the 2.5%, 50%

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(median), and 97.5% quantiles, along with Bayesian *p*-values, which indicate the probability of $\rho > 0$ or $\rho < 0$.

Random samples from the estimated bivariate normal distributions, generated by our models, illustrated how well each model fitted the data. The original data (measurements from urine and plasma for a given variable) were plotted with two super-imposed ellipses covering 50% and 95%, respectively, of the density of the posterior bivariate distribution. These model fits and the original data were presented alongside the density curves, for each associated posterior distribution on ρ.

5.3. Results

5.3.1. Urine

A summary of the Nile crocodile urine results is given in Table 5.1, showing reporting units, lower HPDI, median and upper HPDI concentrations for Na, K, Cl, urea, creatinine, iCa, Mg, P, U/A, osmolality, NH_4^+:and NH_4^+:U/A ratio. Appendix 5.1 shows the Nile crocodile urine results of all the subjects in the study.

Table 5.1. A summary of the captive juvenile Nile crocodile urine (n = 101) results for 11 biochemical variables and the NH_4^+:U/A ratio.

Variable	unit	Lower	Median	Upper
Sodium	mmol/L	3.728	4.631	5.635
Potassium	mmol/L	20.40	22.66	24.97
Chloride	mmol/L	18.56	20.40	22.22
Urea	mmol/L	39.31	42.23	45.10
Creatinine	µmol/L	10.32	16.18	22.89
Ionised Calcium	mmol/L	0.357	0.435	0.518
Magnesium	mmol/L	0.015	0.025	0.038
Phosphorous	mmol/L	5.808	7.010	8.269
Uric acid	mmol/L	0.145	0.173	0.204
Osmolality	mosm/kg	197.4	206.7	216.4
NH_4^+	mg/L	2373	2667	2965
NH_4^+:U/A ratio	n/a	12064	15399	19136

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5.3.2. Plasma

A summary of the plasma results is given in Table 5.2, showing reporting units, lower HPDI, median and upper HPDI concentrations for Na, K, Cl, urea, creatinine, total Ca, iCa, Mg, P, U/A, osmolality, total protein and glucose. Appendix 5.2 shows the plasma results of all the subjects in the study.

Table 5.2. A summary of the captive juvenile Nile crocodile plasma (n = 101) results for 13 biochemical variables.

Variable	unit	Lower	Median	Upper
Sodium	mmol/L	138.3	140.4	142.6
Potassium	mmol/L	3.941	4.080	4.211
Chloride	mmol/L	114.0	116.1	118.2
Urea	mmol/L	0.105	0.151	0.210
Creatinine	µmol/L	24.14	25.62	27.06
Calcium	mmol/L	2.707	2.755	2.803
Ionised Calcium	mmol/L	1.364	1.387	1.412
Magnesium	mmol/L	1.288	1.346	1.403
Phosphorous	mmol/L	1.386	1.462	1.539
Uric acid	mmol/L	0.169	0.192	0.218
Osmolality	mosm/kg	306.4	312.1	317.7
Total protein	g/L	52.70	55.06	57.33
Glucose	mmol/L	6.198	6.766	7.372

5.3.3. Relation of the urine- to the plasma biochemical parameters

The correlation between Nile crocodile urine- and plasma-measured concentrations for eight of the ten biochemical variables was low, with Bayesian credible intervals covering $\rho = 0$ (Figure 5.1), suggesting no association between urine- and plasma-measured concentrations. However, for U/A and Cl, the association between the urine- and plasma-measured concentrations was positive and moderately strong to strong, respectively, suggesting that urine measurements for these two biochemical variables may serve as a proxy for plasma measurements. These results are emphasized by plots of model fits and Bayesian posterior distributions on ρ (Figure 5.2) where Cl and U/A were the only variables that exhibited a positive association (with relatively low variability) between their urine- and plasma-derived concentrations.

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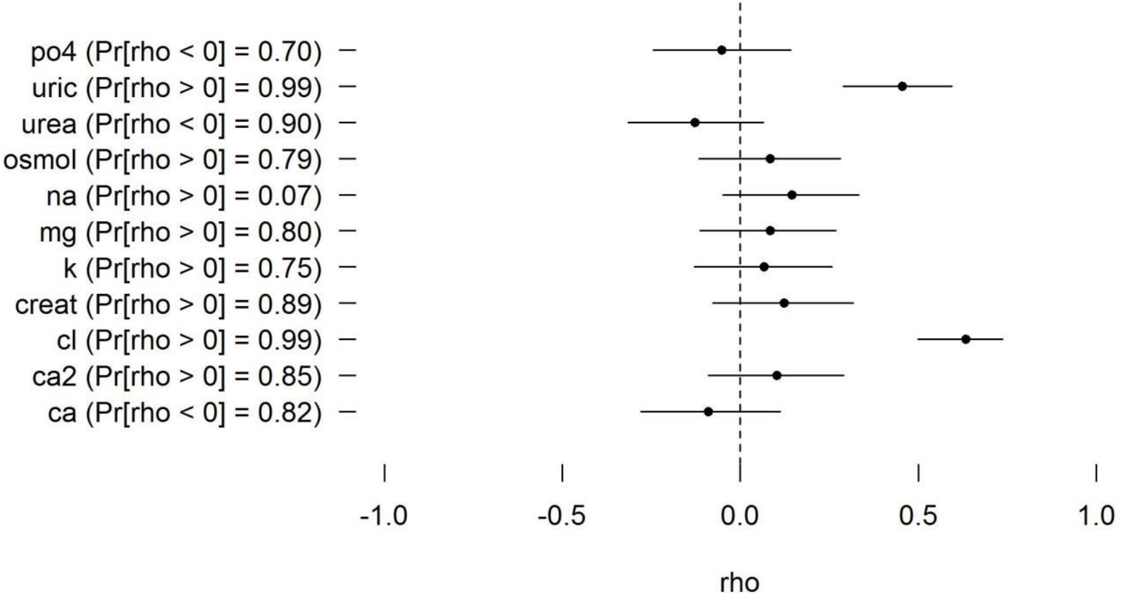

Figure 5.1. Posterior distributions of *p* for each comparison using the 2.5%, 50% (median), and 97.5% quantiles, along with Bayesian *p*-values, indicating the probability of $\rho > 0$ or $\rho < 0$

po4	**represents P**
uric	**represents U/A**
na	**represents Na**
mg	**represents Mg**
k	**represents K**
creat	**represents creatinine**
cl	**represents Cl**
ca2	**represents ionized Ca**
ca	**represents Ca**

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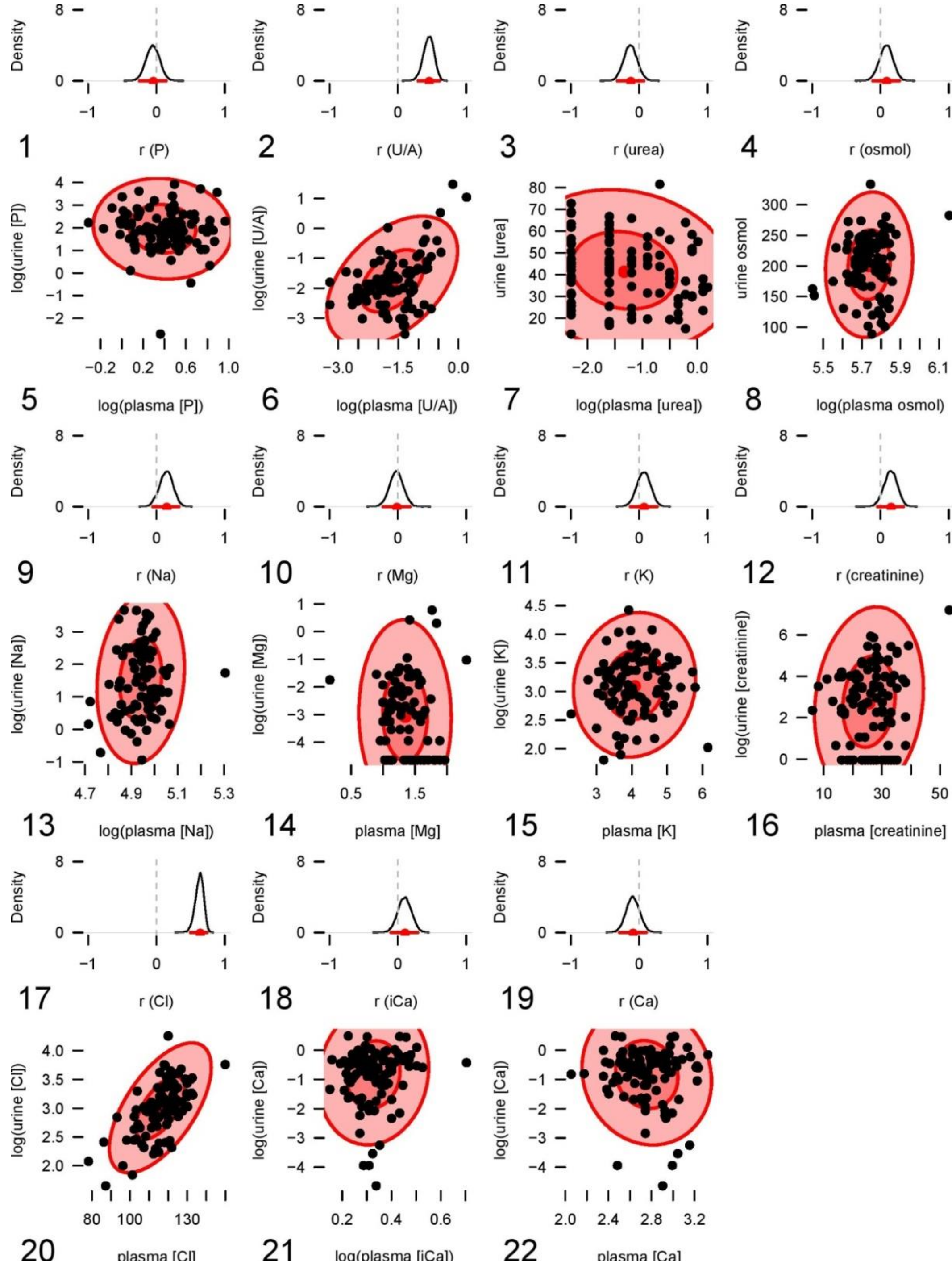

Figure 5.2. Posterior distributions on Pearson's sample product-moment correlation (*r*) including 95% highest posterior density credible intervals (red lines) and posterior median (red dot) [1-4.9-12-17-19], along with model fits (red curves, encompassing 50% [dark shading] and 95% [light shading] of the posterior distribution) and original data (black dots) [5-8, 13-16, 20-22].

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Figure 5.2 legend (cont.): Correlations were between plasma and urine P [1,5], plasma and urine U/A [2,6], plasma and urine urea [3,7], plasma and urine osmolality [4,8], plasma and urine Na [9,13], plasma and urine Mg [10,14], plasma and urine K [11,15], plasma and urine creatinine [12,16], plasma and urine Cl [17,20], plasma and urine iCa [18,21], plasma Ca and urine iCa [19,22].

5.4. Discussion

In humans and mammalian animals, plasma and urinary biochemical concentrations are well-documented and so is the correlation between plasma and urinary concentrations (Harwell, 2013; Frank, 2013; Stechman et al., 2010). Harwell (2013) reported on concentrations of Na, K, Cl, iCa, Mg, P and osmolality in human plasma and urine, and Frank (2013) described normal reference ranges for urea, creatinine, U/A, and NH_4^+. Stechman et al. (2010) documented plasma Na, K, Cl, urea, creatinine, Ca, P, alkaline phosphatase, albumin, cholesterol and glucose; and urinary Na, K, iCa, P, glucose and protein, providing data for the evaluation of homeostasis in mice. Unfortunately, this aspect has not received much attention in crocodilian physiological investigations (Huchzermeyer, 2003; Grigg and Kirshner, 2015; Myburgh, 2014), and in the Nile crocodile, investigation on urine:plasma ratios have been limited to a few biochemical parameters, namely Na, K, Cl and osmolality (Schmidt-Nielsen and Skadhauge, 1967; Lauren, 1985;).

This is the first study investigating a large array of Nile crocodile urine biochemical variables and comparing the urine concentrations with those of corresponding blood variables. The subjects (n = 101) were of a similar age and from a controlled freshwater captive environment. The slaughter procedure at the abbatoir did not include sexing of the animals. Concentrations of urinary Na, K, Cl, iCa, Mg, P, urea, creatinine, U/A, osmolality and NH_4^+; and plasma total protein, glucose, Na, K, Cl, total Ca, iCa, Mg, P, urea, creatinine, U/A, and osmolality, were determined.

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In an attempt to put the results from this study into perspective, Tables 5.3 and 5.4 compares results from available published data on crocodilian biochemistry parameters, including species other than the Nile crocodile.

Table 5.3. Concentrations of biochemical variables in urine of captive juvenile Nile crocodiles (n = 101) compared with other crocodilians.

Variable/ unit	This study Nile crocodile	Other Nile crocodile	Estuarine crocodile	American crocodile	Australian freshwater crocodile	American alligator
Na / mmol/L	3.728-5.653	-	8.6+/-2.9	5.9	1.0+/-0.0	16.1+/-0.5
K / mmol/L	20.40-24.97	3.2-47.1	67.0+/-13.3	2.7	18.4+/-4.29	1.5+/-0.4
Cl / mmol/L	18.56-22.22	10-155	8.7+/-4.8	1.5-30.1	4.0+/-1.82	9.5+/-1.6
Urea* / mmol/L	39.31-45.10	-	-	-	-	0.0-5.82
Creatinine µmol/L	10.32-22.89	-	-	-	-	-
iCa / mmol/L	0.357-0.518	-	-	-	-	-
Mg / mmol/L	0.015-0.038	-	-	-	-	-
P / mmol/L	5.808-8.269	-	-	-	-	-
U/A* / mmol/L	0.145-0.204	0.165 0.065-0.52	-	-	-	1.095-2.46
Osmolality mosm/kg	197.4-206.7	327.6+/-72.5	-	238-269	232+/-19.4 184.5+/-7.8	244.0+/-3.9
NH_4^{+}* mg/L	2373-2667	1542	-	1854-2556	-	1400-2080
NH_4^{+} : U/A ratio	12064-15399	-	-	-	-	-

*Sources: Nile crocodiles (Khalil and Haggag, 1958; Leslie and Spotila, 2000; Huchzermeyer, 2003), estuarine crocodiles (Taplin, 1984b; Kuchel and Franklin, 1998), American alligators (Lauren, 1985), Australian freshwater crocodiles (Taplin et al., 1999), and American crocodiles (Schmidt-Nielsen and Skadhauge, 1966) **Urea, U/A and NH_4^{+}: Nitrogenous exctretion of the liquid part of the urine only, was used to compare with N excretion from this study.

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Table 5.4. Concentrations of biochemical variables in plasma of captive juvenile Nile crocodiles (n = 101) compared with other crocodilians.

Variable/ unit	This study Nile crocodile	Other Nile crocodile	Estuarine crocodile	American crocodile	Australian freshwater crocodile	American alligator
Na / mmol/L	138.3-142.6	143-161 144-180	152.7+/-0.9 158.4+/-3.3	-	149+/-2.5	147.4+/-2.0
K / mmol/L	3.941-4.211	3.8-7.2 3.2-5.5	4.5+/-0.2 4.4+/-0.2	-	3.9+/-0.24	3.4+/-0.5
Cl / mmol/L	114.0-118.2	88.5-120.5 110-140	94.3+/-3.7 116.6+/-4.5	-	113+/-3.8	119+/-3.0 52.5
Urea / mmol/L	0.105-0.210	0.60-2.62	-	-	-	-
Creatinine µmol/L	24.14-27.06	36.5-97.0	-	-	-	-
Ca / mmol/L	2.707-2.803	2.97 2.23-3.00	-	-	-	2.34 2.6 - 4.3
iCa / mmol/L	1.364-1.412	-	-	-	-	-
Mg / mmol/L	1.288-1.403	0.52	-	-	-	-
P / mmol/L	1.386-1.539	0.88-1.96	-	-	-	4.3 1.5 - 3.3
U/A / mmol/L	0.169-0.218	0.065-0.52	-	-	-	-
Osmolality mosm/kg	306.4-317.7	315.3+/-7.3	300.8+/-3.8	289-296	293.15+/-3.1	291+/-6.5
Total protein g/L	52.70-57.33	50.2 41	-	-	-	47 - 77
Glucose/ mmol/L	6.198-7.372	5.9	-	-	-	1.64- 5.38

*Sources: Nile crocodiles (Khalil and HagLeslie and Spotila, 2000; Huchzermeyer, 2003), estuarine crocodiles (Taplin, 1984b; Kuchel and Franklin, 1998), American alligators (Hopping, 1923; Lauren, 1985), Australian freshwater crocodiles (Taplin et al., 1999), and American crocodiles (Schmidt-Nielsen and Skadhauge, 1966).

. Selection criteria was based on factors that may influence the concentrations of certain variables, namely: sampling at different salinities, cloacal vs urethral urine, age/size and optimal feeding conditions. Reporting units were converted to correspond where applicable, however, means and ranges were not changed and shown as originally published. More than one concentration per parameter for a specific species features below each other in the cells in the table.

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Observations of the comparisons of urine and plasma biochemical parameters with other studies (Tables 5.3 and 5.4) were discussed below. Rather big differences in Na, K and Cl urine concentrations were seen between results from this study and results from studies on other groups of Nile crocodiles and other crocodilian species. Insufficient data were available for urinary urea, creatinine, iCa, Mg, P and NH_4^+. Many studies addressed urea as a percentage of total excreted nitrogen, and concentrations were not reported clearly. Urinary osmolality was found to be lower in this study than reported for other Nile crocodiles and slightly lower or equal to findings in other crocodilian species. Results for urinary U/A overlapped with data from other Nile crocodiles but were much lower than concentrations found in American alligators.

Plasma Na, K and Cl concentrations were very similar to results in other groups of Nile crocodiles and other crocodilian species.Urea and creatinine in plasma were lower than concentrations published previously for Nile crocodiles. No significant differences were observed in the plasma Ca concentrations published for Nile crocodiles and American alligators. However, in most published data it was not stated whether total Ca or iCa was measured, hence data was considered to be total Ca concentrations. In female adult (>2m) American alligators seasonal differences were reported for Ca, with a range wider than was found in this study. Plasma Mg concentrations found in other Nile crocodiles differed greatly from Mg concentrations determined in this study. Plasma P concentrations have been found in a slightly narrower range from other Nile crocodile studies. In American alligators, much higher concentrations were reported in one study, while another, in female adult (>2m) alligators showed seasonal differences with a much wider range overlapping results from this study in the lower range. Ranges for osmolality in plasma was found to be very similar to ranges reported for Nile crocodiles and not much different from osmolalities found in other species. The range for plasma U/A in this study was narrower and fitted into the range reported in several Nile crocodile studies. Two variables that were not included in the urine profile, showed plasma total protein concentrations slightly higher than in other Nile crocodiles, but

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fitting into the wide range found in female adult (>2m) American alligators. Total protein investigations included the determination of seasonal variation. Plasma glucose measurements in this study were slightly higher than in other Nile crocodiles, and it was noted that glucose concentrations in American alligators were reported to have seasonal changes.

Taking into consideration that in all the results that were published, there were numerous animal variables and differences in sampling procedures. More studies are required addressing specific age groups, gender differences, salinity of the environment, stress, nutrition, and season.

Valuable information may be gained from urinary biochemical investigtions. Questions that can be asked is:

- U/A and NH_4^+ concentrations are measured to monitor protein intake in farmed crocodilians (Herbert, 1981). Can the excreted ratio of these two parameters, in future, be applied to assess optimal feeding of Nile crocodiles? Economically, it would be very valuable to optimise the protein consumption in farmed crocodiles by means of biochemical testing.
- The role of circulating Ca levels in stress-induced metabolic disorder was reported by Morpurgo et al. (1992), and reports on the mechanisms and impact of stress on captive Nile crocodiles have been addressed (Lance, 1994; Smith and Marais, 1994; Lance et al., 2000). Huchzermeyer (1986) reported on osteoporosis and osteomalacia. Except for Coulson and Hernandez (1964), who included iCa when listing the urine composition of *A. mississippiensis*, nothing was reported regarding iCa excretion in these subjects, which bestows the question on whether stress effects Ca excretion in crocodiles through glucocorticoid release by the adrenals. Glucocorticosteroids will affect the function of osteoblasts and osteoclasts. Could other endocrine problems be diagnosed by a urine sample?
- What is the value of the urine to plasma ratios that showed a positive correlation? In this study, the correlation between urine- and plasma-measured concentrations for eight (Na, K, iCa, Mg, P, urea, creatinine, and osmolality) of

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the 10 biochemical variables suggested no association between urine- and plasma-measured concentrations. However, for Cl and U/A, the association between the urine- and plasma-measured concentrations was positive and moderately strong, suggesting that urine measurements for these two biochemical variables may serve as a proxy for plasma measurements.

A possible explanation for the poor correlation found between urine and plasma biochemical variables in the Nile crocodile could be the post-renal modification of urine in the cloaca (Skadhauge and Duvdevant, 1977; Skadhauge, 1977; Laverty and Skadhauge, 2008). The post-renal modification in the urine by Nile crocodiles will definitely influence the diagnostic value of urine samples – the longer the urine is stored in the cloaca the more it could be affected. Therefore, it was not expected to find matching biochemical variables in the urine and plasma. The physiological role of the coprodeum and urodeum in the post-renal modification of urine was not investigated by Myburgh et al. (2012) and needs further investigation in the Nile crocodile. In addition, it would be very helpful to know the volume of water intake per animal per day (L of water per kg of animal per 24 h); frequency of urination per day; if they prefer to urinate in the water or on land; and the volume of urine production per day (volume of urine per kg body mass per 24 h).

The cloaca of alligatorids has little impact on the composition of accumulated urine and it seems as if alligatorids largely depend on a renal response when exposed to saltwater (Moore et al., 2009; Dantzler and Braun, 1980). This inability of alligatorids to handle excess salt is an indication of their freshwater origin (Pidcock et al., 1997). However, at Cape Canaveral on the east coast of the United States of America, American alligators are permanently found in brackish / estuarine water (personal communication R Lowers, 2008). The respective abilities of crocodiles and alligators to survive in saline waters appear linked at least in part to these differences. The estuarine crocodile is highly competent in salt water while the American alligator appears unable to regulate plasma electrolytes in

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hyperosmotic salt water. For the estuarine crocodile, Kuchel and Franklin (1998) noted that both Na and Cl are almost completely reabsorbed under hyposmotic conditions. This may indicate the necessity for secretory activity by the lingual salt glands.

5.5. Conclusion

In our study, plasma and urinary biochemical concentrations may contribute to the understanding of the normal concentrations (reference intervals) for crocodylians. Our animals were not selected or exposed to any specific conditions. Our results for these variables in young Nile crocodiles. may serve as baseline data for future urine examinations in the Nile crocodile.

Cl and U/A are the parameters with the most potential for future use, and therefore, a follow-up study on the value of Cl and U/A excretion as indicator of homeostasis in the Nile crocodile would be valuable. These two urinary variables were the only of those investigated, to show a positive correlation with plasma concentrations. However, the use of urine as a routine diagnostic sample in crocodiles is a rather unique opportunity to monitor health and requires further investigation regarding the clinical usefulness.

5.6. Acknowledgements

The guidance of Dr. Fritz Huchzermeyer is appreciated and acknowledged. The owner and staff of Izintaba crocodile farm for assistance with this research project.

5.7. Proposed publication from this chapter

Lasya C Bekker, Peter N Laver, Maria M van Niekerk, Amelia Goddard, Jan G Myburgh. Urine and plasma concentrations of routine biochemical analytes in healthy pre-slaughter Nile crocodiles (*Crocodylus niloticus*). Journal of Veterinary Diagnostic Investigations.

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CHAPTER 6: Clinical validation of the analysis of steroids and steroid metabolites in Nile crocodile urine

6.1. Introduction

In humans and mammals, endogenous steroid hormones are biosynthesized from cholesterol, by a complex group of enzymes including hydroxylases, lyases, isomerases and dehydrogenases (Andersson, 2008). The class and quantity of individual steroids produced in plasma depends on which gonads (testes or ovaries) are present, and on the activity of the adrenal gland. Catabolism of steroids occurs mainly in the liver, where the potency of the majority of steroid hormones is reduced by addition of hydroxyl groups, dehydrogenation, reduction and conjugation with sulphuric or glucuronic acid. Inactivation of steroids can also occur through metabolism to compounds that have greatly reduced biological activity. Most steroids are excreted as water-soluble sulphate and glucuronide conjugates via the urine although amino acid (glycine and tauric acid) conjugates are also often found in the bile (Goad, 1975; Andersson, 2008)

In non-mammalian vertebrates, conjugation of steroid hormones by glycine and taurine have been found to be dominant over the formation of sulfates and glucuronides (Norris, 2007). However, not much is documented about the metabolite pathways in reptilians, and limited information is available on the conjugation of steroids in the Nile crocodile (*Crocodylus niloticus*). Considering the species-differences in the metabolism and excretion of steroids, various approaches in sample preparation and analytical methodology were considered for optimum determination of steroid metabolites in the Nile crocodile.

Standard analytical methodology for steroids has been extensively developed for GC methods where hydrolysis of the polar conjugates to expose chemical groups suitable for derivatization to enhance volatility has been the norm (Gomes et al., 2009). Hydrolysis of steroid metabolite conjugates by specific enzymes yields

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typical-conjugate free steroids for further derivatization which facilitates analysis by gas chromatography-mass spectrometry (GC/MS). Various acid and enzymatic hydrolysis methods and preparations have been reported with regard to efficiency (Venturelli et al., 1995; Shibasaki et al., 2001). Although additional conjugates, not only steroid glucuronides and sulfates were expected in crocodilian urine, enzymatic hydrolysis utilizing the most commonly recommended preparation, beta-glucuronidase/sulfatase mixture from *Helix pomatia*, was selected for this study. This enzyme mixture is relatively non-specific and may be able to achieve deconjugation at additional sites (Takeda et al., 1966; Crabbe et al., 2002).

Measurement of intact steroid metabolite conjugates is possible with liquid chromatography combined with tandem mass spectrometry (LC/MS/MS) (Guan et al., 2005), and eliminates the required hydrolysis and derivatization steps needed for the analysis of free steroids by gas chromatography–mass spectrometry (GC/MS) (Buiarelli et al., 2010). Although both high sensitivity and specificity can be achieved with triple quadrupole based LC/MS/MS analyses of steroid hormones and their metabolites, a big drawback is that targeted measurements require previous knowledge of which steroids are conjugated to which polar groups. This makes steroid conjugate identification and quantitation of unknowns more complex and many analytes can be missed, as is experienced with immunoassays. Radioimmunoassay (RIA) measurements are prone to cross-reactivity between several analytes which may be matrix dependent and can suffer from compromised specificity (Krone et al., 2010). Furthermore, enzyme immunoassays (EIA's) may lack accuracy and specificity due to structurally similar compounds (Anfossi et al., 2014).

Liquid chromatography combined with triple quadrupole, time of flight, Fourier transform ion cyclotron resonance (FT-ICR), and ion trap mass spectrometry, are useful instrument configurations to employ in the elucidation of fragmentation pathways of steroids with small differences in structure (Guan et al., 2005). Liquid chromatography time-of-flight mass spectrometry (LC-TOF/MS) achieves high

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mass accuracy in the quantitative analysis of steroids and their metabolites (Touber et al., 2007; Rousu and Tolonen, 2012). Franke et al. (2011) reported that LC-MS with Orbitrap technology has proven to be superior regarding productivity and sample matrix, but problematic in the measurement of non-polar steroids such as pregnanediol and DHEA, when using electrospray ionization (ESI) due to poor analyte ionisation.

GC/MS methodology, preceded by thin layer chromatography (TLC) (Mitchell and Shackleton, 1969; Shackleton et al., 1972) and gas chromatography (GC) with conventional detectors e.g. GC-FID (Hurter and De Villiers, 1978), in the measurement of steroids and their metabolites, emerged approximately 40 years ago (Shackleton, 1976). Both gas chromatography-isotope ratio mass spectrometry (GC-IR/MS) (Piper et al., 2008, Ahrens and Butch, 2013) and gas chromatography time-of-flight mass spectrometry (GC-TOFMS) (Revelsky et al., 2011) are employed in the detection of anabolic steroids in doping control laboratories. The recent development of GC/MS with Orbitrap technology (Peterson et al., 2010, Peterson et al., 2014), has added to the array of available high resolution, accurate mass methodologies for steroid analysis.

Negative ion chemical ionization (NCI) GC/MS has been employed in steroid and steroid metabolite analysis (Nakamura et al., 2001; Fine et al., 2003) but difficulties with methodology and instrumentation rendered this method problematic which compromised further developments (Houghton et al., 1988). GC/MS with electron impact ionization (EI), with comprehensive (full scan mode/TIC) or targeted analysis (selected ion mode/ SIM) followed by automated library searching can be performed, which was more successful and has been considered the method of choice for the analysis of free steroids. (Griffiths et al., 2006).

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Evaluation of GC *vs* LC as front end to MS or tandem MS (MSMS) detection of several steroids in the diagnosis and management of complex diseases, show data regarding the performance and limitations of these methods. Tandem mass spectrometric (MS/MS) technology enhances sensitivity and selectivity by reducing background noise, and is by far the analytical method of choice when quantitation of trace levels is required (McDonald et al., 2011).

High maintenance and running cost of equipment, together with the requirement of dedicated, highly skilled analytical staff, somewhat places high resolution accurate mass spectrometry and tandem mass spectrometry methodology out of the reach of most routine clinical and veterinary laboratory establisments (Fine et al., 2003; Buiarelli et al., 2010; Peterson et al., 2010). The decision to use GC/MS (single quadrupole) with EI as analytical instrument for this study, was made on the basis of operator background and experience, and the wide availability of this instrumentation in analytical laboratories.

Separation of steroids in a gas chromatographic system requires formation of volatile derivatives of the analytes (Bekker, 2004; Gomes et al., 2009). The selection of derivatization reagents are based on the requirements of rapid and complete derivatization, high recovery as well as predicatable fragmentation for structural elucidation of the steroids. Various derivatizing reagens have been used to prepare suitable volatile derivatives of extracted steroids. Methoxime trimethylsilyl (MO-TMS) derivatization has been employed in human steroid profiling for almost 45 years (Thenot and Horning, 1972). Enol/TMS formation employing N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) and trimethyliodosilane (TMIS) (Haber et al., 2001, Le Bizec et al., 2004), tert-butyldimethylsilyl (TBDMS) (Houghton et al., 2006) and heptafluorobutyric acid (HFBA) (Tang et al., 1996) have all been used with success.

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6.2. Materials and methods

6.2.1. Subjects

Juvenile Nile crocodiles of approximately two years old (total length: 1.8 to 2m), were selected as subjects at Izintaba Crocodile Farm during 2006. Collection of the urine samples took place prior to slaughter and was based on the technique described by Myburgh et al. (2012), by inserting a dog urinary catheter through the cloaca. A pilot study was conducted to optimize preparation and analysis conditions from aliquots of these urine collections. Initial results revealed very low concentrations due to the dilution effect, which required larger volumes of urine per analysis. To obtain sufficient volumes of urine for analysis futher samples from several matched subjects were pooled to improve sensitivity. Table 6.1 lists the samples employed in the method optimization.

Table 6.1. Single and pooled captive juvenile Nile crocodile urine samples employed in the optimization of analytical conditions.

Sample name	Volume received	Volume extracted	Comment/result
croc2	4.1 ml	3.9 ml	Dual injections using FID and MS detection showed few steroid peaks with low s/n. Internal standard (IS) peaks dominated, leading to re-planning the amount of IS in future samples.
croc5	10.5 ml	10.2 ml	
croc7	14.5 ml	14.0 ml	
Remaining matched subject pool: ”croc70”		70 ml	Many peaks acquired with good s/n, allowing qualitative investigation. Amount of IS still needed to be reduced.

6.2.2. Materials

- Sodium acetate, sodium sulphate, acetic acid, methanol (MS grade), and ethyl acetate (*pro analysi)*, was obtained from MERCK (Darmstadt, Germany).
- Glusulase, a preparation from the intestinal juice of the snail *Helix Pomatia*, containing β-glucuronidase (no less than 90 000 units/ml) and sulfatase (no less than 10 000 units/ml), was manufactured by duPont (Boston, MA, USA).
- BOND ELUT ™ octadecyl bonded silica (C18), 6 ml, 500 mg and aminopropyl

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unendcapped bonded silica (NH2), 3 ml, 200 mg solid phase extraction (SPE) cartridges were purchased from Varian (Harbor City, CA, USA).

- O-Methoxylhydroxylamin Hydrochloride was obtained from Sigma Aldrich (Steinheim, Germany).
- Pyridin, silylation grade, and N-Trimethylsilylimidazole (TMSI), was supplied by PIERCE (Rockford, IL, USA).
- Gas was supplied by AFROX (Benoni, SA). Helium, ultra high purity (UHP) was employed as carrier gas. Samples were dried by nitrogen gas (IG).
- Steroid standards were obtained from SIGMA (St. Louis, MO, USA) and Steraloids Inc. (Wilton, NH, USA). Table 6.2. lists the trivial name and abbreviation of each steroid standard employed, adjacent to the structural name.

Table 6.2. Steroid standards employed for calibration, showing trivial name, abbreviation and structural name.

Trivial name (abbreviation)	Structural Name
Androsterone (An)	5α-androstan-3α-ol-17-one
Etiocholanolone (Et)	5β-androstan-3α-ol-17-one
Dehydroepiandrosterone (DHEA)	5-androsten-3β-ol-17-one
11-OH Androsterone (HAn)	5α-androstan-3α,11β-diol-17-one
Pregnanediol (Pd)	5β-pregnan-3α,20α-diol
Pregnanetriol (Pt)	5β-pregnan-3α,17α,20α-triol
11-Deoxytetrahydrocortisol (THS)	5β-pregnan-3α,17α,21-triol-20-one
Tetrahydrocortisone (THE)	5β-pregnan-3α,17α,21-triol-11,20-dione
Tetrahydrocorticosterone (THB)	5β-pregnan-3α,11β,21-triol-20-one
Tetrahydrocortisol (THF)	5β-pregnan-3α,11β,17α,21-tetrol-20-one
Cholesteryl n-butyrate (C n-But)*	5-cholesten-3β-ol n-butyrate

*Internal Standard

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6.2.3. Instrumentation

Sample preparation equipment: A 24-position extraction manifold, manufactured by Supelco (Bellefonte, PA, USA) was employed. A Zymark Turbo Vap® LV evaporation system (Zymark Corporation, Hopkinton. MA), equipped with in-line filters and a stainless steel manifold, was used. The incubation- and derivatization-steps were achieved with a 15V Labotec oven/incubator with adjustable temperature.

Several GC systems were used wih common features. Chromatographic separations were performed on J&W Scientific, DB-1 capillary column (30 m X 250 µm I.D., 0.1 µm film) (Agilent Technologies, Palo Alto, CA, USA). In the initial optimisation and calibration study, an HP 6890 gas chromatograph, with a split/splitless injection system was used. GC-MS detecion was performed on an HP 5973 mass selective detector (Agilent technologies, Palo Alto, CA, USA). The quality control standards was included in batches of crocodile samples, were analyzed on a similar column as above, employing a Shimadzu 2010 GC, equipped with a split/splitless injector, and a QP2010S mass selective detector (Shimadzu, Kyoto, Japan). Data collection and integration was achieved with HP Chem Station software (Agilent), or LabSolutions software (Shimadzu), respectively.

6.2.4. Preparation procedures

1. A 0.2 M sodium acetate/acetic acid buffer, pH 4.6, was prepared as follows: Sodium acetate (1.64 g) was dissolved in 100 ml de-ionized water, to produce a 0.2 M sodium acetate solution, and 1.15 ml acetic acid made up to 100 ml with de-ionized water to obtain a 0.2 M acetic acid solution. The two solutions were mixed while stirring, monitoring the pH and adjusted to 4.6.
2. Stock standard solutions (1 mg/ml) of steroid metabolites were prepared by dissolving 10 mg of the analytical standard in 10 ml methanol or ethyl acetate. Calibration mixtures (working standard concentrations) were spiked with pre-determined volumes of the solutions (see Table 6.3). These were stored at ambient temperature in airtight light excluding containers.

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3. The internal standard (200 μg/ml) was prepared as follows: 20.0 mg cholesteryl n-butyrate (C n-But) was made up to 100 ml with ethyl acetate,
3. A column performance and retention time standard, A 200 µg/ml C_{23} solution was prepared in a similar way as C n-But.
4. Derivatization reagent, *O*-Methoxylhydroxylamine hydrochloride salt was dried at 60°C for 1 h and used to prepare 10% solution in pyridine (0.5 g/5 mL).

6.2.5. Extraction Procedure

For each Nile crocodile urine sample and standard spiked sample, one Varian Bond Elut C18 (octadecyl bonded silica) solid phase extraction cartridge was prepared on a vacuum extraction manifold by priming with 10 ml methanol, followed by aspiration with 10 ml de-ionized water without allowing the cartridge to run dry. The first solid phase extraction (SPE) was performed by loading each sample onto a designated cartridge, drawing the sample through slowly followed by washing with 10 ml de-ionized water. The steroids were eluted into tubes with 4 ml methanol. The flow rate of the vacuum connected to the extraction manifold was set to 1 ml/min and kept constant during the prime and extraction steps. At the end of each wash step (after loading of the samples onto the cartridges) the flow rate was slightly elevated. The eluates were dried under nitrogen gas at 37˚C on a Zymark Turbo Vap system.

In the hydrolysis step, each dried eluate was re-dissolved in 100 μl of methanol, after which 5 ml of a sodium acetate/acetic acid buffer (0.2 M, pH 4.6) was added. This was followed by the addition of 200 μl glusulase, and the mixture was vortex mixed. The tubes were stoppered and enzymatic hydrolysis was allowed to take place overnight at 50°C.The hydrolyzed mixtures were centrifuged for 6 min at 1750 x *g*. and the supernatants were subjected to a second SPE step. One NH2 SPE cartridge was prepared for each sample as follows: One gram dry sodium sulphate (Na_2SO_4) was placed into each cartridge on top of the aminopropyl layer. An extension adapter was connected to each NH2 cartridge. The matched previously used C18 cartridges were primed as for the first extraction with 10 ml methanol and washed with 10 ml de-ionized water. The supernatants of the

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hydrolyzed mixtures were aspirated through the C18 cartridges, which were then washed with 10 ml de-ionized water. The C18 cartridges were then connected to the top of the pre-prepared NH2 cartridges and again connected onto the vacuum extraction manifold as shown in Figure 6.1. The free steroids were eluted with 6 ml ethyl acetate, into final tubes containing the internal standard, C n-But (50 µg) and C_{23} (20 µg). The eluates were completely dried under nitrogen gas and derivatized. Alternatively, the dried eluates could be stored at -20°C for later derivitization.

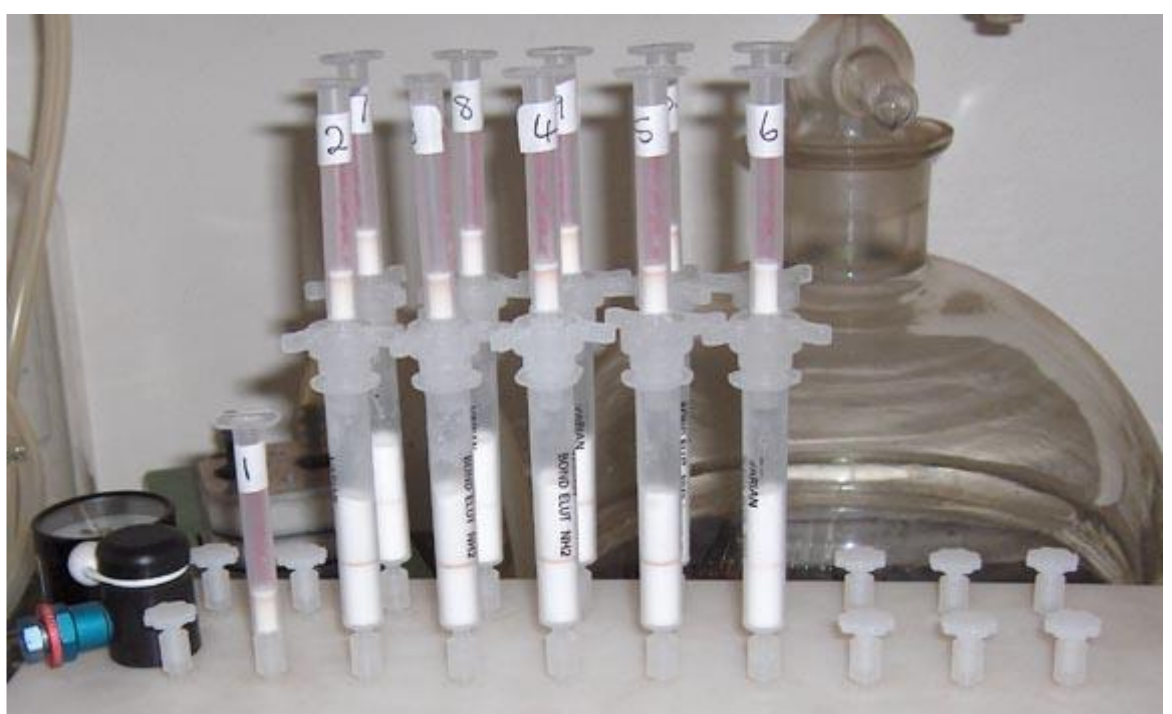

Figure 6.1. The second solid phase extraction (SPE) step showing the octadecyl bonded silica (C18) SPE cartridges connected on top of the pre-prepared aminopropyl unendcapped bonded silica (NH2) SPE cartridges containing one gram Na_2SO_4, connected onto the extraction manifold.

Derivatization was achieved with a two-step procedure: To facilitate methoxylation, 100 µl of a 10% *O*-methoxylhydroxylamine hydrochloride solution was added to the dried residue. The mixture was incubated at 60ºC for 15 min. Silylation followed, by addition of 100 µl N-Trimethylsilylimidasole (TMSI) to the incubation mixture, and further incubation 100ºC for 2 h. The sample was allowed to cool before opening the tube and transfere to a vial, which was stored at -20°C until

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performing GC-analysis. Following storage, the mixture was allowed to reach room temperature before injection.

6.2.6. Chromatographic conditions

The ion source of the mass spectrometer was set to an electron voltage of -70 eV, with the transfer line temperature set at 280°C, source temperature set at 230°C, and the quadrupole temperature at 150°C. Scan mode was selected for the mass range 50 to 550. Helium (5,5 N) was employed as carrier gas, with a flow rate of 1.2 ml/min. The split method of injection with a split ratio of 30:1 was performed in the chromatographic separations. A 1 µl sample was injected by auto injector onto the GC column. The carrier gas was employed in the splitter-system. The inlet temperature was set at 250°C. The temperature program was as follows: initial isotherm for 3 min at 215°C; program rate: 1.6°C/min up to 226°C; second isotherm 3 min at 226°C; second program rate: 1.6°C/min up to 300°C; final isotherm of 5 min before returning to starting conditions.

6.2.7. Validation of method

Validation parameters that were previously addressed in the MSc thesis of Bekker (2004), covering extraction efficiency (breakthrough volumes of SPE cartridges), optimization of enzymatic hydrolysis conditions, spectrophotometric evaluation of enzyme activity, hydrolysis *vs.* no hydrolysis, repeatability, variation within and between subjects, and stability of derivatives left at room temperature vs. kept at -20˚C, accuracy and precision were not repeated in this study. The validation parameters confirmed in this study were linearity of calibration, recovery, determination of lower and upper limits of quantitation, and quality control.

6.2.8. Calibration of analytes

A urine pool was centrifuged in a Beckman Coulter Allegra™ X22R centrifuge (Beckman Instruments Inc., Fullerton, CA, USA), at 1750 x *g* for 6 min, to remove deposits. Standard steroid metabolite solutions were spiked into 5 ml aliquots of the clean pooled crocodile urine sample, to prepare standard mixes within the concentration range 0.2 to 20 µg (40 ppb to 4 ppm).

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Table 6.3. gives a summary of the amount of steroid standard added to each calibration sample. Each mixture was extracted, hydrolyzed, and derivatized as in the procedure described in Section 6.2.5., and injections of these samples were performed on GC/MS. To S0, which served as "blank", only internal standards were added. From these data, calibration curves were established for each analyte, to enable determination of linearity, limit of detection, limit of quantitation, accuracy, and precision.

Table 6.3. Amounts in µg, of steroid standards added to aliquots of a pooled urine sample in preparation of six calibration mixtures.

Steroid metabolite	µg S-0	µg S-0.2	µg S-1	µg S-10	µg S-15	µg S-20
Androsterone	0	0.2	1	-	15	20
Etiocholanolone	0	0.2	1	10	15	20
Dehydroepiandrosterone	0	0.2	1	10	15	20
11-hydroxy Androsterone	0	0.2	1	10	15	20
Pregnanediol	0	0.2	1	10	15	20
Pregnanetriol	0	0.2	1	10	15	20
Tetrahydro substance S	0	0.2	1	10	15	20
Tetrahydrocortisol	0	0.2	1	10	15	20
Tetrahydrocorticosterone	0	0.2	1	10	15	20
Tetrahydrocortisol	0	0.2	1	10	15	20
Cholesteryl n-butyrate (IS)	20	20	20	20	20	20

6.2.9. Quality control

The unavailability of a commercial control providing concentration values for all the steroid metabolites in the profile necessitated the inclusion of a standardised quality control from pooled human urine. This was divided into aliquots and a recorded volume of urine was extracted with every batch that included crocodilian samples (and analysed with these samples as discussed in Chapter 7) on the extraction manifold.

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6.2.10. Quantitation procedures

6.2.10.1. Calibration

Peak areas (PA), were obtained as integrated signal intensity multiplied by time values by the chromatographic software. Linear calibration graphs of relative peak area *vs* concentration were obtained for each analyte from the series of standards injected. Recoveries were determined from means of the concentrations of standards that were obtained from solvent versus sample matrix and "post-extraction" stage addition of standards for each analyte into recovery assays.

The lower limit of quantitation (LLQ) (the minimum accurate quantitative concentration) was defined as the concentration resulting in a peak having a signal-to-noise ratio (S/N) equal to or higher than 10:1. The limit of detection (LOD) was defined as any peak that had a S/N not less than 3:1.

6.2.10.2. Quality control and crocodilian urine samples

Quantitation of quality control (QC) samples and Nile crocodile urine samples was performed by means of ratio of peak areas of analyte versus internal standard (relative peak area), and identification of mass spectra were based on mass and relative retention times versus column standard. This was done to facilitate comparison between results of urinary steroid analyses on different GC/MS systems at different facilities. Calculations of individual QC sample concentrations, providing a µmole steroid metabolite/day result; equation described in the equation in Equation 6.1.

$$Analyte\ (\mu moles/day) = \frac{PA_{steroid\ metabolite}\ x\ control\ volume\ x\ IS\ amount}{PA_{IS}\ x\ extracted\ volume\ x\ MW_{steroid\ metabolite}}$$

Equation 6.1. Equation used for the quantitation of excreted steroid metabolites in µmole/day.

PA (peak area), was measured as integrated counts by the chromatographic software.

Control urine volume and volume of urine extracted measured in ml.

Amount of internal standard (IS) was given in µg.

Molecular weight (MW) was given in µg/µmole.

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Crocodilian urine samples and random urine samples, nmole steroid metabolite per µmole creatinine was calculated as described in the equation in Equation 6.2.

$$Analyte\ (nmoles/\mu mole\ creatinine) = \frac{PA_{steroid\ metabolite}\ x\ \ IS\ amount\ x\ 1000\ mL}{PA_{IS}\ x\ extracted\ volume\ x\ MW_{steroid\ metabolite}\ \ x\ \mu mole\ creatinine}$$

Equation 6.2. Equation used for the quantitation of excreted steroid metabolites in nmole steroid metabolite/µmole creatinine.

PA (peak area), was measured as integrated counts by chromatographic software.

Amount of internal standard (IS) was given in ng.

Molecular weight (MW) was given in ng/nmole.

Volumes were in mL

6.3. Results

Five of the EI (electron impact) m/z (mass:charge) spectra obtained from the peaks in the TIC of the pooled Nile crocodile urine (Figure 6.3) showed similarities to m/z spectra obtained from endogenous human steroids. These were the spectra of androsterone/etiocholanolone, pregnanediol, pregnanetriol and 16α-OH-DHEA. Relative retention times of all of these peaks, however, did not match those of standards. Figures 6.2 and 6.3 show total ion chromatograms obtained in a human urine sample, and the pooled crocodile urine sample, respectively.

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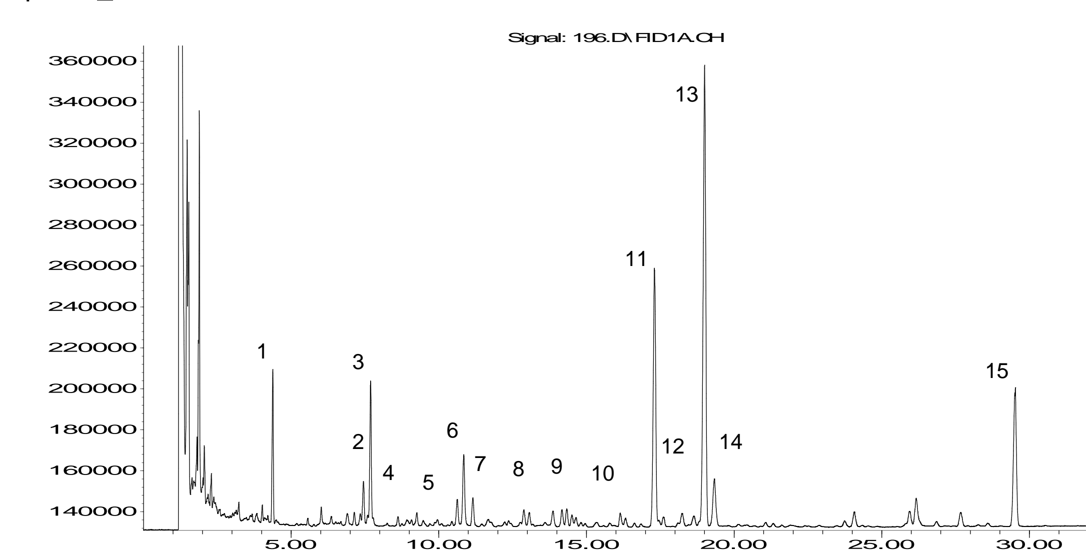

Figure 6.2. TIC of a sample from a 24h human urine collection, showing MO-TMS derivatives of: 1. C_{23}, 2. androsterone, 3. etiocholanolone, 4. DHEA, 5. KAn, 6. HAn, 7. HEt, 8. pregnanediol, 9. pregnanetriol, 10. THS, 11. THE, 12. THB, 13. THF, 14. α-THF, 15. C n-But (IS).

Table 6.4 lists the peaks as numbered in Figure 6.2, and the dominant masses in their spectra. Identification was achieved with NIST and relative retention time.

Table 6.4 Peaks obtained in the TIC of the 24h human urine collection as numbered in Figure 6.2, and the dominant masses in their spectra.

Peak Nr	Steroid Metabolite Trivial Name (abbr.)	Dominant masses in EI m/z spectrum	Peak Nr	Steroid Metabolite Trivial Name (abbr.)	Dominant masses in EI m/z spectrum
1	C_{23}	57; 71; 85; 99	9	Pregnanetriol	255; 435; 345
2	An	270; 360; 213	10	THS	564; 474; 384
3	Et	270; 360; 213	11	THE	578; 488; 609
4	DHEA	268; 358; 260	12	THB	188; 474; 564
5	KAn	374; 284; 390	13	THF	652; 562;472
6	HAn	448; 358; 268	14	α-THF	652; 562;472
7	HEt	448; 358; 268	15	C n-B	368; 353; 260
8	Pregnanediol	117; 269; 347			

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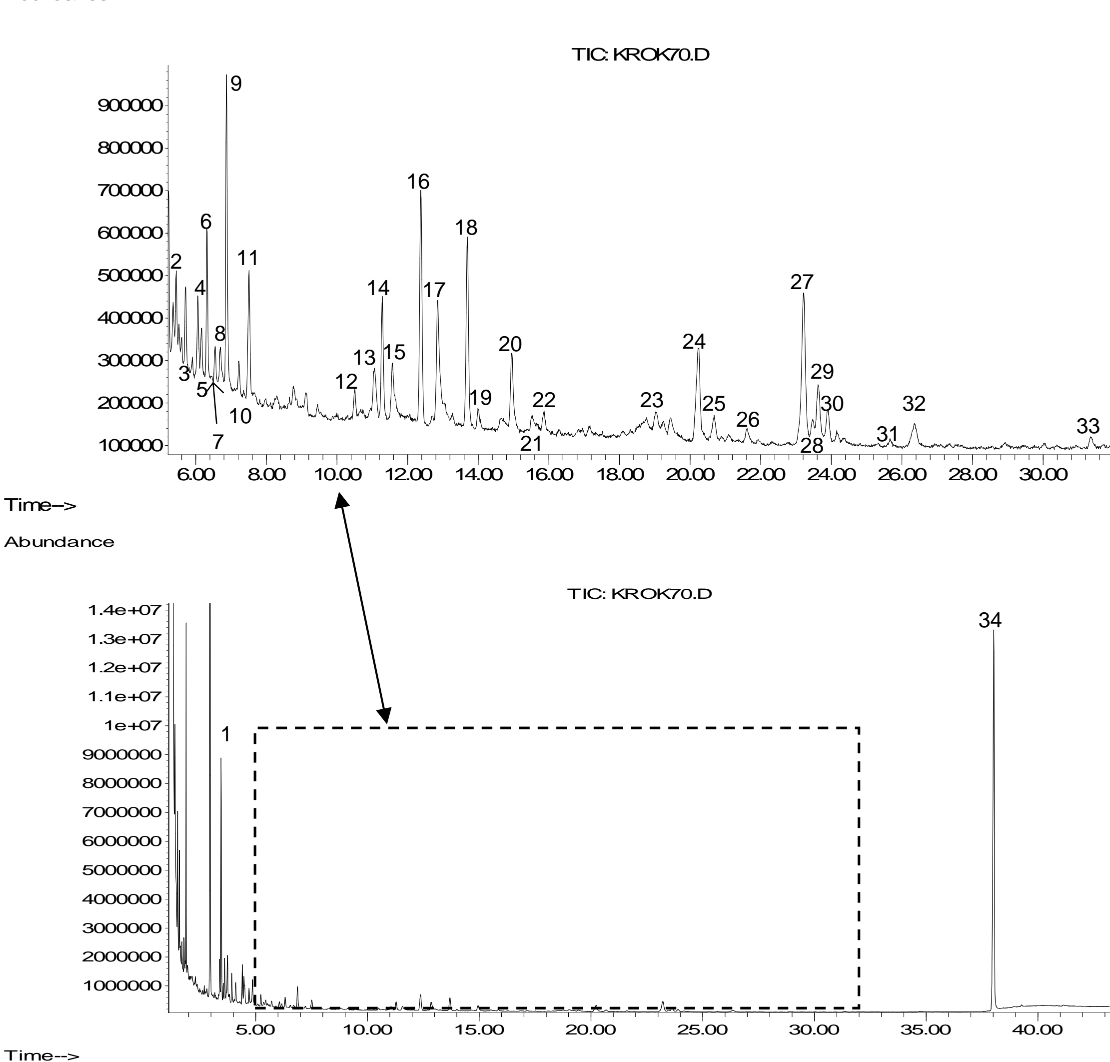

Figure 6.3. TIC of a pooled Nile crocodile urine sample after selective extraction and derivatization. Information on numbered peaks in the top section (an extract from the chromatogram in the bottom section), is shown in Table 6.5

In the pooled Nile crocodile urine sample, more than 30 unique mass peaks were obtained of which more than 25 did not match known steroid or steroid metabolite derivative spectra from the NIST library. These analytes are still unidentified. Library searches against the NIST database could identify only five steroid peaks. Table 6.5 lists the peaks as numbered in Figure 6.3, the dominant masses in their spectra, and similarity of the spectra to those of known steroid metabolites.

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Table 6.5. Peaks obtained in the TIC of the pooled Nile crocodile urine sample sorted according to retention time as identified in Figure 6.3.

Peak nr.	Dominant masses in EI m/z spectrum	Retention time (min)	Match with known steroid spectra
1	85/113/324	3.448	C23*
2	282/254/131/224/310	5.357	unknown
3	146/168/224/299/430	5.446	unknown
4	242/158/361/283/217	6.056	unknown
5	256/241/129/215/346	6.156	unknown
6	182/150/237/327	6.311	unknown
7	242/283/185/213/320/391	6.544	unknown
8	152/131/283/242/298	6.700	unknown
9	208/152/131./283/167/298	6.866	unknown
10	281/179/196/460/253/213	7.221	unknown
11	217/434/143/344/241	7.510	unknown
12	196/271/448/217/181	10.506	unknown
13	270/360/213/239/129/173/433	11.061	An/Et
14	149/167/279/113	11.283	unknown
15	255/435/147/117/215/345	11.560	Pregnanetriol
16	117/269/347/449	12.370	Pregnanediol
17	182/166/150/273/221/ 339	12.847	unknown
18	448/268/213/125/358/182	13.691	16a-OH-DHEA
19	314/299/271/209	13.990	unknown
20	261/300/315/284/405/344	14.945	unknown
21	125/244/153/193/418	15.522	unknown
22	270/361/238/330/	15.866	unknown
23	213/449/243/332/359	19.028	unknown
24	531/342/472/444	20.238	unknown
25	196/271/181/402/492	20.682	unknown
26	531/342/472/369/253/443	21.682	unknown
27	457/488/429/268/339/398	23.212	unknown
28	373/283/147/463/175/343	23.478	unknown
29	129/329/368/353/458/443	23.622	unknown
30	196/398/271/488/504	23.889	unknown
31	299/255/389/281/477	25.664	unknown
32	457/371/273/488/197	26.352	unknown
33	209/245/151/532/181	31.345	unknown
34	368/353/147/247/261	38.026	C n-But**

*C23 is added to monitor column efficiency

** Internal standard

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6.3.1. Calibration results

Table 6.6 shows the values obtained in the calibration procedure, for the steroid metabolites employed in this investigation. The TIC of the pooled Nile crocodile urine aliquot spiked with 10 µg steroid standards, is shown in Figure 6.4

Table 6.6. Correlations (r^2), recoveries, LLQ's and LOD's and linearity in the calibration of the steroid metabolites.

Steroid metabolite	r^2	Recovery %	LLQ (µg)	LOD (µg)	Highest linear concentration (µg)
Androsterone	0.9735	83.3	0.2	0.2	20
Etiocholanolone	0.9365	113.8	0.2	0.2	15
Dehydroepiandrosterone	0.9485	107.9	0.2	0.2	15
11-hydroxy Androsterone	0.9866	83.6	1.0	0.2	20
Pregnanediol	0.9886	92	0.2	0.2	20
Pregnanetriol	0.9822	82	0.2	0.2	15
Tetrahydro substance S	0.9985	100.3	0.2	0.2	10
Tetrahydrocortisone	0.9984	100.2	1.0	0.2	15
Tetrahydrocorticosterone	0.9945	109.3	0.2	0.2	20
Tetrahydrocortisol	0.9691	112.2	0.2	0.2	15

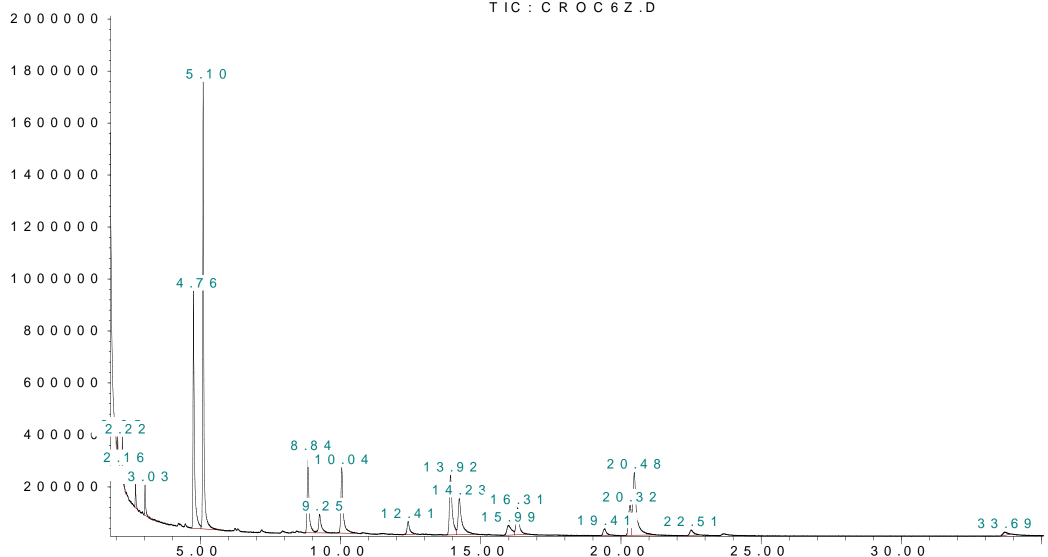

Figure 6.4. TIC of the pooled Nile crocodile urine aliquot spiked with 10 µg steroid standards.

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6.3.2. Quality Control Results

An internal standardised quality control was analysed with each batch. Mean concentrations and SD for each analyte are listed in Table 6.7.

Table 6.7. Means and SD's obtained for the set of controls extracted and analysed with each batch.

Steroid metabolite	Mean µmole/day	n	SD
Androsterone	2.31	7	0.44
Etiocholanolone	2.71	7	0.42
Dehydroepiandrosterone	0.26	7	0.14
11-hydroxy Androsterone	3.09	7	0.71
Pregnanediol	2.13	7	1.36
Pregnanetriol	2.08	7	0.89
Tetrahydro substance S	0.43	7	0.22
Tetrahydrocortisone	4.90	7	1.92
Tetrahydrocorticosterone	0.28	7	0.1
Tetrahydrocortisol	2.74	7	1.36

6.4. Discussion

The sample collection technique was relatively simple and non-traumatic for the animals. Although all the samples were collected from healthy farm crocodiles, in future urine of crocodiles from polluted river areas could be analyzed. This will aid in the investigation to evaluate the significance of urine analysis as an indicator of endocrine disruption.

Qualitative investigation of the pooled crocodile urine sample was problematic in the sense that many peaks were not identified from the NIST library and complete confirmation of structures were not possible with the equipment available. The near-matching spectra of peaks which did not have relative retention times matching those of standards run under the same chromatographic conditions, could suggest similarity of the base molecules, but different molecular weights than those of the known steroid metabolites.

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Potential conjugation by the amino acids e.g. taurine and glycine will need to be assessed (it is all speculation). The best way to proceed with the assessment will be LCMSMS (high resolution) of non-hydrolysed urine. This should be followed up with the appropriate hydrolysis i.e. targeting the existing conjugates to obtain the steroids and steroid metabolites in free form. Accurate mass GC/MS (Orbitrap or TOF GC/MS) determination of the MO-TMS derivatives to elucidate structures.

The validation of the GC/MS method for Nile crocodile urine was successfully completed, by determining lower limits of quantitation and limits of detection for each analyte, establishing linearity's up to the highest calibration level, correlations exceeding 0.9365, and recoveries between 82% and 113.8%. The LLQ's of all the analytes under investigation was established at the lowest prepared concentration, 0.2 µg, except for HAn and THE, which showed significant peaks at a concentration of 1.0 µg. Quality control samples from each batch showed steroid metabolite concentrations to all be within two standard deviations of one another.

6.5. Conclusion

Steroid profiling in humans may be indicative of numerous endocrine dysfunctions (incl. reproductive and thyroid disorders), enzyme deficiencies/ excesses, hypo- and hypercortisolism (incl. tumours of the adrenal gland), and even starvation (Honour, 2001; Bekker, 2004). Qualitative and quantitative investigation of steroids in crocodiles from clean and polluted areas, may serve as a diagnostic tool to investigate similar problems in crocodilians and as a sentinel of water quality in areas where potential endocrine disruptor pollution would be difficult to trace. Considering species-differences in metabolism and excretion of steroids, various approaches in sample preparation and analytical methodology needed to be evaluated for optimum determination of steroid metabolites in the Nile crocodile.

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Potential studies that could follow this investigation are: diagnostic testing for the detection of endocrine disruptive compounds other than steroids; and the establishment of a crocodilian steroid metabolite database, including the alligator and other crocodilian species.

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CHAPTER 7: Steroid profiling of crocodilian urine

7.1. Introduction

In humans, the use of urine samples in the evaluation of gonadal and adrenal endocrine function is well-documented (Hähnel et al., 1985; Shackleton, 1986; Honour et al., 1991; Honour and Brook, 1997). Weykamp et al. (1989) published reference intervals for all the steroid profile variables determined for both sexes in six different age catagories, determined with GC and flame ionization detection (GC/FID). Steroid profiling by GC/MS has been used to confirm a diagnosis of hormonal abnormalities, including reproductive and thyroid disorders, enzyme deficiencies/excesses, hypo- and hypercortisolism, starvation, stress and hypertension (Fenessey et al., 1983; Honour, 1997; Lee et al., 1998; Wolthers and Kraan, 1999; Honour, 2001; Bekker et al., 2001; Bekker, 2004).

Non-invasive endocrinology has been widely employed in wildlife studies, mainly for quantitation of hormones in urine (Monfort et al., 1991), faeces (Monfort et al., 1998; Brown et a.l, 2001; Turner et al., 2002;), hair (Meyer and Novak, 2012), aquatic media and saliva samples (Narayan, 2013; Kersey and Dehnhard, 2014).

Numerous endocrine studies conducted on crocodilians were primarily performed on blood or faeces specimens.In an attempt to reveal information on the evolution of reproductive hormones in ectothermic vertebrates, Norris and Jones (1987), investigated gonadal and testicular function in reptiles and amphibians. The existence of a feedback relationship between gonadotropin secretion and sex steroids produced by the gonads was confirmed. It was also revealed that LH and FSH are two chemically distinct gonadotropins in crocodilian reptiles, with a marked homology to those in mammals and birds, unlike the the single “complete” gonadotropin believed to be present in squamates (snakes and lizards). Licht (1979) also reported a general non-specificity to LH and FSH in reptilian reproduction studies.

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Guillette et al. (1997) studied the reproductive cycle of the female American alligator (*Alligator mississippiensis*) residing in central Florida, USA. In the plasma, vitellogenin was confirmed by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1D SDS-PAGE), and oestradiol, progesterone, testosterone and corticosterone were quantitated by RIA. Total protein and inorganic ions (Ca, P) were measured spectrophotometrically. Monthly/seasonal variations in analyte concentrations were determined, as well as correlations between the individual parameters. Seasonal patterns of testosterone (T) and dehydroepiandrosterone (DHEA) were assessed in adult male American alligators. Differences in peak concentrations during breeding and non-breeding seasons, and across consecutive years, were determined in two size classes (Hamlin et al., 2011). Further investigations of parameters reflecting the endocrine systems of the American alligator and the Nile crocodile (*Crocodylus niloticus*) have been done extensively (Lance et al., 2003; Huchzermeyer, 2003). Ganswindt et al. (2013) assessed adrenocortical function in captive Nile crocodiles, by measuring faecal corticosterone levels before and after performing an adrenocorticotrophic hormone (ACTH) challenge.

As far as could be ascertained, steroid profiling has not been performed on urine from a Nile crocodile before. Evaluation of seasonal differences in steroid hormones and metabolites may add new information with respect to the endocrine axis of both male and female crocodiles. Excretion of certain steroid metabolites, and in particular the ratios at which they are excreted, may hold useful information to be correlated with gene-expression studies currently also under way in both captive and wild crocodiles. Moreover, assessment of urinary steroid profiles could provide important information about the functioning of the Nile crocodile endocrine system and may reflect exposure to aquatic pollutants. Urinary steroid profiling of crocodilian urine could add a new dimension to the role of crocodiles as sentinels of pollution, in general, and the diagnostic value of a urine sample in individual animals. The diagnostic value of a crocodile urine sample is at this stage mostly underestimated; however, it will most likely become more important in future, as effective techniques for sample collection have been developed.

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This investigation was aimed primarily at Nile crocodiles, but the opportunity arose to obtain urine samples from other crocodilian species, and analyses of urine samples from a female and male spectacled caiman (*Caiman crocodilus*), and a dwarf crocodile (*Osteolaemus tetraspis*) and American alligators were also performed.

7.2. Materials and methods

7.2.1. Sample collection and Subjects

In 2012, Myburgh et al. reported a simple and effective technique for the collection of crocodile urine, using a canine urinary catheter, making urine samples for diagnostic and research purposes a feasible option. With this non-traumatic technique a relatively clean urine sample can easily be collected. The history of each subject and sample collection background is shown below.

7.2.1.1. Subject 1: A reproductively active female Nile crocodile

Sample no. 1: A permit was issued by Mpumalanga Tourism and Park Agency (MTPA) for a mature female Nile crocodile to be shot on a commercial crocodile farm (Crocgrove, Mpumalanga Province, South Africa) after her mandible was fractured bilaterally. This mature female Nile crocodile of 3.54 m total length (TL) had been in captivity for more than 30 years. The animal was shot through the brain with a high caliber rifle by an official from Mpumalanga Tourism and Park Agency (MTPA).

Urine was collected from the animal, immediately after she was shot, by using the method described by Myburgh et al. (2012). The urine was collected into sterile plastic containers. The urine sample was transported, on ice, back to the Ecotoxicology Laboratory of the Department of Paraclinical Sciences, Faculty of Veterinary Science, Pretoria. The necropsy noted nothing abnormal, except for the fractured mandible. Sixty two eggs were counted in the uterus.

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7.2.1.2. Subject 2: Pooled sample of adult Nile crocodile urine

Sample no. 2: Five batches of crocodile urine samples (from hatchlings, yearlings, juveniles, sub-adults and adults) collected in Zambia, was received from Prof. Alison Leslie. Classification was based on snout-vent length (SVL), as some of the crocodiles had bits of tail missing. Subjects with a SVL of 1159 mm and higher were classified as adults. Due to the small volumes per sample (aliquots were received in micro tubes) the adult samples were pooled to enhance sensitivity for analyses.

7.2.1.3. Subject 3: Female Nile crocodile; Izintaba Crocodile Farm

Sample no. 3: Urine was collected from Princess, an emaciated and chronically sick adult female Nile crocodile at Izintaba Crocodile farm.

7.2.1.4. Subject 4: Juvenile male Nile crocodile; Kruger National Park

Sample no. 4: Urine was collected from a juvenile male Nile crocodile in the Olifants Gorge, Kruger National Park. The recorded measurements were as follows: Body length 1.1 meter; SVL 520 mm; Girth 310 mm, Neck girth 200 mm; Tail girth 210 mm.

7.2.1.5. Subject 5: Sub-adult male Nile crocodile; Loskop Dam

Sample no. 5: Urine was collected from a sub-adult male Nile crocodile at Loskop dam. The total body length of the subject was 2.43 meter; with a head length of 340 mm, head width of 190 mm, and SVL of 1280 mm.

7.2.1.6. Subject 6: Sub-adult male Nile crocodile; Flag Boshielo Dam

Sample no. 6: Urine was collected from a sub-adult male Nile crocodile at Flag Boshielo dam. The total body length of the subject was 2.52 meter; with a head length of 330 mm, head width of 210 mm, and SVL of 1280 mm.

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7.2.1.7. Subject 7: Female spectacled caiman; Subject 8: Male spectacled caiman; Subject 9: Male dwarf crocodile

Sample no's. 7,8,9: Urine samples were obtained from a female spectacled caiman, a male spectacled caiman and a male dwarf crocodile in the National Zoological Gardens, Pretoria.

7.2.1.8. Subject 10: American alligators

Samples no. 10: The aim of this study was to evaluate the urine of pre-selected groups of crocodilians with the standardized GC/MS method for the detection of steroids and their metabolites. Stored urine samples from American alligators were selected in the research laboratory of the Department of Zoology, University of Florida, Gainesville, FL, USA. The selection was primarily based on whether samples were of sufficient volumes for steroid metabolite extraction. Urine samples from 22 alligators of which the plasma samples had been previously analysed by the RIA technique: seven from males, who had elevated testosterone levels, seven from females with high progesterone, and eight from females with abnormal oestradiol levels, were selected. Steroid metabolite standards, as well as testosterone and oestradiol standards, were prepared at three concentration levels. A human control urine sample was extracted with each batch to monitor sample preparation efficiency. Appendix 7.1 lists information on the samples.

7.2.2. Sample preparation

In the laboratory selected volumes of the urine samples of subjects 1 to 9 were prepared for GC/MS analysis of steroids and steroid metabolites, employing the sample preparation technique described in Chapter 6. Information on volumes that were extracted and considerations regarding certain samples is shown in Table 7.1. Individual urine samples were selected, batched and extracted at the same time as a pooled human urine control sample, which served as method quality control.

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Table 7.1. Information on sample preparation of the different subjects.

Sample no./subject	Volume extracted (ml)	Comment
Human control sample	25	An aliquot of a 24 h urine collection stored in 30 ml aliquots and stored at $-20^{\circ}C$ was thawed and extracted with every batch.
1: Reproductively active female Nile crocodile, Crocgrove crocodile farm	10	A duplicate extraction was performed, and two different silylation chemicals were evaluated, TMSI (which was used throughout the project) and MT-BSTFA.
2. Pooled sample of adult Nile crocodile urine	13	Extraction of small volumes of urine presented sensitivity problems, therefore the adult urine samples were pooled and a single extraction on the total volume performed.
3: Female Nile crocodile, Izintaba Crocodile Farm	27	Blood was also drawn from this crocodile, which made a volume of 8 mL plasma available; for this the same extraction procedure as for urine samples was preformed, omitting enzymatic hydrolysis.
4: Juvenile male Nile crocodile; KNP	9	The sample preparation technique described in Chapter 6 was employed for all subjects.
5: Sub-adult male Nile crocodile, Loskop Dam	25	
6: Sub-adult male Nile crocodile, Flag Boshielo Dam	21	
7: Female spectacled caiman	13.6	
8: Male spectacled caiman	22	
9: Male dwarf crocodile	16	
10: American alligators	See appendix 7.1	The sample preparation and analyses were performed at the College of Medicine, University of Florida, Gainesville FL, USA. The sample preparation technique described in Chapter 6 was employed. The only exceptions (different sample preparation equipment used) were C18 cartridges (3 ml, 200 mg) and NH2 cartridges (3 ml, 500 mg) obtained from Thermo Scientific Runcorn, UK, and the use of a Reacti-Vap III Evaporator system (Thermo Scientific, Runcorn, UK) for the drying of the eluates under nitrogen gas.

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7.2.3. Instrumentation

7.2.3.1. Subjects 1 to 9

The gas chromatographic mass spectrometric (GC/MS) analysis was performed with a Shimadzu 2010 GC, with a split/splitless (SSL) injection system, electron impact ionization (EI) and a single quadrupole mass spectrometer, MS-QP2010 (Shimadzu, Kyoto, Japan). The ion source of the mass spectrometer was set to an electron voltage of -70 eV. Separation was achieved with a J&W Scientific DB-5 Capillary GC column (30 m, 0.32 mm id, 0.1 µm film) (Agilent Technologies, Palo Alto, CA, USA). Data collection and integration were performed with Lab Solutions software (Shimadzu, Kyoto, Japan).

Chromatographic conditions: Helium (5,5 N) was employed as carrier gas, with a flow rate of 1.2 ml min^{-1}. The ion source of the MS was operated at -70 eV. Injections were performed in split mode with a split ratio of 30:1. A volume of one µl of the derivatised sample was injected onto the capillary GC column. The inlet temperature was set at 250°C. The temperature program was as follows: initial isotherm 215°C for 3 min; program rate: 1.6°C min^{-1} up to 300°C, 30°C min^{-1} up to 310°C, final isotherm of 3.5 min.

7.2.3.2. Subject 10

Gas Chromatographic/Mass Spectrometric (GC/MS) analyses was performed with a Thermo Polaris Q GC-MS, equipped with a SSL injection system, EI and an ion trap mass spectrometer (Thermo Fisher Scientific, Texas, USA). The ion source of the mass spectrometer was set to an electron voltage of -70 eV. Separation was achieved with a TRACE TR5-MS capillary column (30 m X 0.25 mm id X 0.25 µm film), (Thermo Fisher Scientific, Runcorn, UK). Data collection and qualtitative reporting were done using Thermo Scientific Xcalibur software.

GC conditions: Helium (5,5 N) was employed as carrier gas, with a flow rate of 1.2 ml min^{-1}. Injections were performed in split mode with a split ratio of 30:1. One µl of sample was injected by auto injector onto the GC column. The inlet temperature

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was set at 250°C. The temperature program was as follows: initial isotherm 215°C for 3 min; program rate: 1.6°C min^{-1} up to 226°C; second isotherm at 226°C for 3 min; second program rate: temperature ramped at 1.6°C min^{-1} up to 300°C; final isotherm of 5 min.

7.3. Results

7.3.1. Human control sample

Figure 7.1. shows a Total Ion Chromatogram (TIC) obtained by analysis of a 24h human urine collection (volume 2940 mL), serving as between-batch control.

The steroid metabolites identified by the EI m/z spectra, retention times and calculated concentrations, are listed in Table 7.2. Concentrations were calculated according to Equation 6.1 to give µmole steroid per day, and according to Equation 6.2 to give nmole steroid per µmole creatinine.

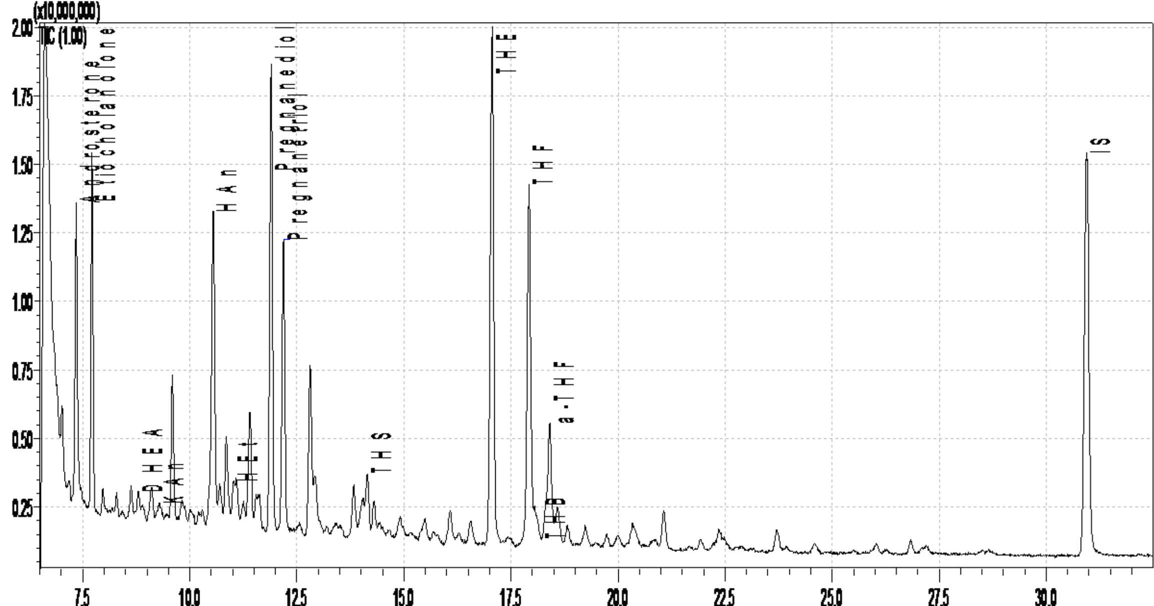

Figure 7.1. TIC of the urine sample of a female human control after selective extraction and derivatisation.

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Table 7.2. Steroid metabolites identified by retention time and EI m/z mass spectra of the MO-TMS derivatives in the TIC of the female human control urine sample.

Steroid Metabolite Trivial Name	RT (min)	Dominant masses in EI m/z spectrum	Concentration nmole steroid/ µmole creatinine	Concentration µmole steroid/ day
Androsterone	7.348	270; 360; 213	256	2.63
Etiocholanolone	7.715	270; 360; 213	297	3.06
DHEA	8.795	129; 268; 358	17	0.17
11-OH Androsterone	10.546	448; 358; 268	354	3.64
Pregnanediol	11.900	117; 269; 347	463	4.76
Pregnanetriol	12.188	255; 435; 345	269	2.77
Tetrahydro substance S	14.141	564; 474; 384	54	0.56
Tetrahydrocortisone	17.064	578; 488; 609	569	5.85
Tetrahydrocortisol	17.922	652; 562;472	344	3.54
Tetrahydrocorticost.	18.407	188; 474; 564	47	0.48
Cholesteryl n-butyrate	30.949	368; 353; 260	-	-

*Internal standard

7.3.2. Subject 1: A reproductively active female Nile crocodile

In the TIC of the urine of the female adult Nile crocodile (No. 1), shown in Figure 7.2, twenty-two peaks were observed, excluding the internal standard peak. Nine of these peaks were positively identified by comparison with relative retention times of standards and EI m/z spectra in the NIST08 and Wiley8 libraries. For this subject, urinary creatinine determination was performed on an Integra Cobas Roche autoanalyser (Roche Diagnostics, Mannheim, Germany) at the AMPATH laboratories, Pretoria. The creatinine concentration was used to facilitate quantitation of the steroids identified in the profile as described in Chapter 6, Equation 6.2.

The steroid metabolites identified in this profile by the EI m/z spectra, retention times and calculated concentrations, are listed in Table 7.3. Concentrations were calculated using analyte peak area counts relative to the internal standard peak area counts (Equation 6.2) and expressed as nmole steroid per µmole creatinine.

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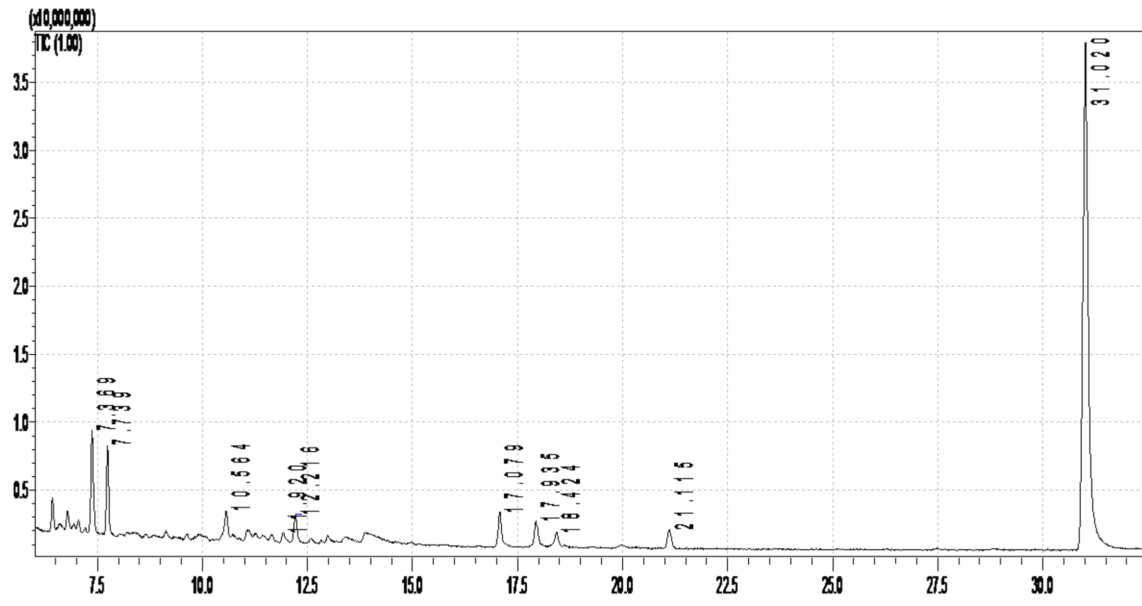

Figure 7.2. TIC of the urine sample of the female adult Nile crocodile (No 1) after selective extraction and derivatisation.

Table 7.3. Steroid metabolites identified by retention time and EI m/z mass spectra of the MO-TMS derivatives in the TIC of the female adult Nile crocodile urine sample (No 1).

Steroid Metabolite Trivial name	Retention Time (min)	Dominant masses in EI m/z spectrum	Concentration nmole steroid/ µmole creatinine
Androsterone	7.375	270; 360; 213	17.37
Etiocholanolone	7.742	270; 360; 213	13.46
11-OH Androsterone	10.558	448; 358; 268	6.35
Pregnanediol	11.925	117; 269; 347	1.37
Pregnanetriol	12.208	255; 435; 345	4.45
Tetrahydrocortisone	17.075	578; 488; 609	5.76
Tetrahydrocortisol	17.933	652; 562;472	4.13
Cholesteryl n-butyrate *	31.020	368; 353; 260	-

*Internal standard

7.3.3. Subject 2: Pooled sample of adule Nile crocodile urine

Sample No. 2: The TIC in Figure 7.3a showed a prominent peak at RT 12.052 min, as well as seven more peaks of which none could be positively identified using NIST. Figure 7.3b. shows the EI m/z spectrum of the peak at 12.052 min. Quantitation was not performed due to lack of identification of the peaks.

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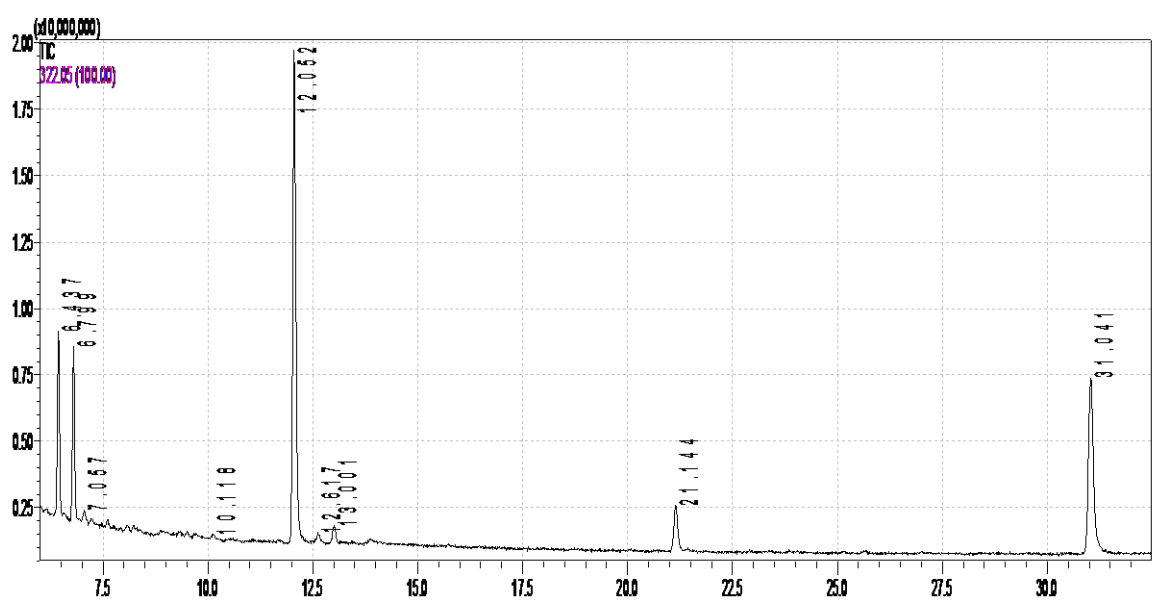

Figure 7.3.a. TIC obtained from pooled urine from collections of adult Nile crocodiles (No 2))

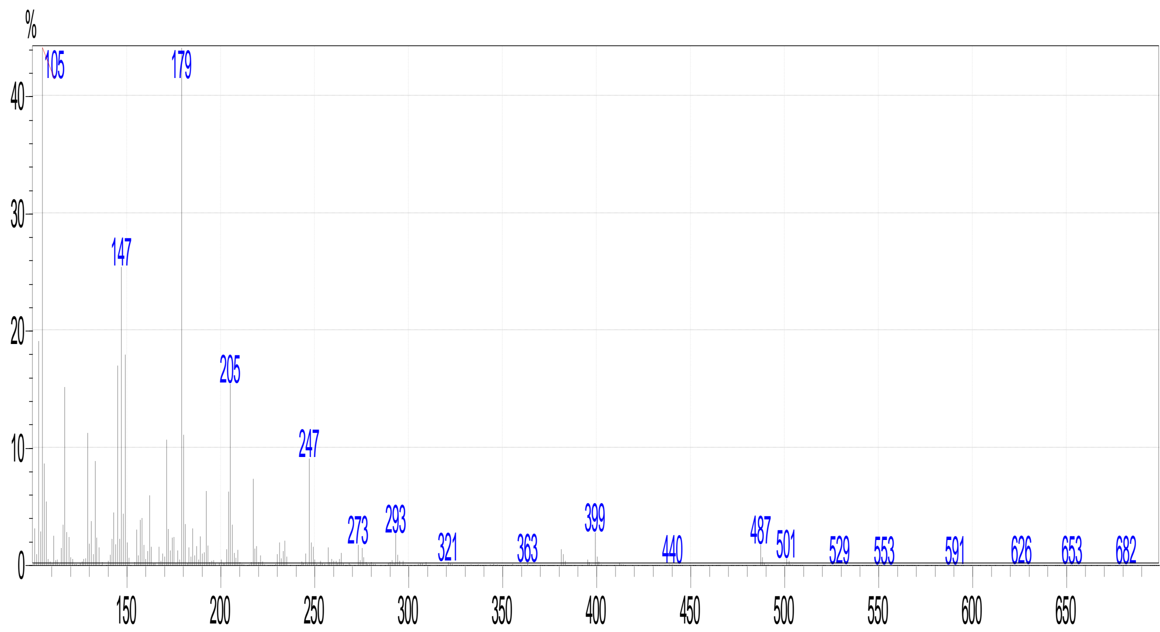

Figure 7.3.b. EI m/z mass spectrum of the unknown peak at RT 12.052 min in the chromatogram of the pooled adult Nile crocodile urine (No 2).

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7.3.4. Subject 3: Female Nile crocodile; Izintaba Crocodile Farm

Figure 7.4.a shows the TIC obtained with GC-MS analysis of the urine sample of the female Nile crocodile (No 3). A few prominent peaks were observed of which none could be identified with NIST

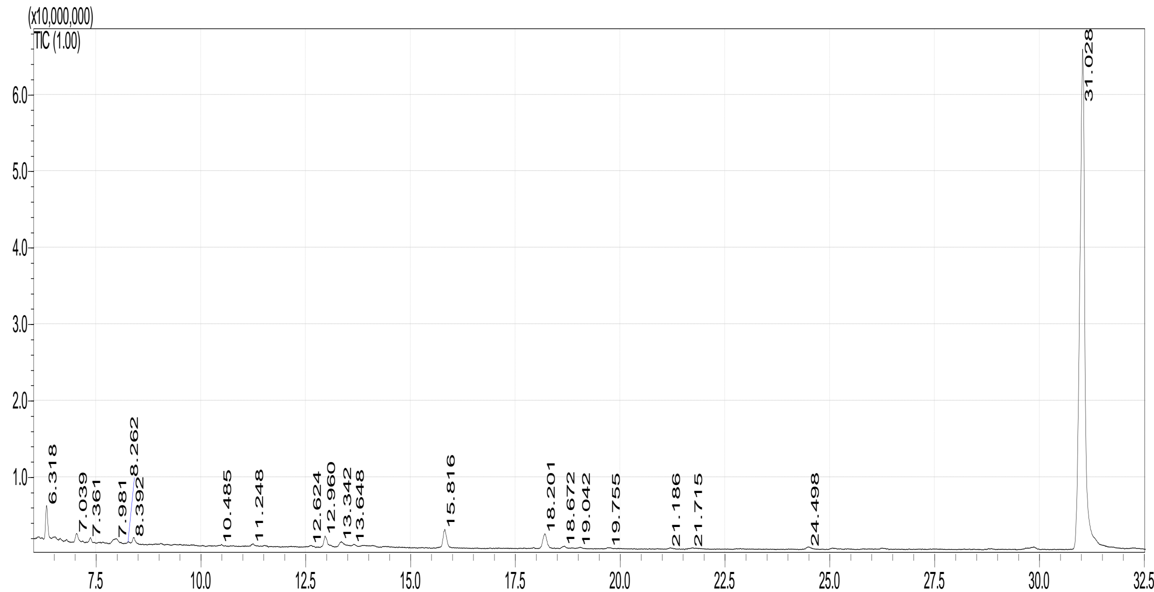

Figure 7.4.a. TIC of urine from the emaciated and chronically sick adult female Nile crocodile (No 3) showing the retention times of the detectable peaks.

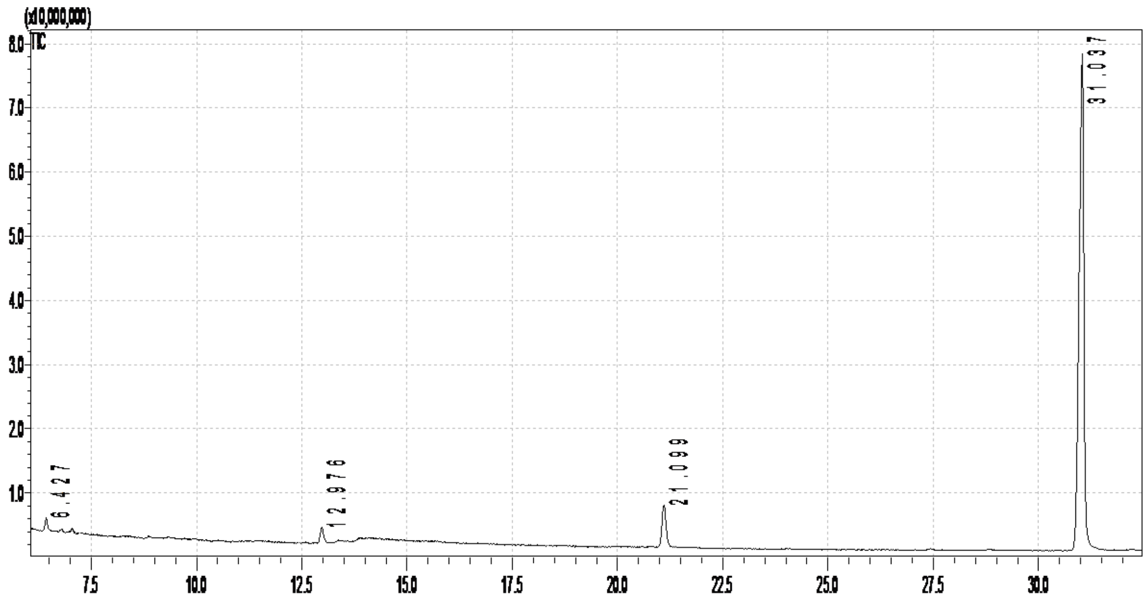

Figure 7.4.b. TIC of plasma from the emaciated and chronically sick adult female Nile crocodile (No 3) showing the retention times of the detectable peaks.

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7.3.5. Subject 4: Juvenile male Nile crocodile; Kruger National Park

Sample No. 4: A very prominent peak observed at RT 12.934 min, as well as a prominent peak at RT 21.058 min were observed. No other dominant peaks were present in the TIC (Figure 7.5.a), except for the internal standard at 30.926 min. The spectrum of the peak at 12.934 min is shown in Figure 7.5.b, and the spectrum of peak at 21.058 min is shown in Figure 7.5.c.

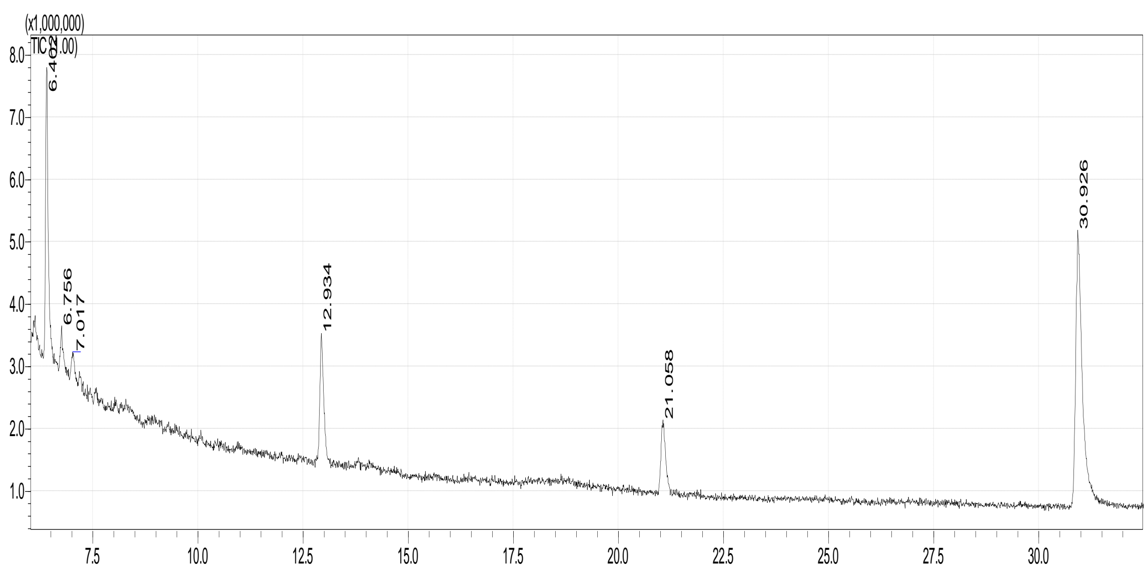

Figure 7.5.a. TIC of the urine sample of a juvenile male Nile crocodile from the Olifants Gorge in the Kruger National Park (No 4).

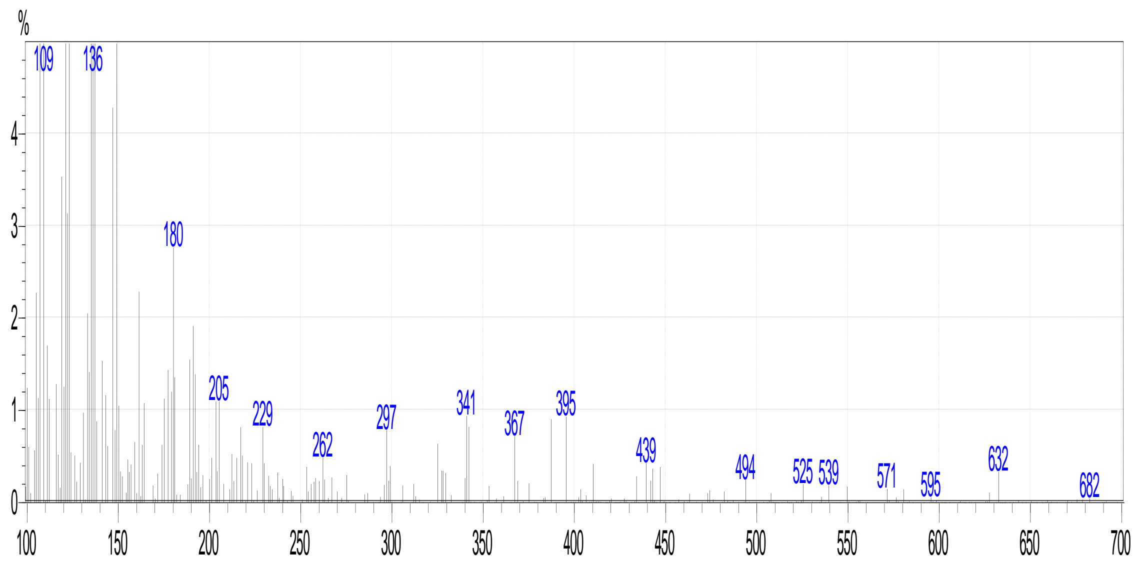

Figure 7.5.b. EI m/z mass spectrum of the peak at RT 12.934 min.

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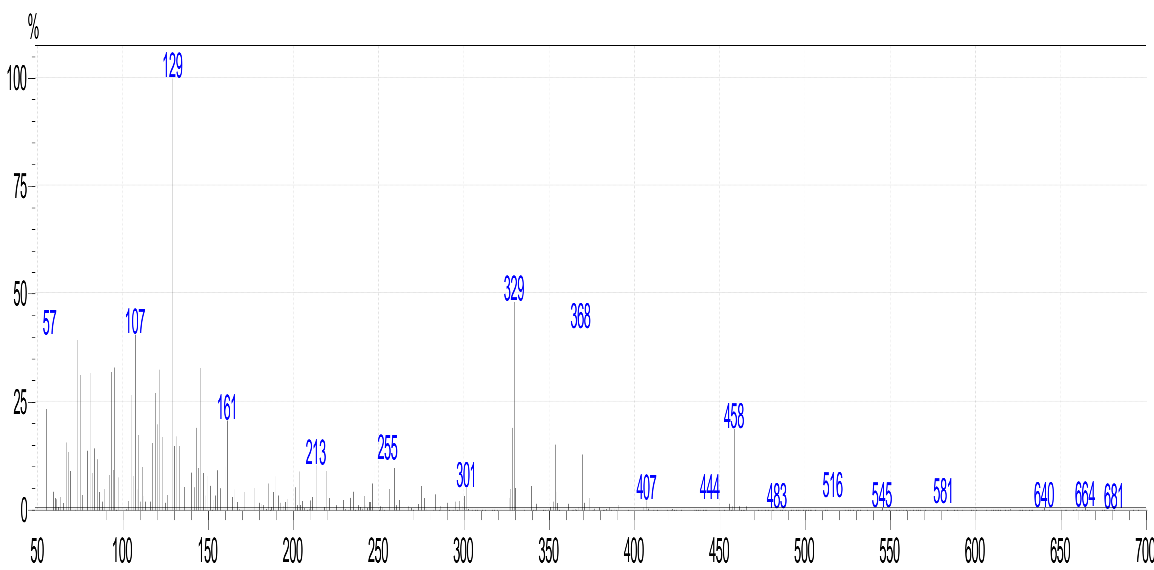

Figure 7.5.c. EI m/z mass spectrum of the peak at RT 21.058 min.

7.3.6. Subject 5: Sub-adult male Nile crocodile; Loskop Dam

Figure 7.6 shows the TIC of the urine sample from the sub-adult male crocodile (No 5). A few prominent peaks were observed of which none could be identified with NIST, therefore quantitation was not performed.

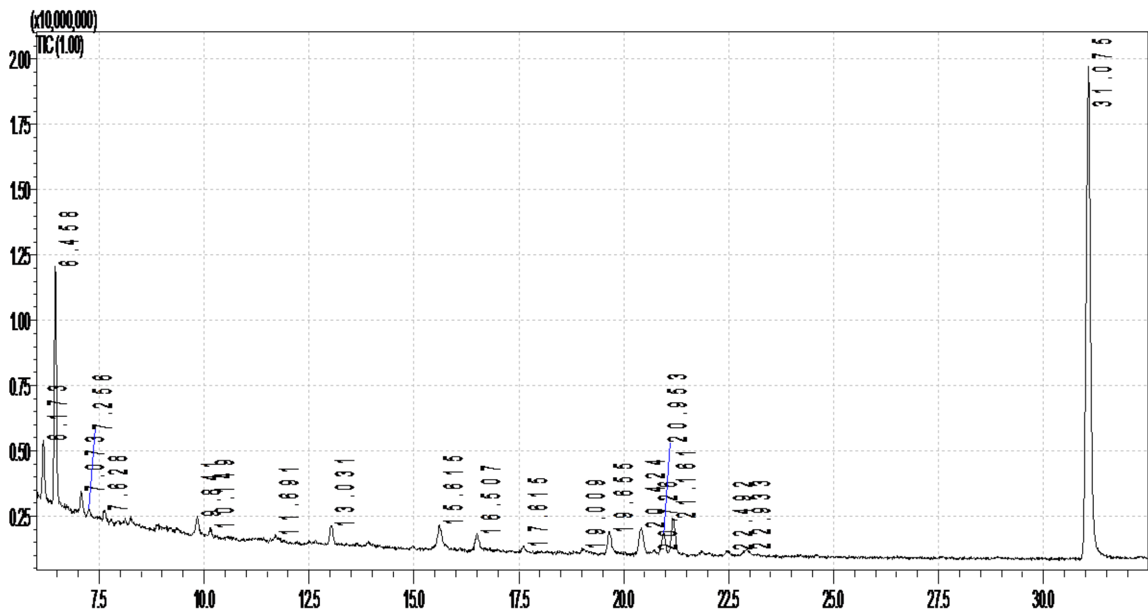

Figure 7.6. TIC of the urine sample from the sub-adult male Nile crocodile from Loskop Dam (No 5).

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7.3.7. Subject 6: Sub-adult male Nile crocodile; Flag Boshielo Dam

Sample No. 6: Figure 7.7 shows the TIC of the prepared sample after GC/MS analysis.

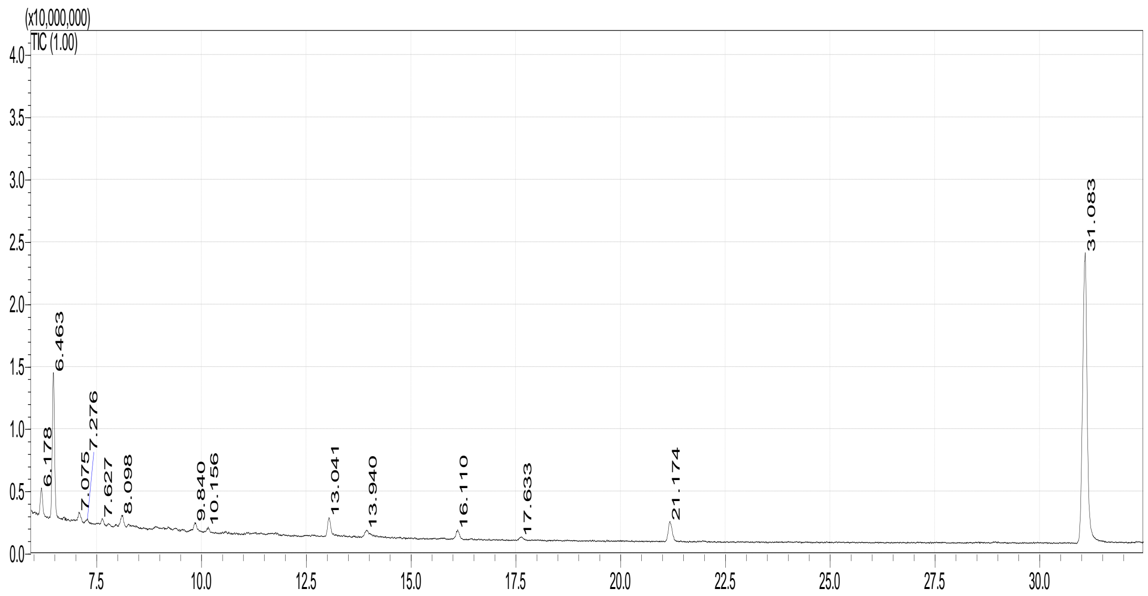

Figure 7.7. TIC of the urine from the sub-adult male Nile crocodile from Flag Boshielo Dam (No 6).

7.3.8. Subject 7: Female spectacled caiman

Sample No. 7: Figure 7.8 shows the TIC of the prepared sample after GC/MS analysis.

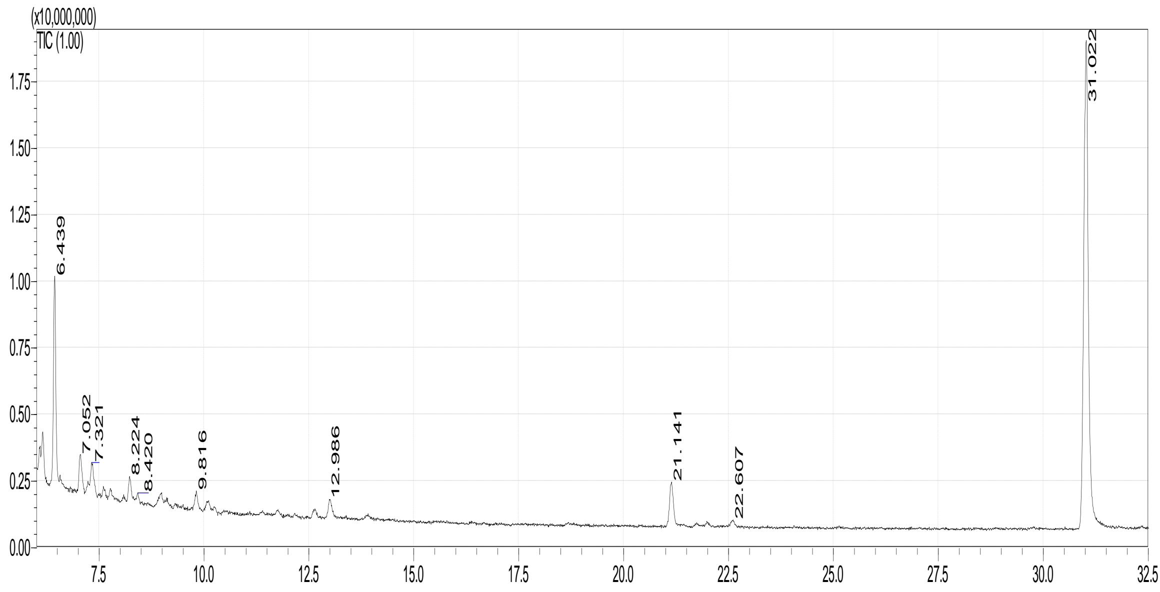

Figure 7.8. TIC of the urine sample from the female spectacled caiman (No 7).

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7.3.9. Subject 8: Male spectacled caiman

Sample No. 8: Figure 7.9 shows the TIC of the prepared sample after GC/MS analysis.

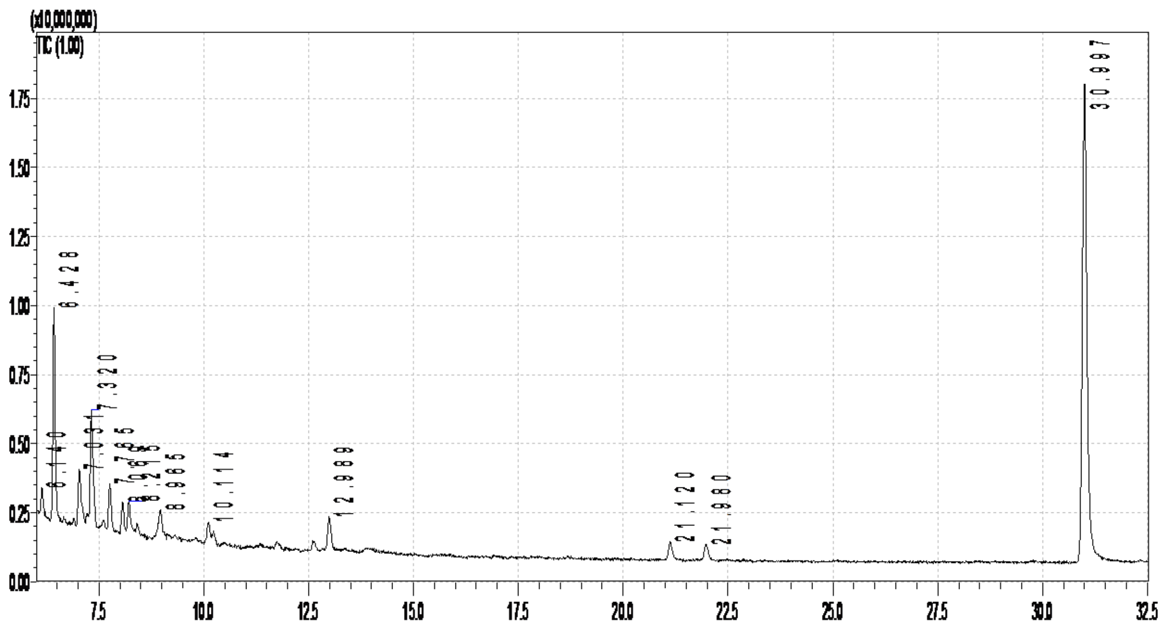

Figure 7.9. TIC of the urine sample from the male spectacled caiman (No 8).

7.3.10. Subject 9: Male dwarf crocodile

Sample No. 9: Figure 7.10 shows the TIC of the prepared sample after GC/MS analysis.

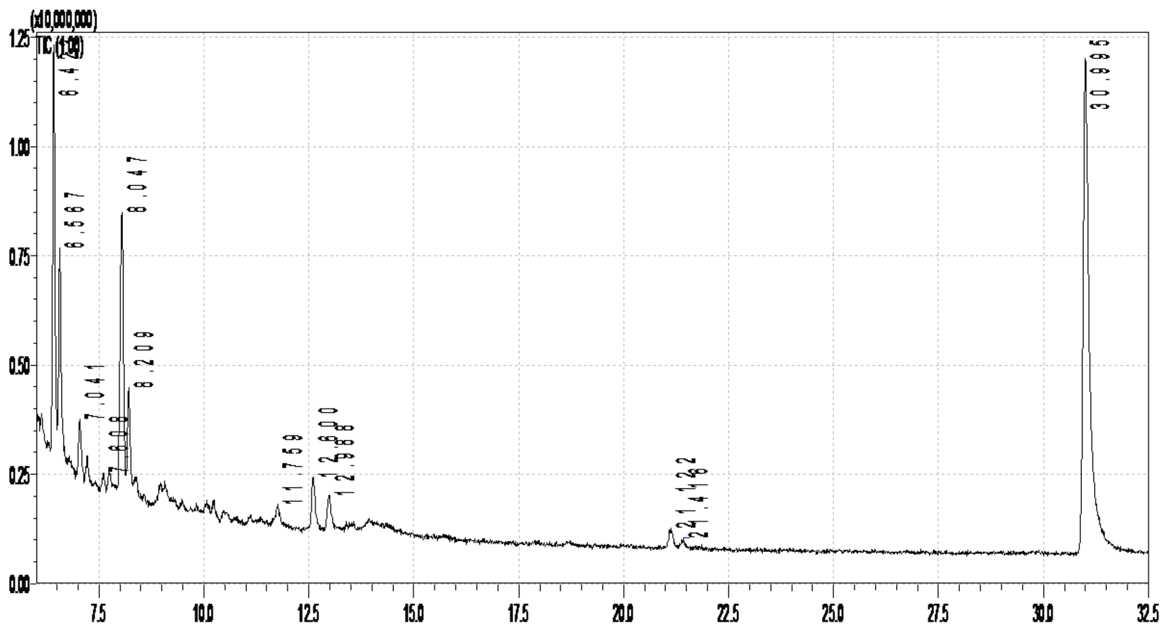

Figure 7.10. TIC of the urine sample from the male dwarf crocodile (No 9).

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Table 7.4 lists the dominant masses in the EI m/z spectra of the MO-TMS derivatives of the known steroid metabolites employed as standards in this study.

Table 7.4. Steroid metabolite name, trivial name, retention times and masses in EI m/z spectra of the MO-TMS or TMS derivatives

Steroid Metabolite	Trivial name	RT (min)	Dominant masses in EI m/z spectrum
5α-androstan-3α-ol-17-one	Androsterone	7.348	270; 360; 213
5β-androstan-3α-ol-17-one	Etiocholanolone	7.742	270; 360; 213
5-androsten-3β-ol-17-one	Dehydroepiandrosterone	8.795	268; 358; 260
5α-androstan-3α,11β-diol-17-one	11-OH Androsterone	10.546	448; 358; 268
5β-pregnan-3α,20α-diol	Pregnanediol	11.900	117; 269; 347
5β-pregnan-3α,17α,20α-triol	Pregnanetriol	12.188	255; 435; 345
5β-pregnan-3α,17α,21-triol-20-one	Tetrahydrosubstance S	14.141	564; 474; 384
5β-pregnan-3α,17α,21-triol-11,20-dione	Tetrahydrocortisone	17.064	578; 488; 609
5β-pregnan-3α,11β,17α,21-tetrol-20-one	Tetrahydrocortisol	17.922	652; 562;472
5β-pregnan-3α,11β,21-triol-20-one	Tetrahydrocorticosterone	18.407	188; 474; 564
5-cholesten-3β-ol n-butyrate	Cholesteryl n-butyrate	30.949	368; 353; 260

*Internal standard

Table 7.5. contains dominant peaks from all subjects, retention times, and the most dominant EI m/z masses in the spectra of the peaks. A few common masses were observed, e.g. in subjets 8 and 9, for peaks at approximately 8 min, the masses 217/434/393 are found.

The peaks that were positively identified as TMS or MO-TMS derivatives of steroid metabolites (by the NIST library and relative retention times) in Subject 1 (see Table 7.3), are omitted from Table 7.5. Peaks and EI M/z masses of the internal standard and C23 are also not shown in Table 7.5.

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Table 7.5. Retention times and masses constantly observed in the EI m/z mass spectra of subjects 1 to 9

Sample nr.	Dominant peaks (RT: min)	Dominant masses	Sample nr.	Dominant peaks (RT: min)	Dominant masses
1	7.042	149/260/348	5	20.953	562/652/472
1	7.200	241/331/688	5	22.492	267/357/327
1	8.667	448/266/356	5	22.933	578/457/488
1	9.133	448/361/339	6	6.178	413/385/355
1	9.625	315/300/405	6	6.463	313/445/370
1	11.092	191/434/358	6	7.075	149/167/279
1	11.267	196/434/357	6	8.098	217/143/434
1	11.658	239/432/329	6	9.840	281/147/369
1	12.363	255/363/399	6	13.041	342/273/601
2	6.437	313/280/445	6	13.940	368/260/328
2	6.799	295/533/311	6	16.110	523/253/343
2	12.052	179/247/293	7	6.439	313/356/445
3	6.318	341/301/267	7	7.052	149/167/279
3	7.039	149/167/478	7	7.321	147/255/323
3	7.361	375/345/432	7	8.224	371/239/459
3	7.981	348/260/281	7	9.816	281/369/607
3	8.392	357/297/483	7	12.986	180/281/427
3	11.248	491/435/344	7	22.607	325/351/456
3	12.624	270/376/302	8	6.140	147/413/281
3	12.976	341/299/368	8	6.428	313/445/356
3	13.342	366/247/253	8	7.031	434/254/379
3	15.816	131/247/487	8	7.320	255/344/434
3	18.201	171/311/575	8	7.765	344/254/434
3	21.186	234/470/237	8	8.069	217/434/393
3	21.715	298/281/237	8	8.215	371/239/459
3	24.498	341/281/461	8	8.965	434/255/524
4	6.402	117/147/130	8	10.114	360/281/450
4	12.934	342/395/297	8	12.989	192/287/407
5	6.173	413/385/448	8	21.980	283/373/463
5	6.458	313/445/370	9	6.423	323/311/445
5	7.073	149/167/279	9	6.567	323/311/504
5	9.841	147/281/369	9	7.041	434/279/419
5	10.149	327/385/459	9	8.047	217/434/241
5	13.031	343/295/350	9	8.209	371/239/459
5	15.615	282/370/460	9	11.759	291/255/524
5	19.009	457/381/430	9	12.600	399/267/487
5	20.424	578/457/488	9	12.988	291/252/355
5	20.726	341/397/552	9	21.416	355/455/370

*most dominant mass

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7.3.11. Subject 10: American alligators

Samples No. 10: Five steroid profiles from the group of seven male alligators were analyzed successfully. Noisy backgrounds were eliminated by doing both background extraction of the whole total ion chromatogram (TIC), subtracting the blank TIC, as well as the noise adjacent to each peak. Urinary creatinine concentrations were not determined, therefore, urine samples from the group of male alligators were qualitatively investigated instead of quantitatively. The qualitative results are shown in Table 7.6. and the identified steroid metabolites are indicated.

Retention times and NIST library matches with the standard EI m/z steroid spectra were used to locate and identify the steroid metabolites in the samples. Qualitative investigation showed the presence of various steroid metabolites. Analytes that were identified include etiocholanolone (Et), 11-hydroxy Androsterone (HAn), 11-hydroxy Etiocholanolone (HEt) and 11-Deoxytetrahydrocortisol (THS).

Table 7.6. Qualitative results obtained from standards and alligator urine samples (No 10) after GC-MS analysis.

Sample name	Sample ID	Collection Date	Steroids identified
Cal. 1/2/3	Steroid L1/2/3	7/29/2009	An, DHEA
Qual S1	S1 ID	7/29/2009	Et, Kan, HAn, Pd, a-THF
Qual S2	S2 ID	7/29/2009	THS, Pt, THB, THE, THF
Blank	Blank	7/29/2009	n/a
Alligator5143	NASA5143	4/18/2008	HAn
Alligator3092	NASA3092	4/15/2009	Et , HAn
Alligator3108	NASA3108	4/20/2009	HAn, Het
Alligator3083	NASA3083	4/13/2009	HAn, THS
Alligator5310	NASA5310	11/13/2008	Het
test39/40/41	KtrlZa/b/c	7/29/2009	An, Et, DHEA, Kan, HAn, Pd, Pt, THS, THE, THF,a-THF
test46	TestosE2 L3	7/29/2009	Testosterone, Oestradiol

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7.4. Discussion

Observations in the results obtained by GC/MS analysis in subjects 1 to 10 are discussed separately below. However, mention is made of any peaks or spectra that are found coinciding within subjects, and whether there are any similarities in the various chromatograms.Table 7.5. lists the major peaks found in the steroid profiles of subjects 1 to 9, with retention times and masses constantly observed in the EI m/z mass spectra.

The peak observed in all the chromatograms at approximately 6.4 min is C23 and the peak at approximately 31 min, the MO-TMS derivative of the internal standard, cholesteryl n-Butyrate. Both were added during sample preparation, and were confirmed to. match the confirmed system retention times of standards and EI m/z spectra with the NIST library search.

The peak at approximately 21 min that is present in TIC of the human control urine sample, and also common in most of the subject's urine samples, was identified in the EI m/z mass spectra as the monotrimethylsilyl derivative of cholesterol (masses 129/329/368/458/353). A peak at approximately 8 min eluted in some of the samples, with a dominant mass of 217 in all the spectra.

Qualitative investigations were performed for all the subjects except No 1, where positive identification of steroid metabolite derivatives by retention time as well as EI m/z mass spectra, and a urinary creatinine concentration made quantification possible.

Subject 1 (No 1): A reproductively active female Nile crocodile -This female crocodile lived in an environment that can be described as relatively unpolluted. The number of eggs the subject produced indicated that she was highly fertile with a functional endocrine system. Seven steroid metabolites that are routinely included in steroid profiles on human urine, were identified in the TIC (Figure 7.2). The urinary creatinine concentration of this sample was determined in the Clinical Pathology Laboratory at Onderstepoort Veterinary Hospital. This enabled

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quantitation (Table 7.3) by means of relative abundance of the peaks to that of the internal standard, as described in Chapter 6.

Subject 2 (No 2): Pooled sample of adult Nile crodocile urine - No similarities were observed in the spectra of related retention times of the previous subject or the control human sample. The mass m/z 205/207, referred to as column bleed; its presence is observed in the baseline as well as in the peaks. Although background subtraction was performed, 205/207 still features in some of the spectra, as seen in Figure 7.3b. Library searches did not deliver conclusive data on the possible structures of these five unknown compounds.

Subject 3 (No 3): Female Nile crocodile; Izintaba Crocodile Farm - Several peaks feature in the TIC of the urine sample, of which none could be identified by NIST library searches. The TIC of the plasma sample (Figure 7.4.b) shows two distinct peaks; one at RT 12.976 min, unidentified, and the other at RT 21.099 min, identified by NIST library search as the monotrimethylsilyl derivative of cholesterol. The EI m/z spectrum of the peak at RT 21.186 min in the chromatogram of the urine sample had no similarity to the peak at 21.099 min in the plasma sample, and the peak at 24.498 min did not match the NIST spectrum of the corticosterone methyloxime tms derivative, which was found to elute at approximately 24.690 min under the same chromatographic conditions (see Chapter 8). EI m/z masses in the spectra of all the unidentified peaks in the profile of this subject are shown in Table.7.5.

Subject 4 (No 4): Juvenile male Nile crocodile; Kruger National Park - The sample of this Nile crocodile from Kruger National Park was collected during a pansteatitis outbreak in 2008 (Myburgh, 2014; Ferreira and Pienaar, 2014). In the TIC, a dominant peak is observed at 12.934 min The EI m/z spectrum of this peak shows no similarity to the spectrum of the dominant peak eluting at 12.052 min in the TIC of subject 2. The only other peak in this steroid profile was the relatively abundant peak at 21.058 min, matching the spectrum of the monotrimethylsilyl derivative of cholesterol. Would this the excessive excretion of

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cholesterol have something to do with stress response in crocodiles, or could it be an indication of glomerular damage in an individual crocodilian?

Subject 5 (No 5): Sub-adult male Nile crocodile; Loskop Dam - Although peaks eluted at retention times very close to many of the known steroid metabolites, difficulty was experienced in matching of the EI m/z spectra of these peaks to those of standard steroid metabolite derivatives. Peaks near the approximate retention times where the androgen metabolites would be expected; 7.37 min (An) and 7.74 min (Et), showed no 270/360 m/z ions in the EI spectra, neither did peaks near 10.6 min show m/z 448/358/268 that would identify HAn. Retention times 20.424 and 20.953 min, respectively, show base peaks m/z 578 and 652, which were also the base peaks for tetrahydrocortisone (THE) and tetrahydrocortisol (THF/a-THF) metabolites. However, the confirmed retention times for this system and conditions for the analytical standard THE was approximately 17.07 min, and for THF, 17.93 min. This observation leads to the speculation that steroid metabolites with structures and molecular weights related to the mineralocorticoids, were present in this sample. The presence of the internal standard at 31.075 min serve to confirm that the temperature program and chromatographic conditions were consistent with injections of steroid metabolite standard mixes.

Subject 6 (No 6): Sub-adult male Nile crocodile; Flag Boshielo Dam – The TIC of the urine sample shows several peaks. The monotrimethylsilyl derivative of cholesterol was identified on NIST for the prominent peak at 21.174 min. None of the other peaks (RT's and EI m/z spectra listed in Table 7.5) could be identified by retention times matching to standards or NIST library searches.

Subject 7 (No 7): Female spectacled caiman – The very prominent peak at 21.141 min was confirmed to be the monotrimethylsilyl derivative of cholesterol observed in the TIC, The EI m/z spectra of the rest of the peaks in the profile shows no resemblance to those of steroid standard peaks. One of the unidentified peaks at RT 8.224 min, shows the same dominant masses (371/239/459) in the EI m/z spectrum as in the spectrum of a peak eluting at 8.215 min in the steroid profile of

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the male spectacled caiman, and at 8.209 min in the TIC of the male dwarf crocodile. This combination of masses were not observed in the EI m/z spectra of the peaks in any of the Nile crocodile urine samples.

Subject 8 (No 8): Male spectacled caiman - The peak eluting at 21.120 min was not as prominent as the peak at 21.141 min in the profile of the female spectacled caiman, but it was also confirmed to be the monotrimethylsilyl derivative of cholesterol The EI m/z spectra of the rest of the peaks in the profile show no resemblance to those of steroid standard peaks. However, a resemblance to the spectrum a peak eluting at 8.215 min to that of the peak at 8.224 min in the TIC of the female spectacled caiman, and 8.209 min in the steroid profile of the male dwarf crocodile was noticed.

Subject 9 (No 9): Male dwarf crocodile – Two very prominent peaks eluting early in the chromatogram, at RT 8.047 min and at RT 8.209 min, both showed EI m/z spectra similar to peaks at approximately the same time in the spectacled caiman species, and even one Nile crocodile subject. The peak eluting at 21.122 min was confirmed to be the monotrimethylsilyl derivative of cholesterol The EI m/z spectra of the rest of the peaks in the profile show no resemblance to those of steroid standard peaks.

Subject 10 (No 10): American alligators - The urine samples of five male American alligators were extracted, hydrolysed and derivatized as described in Chapter 6. Qualitative GC/MS investigation showed the presence of various steroid metabolites. It was notable on the chromatograms that quite a few prominent peaks eluted in the region of the known human androgen metabolites, which may fit in with the high plasma testosterone concentrations determined by RIA. However, many of these peaks could not be identified. Analytes that were identified by matching retention times and NIST library search include Et, HAn, Het and THS. The successfully analysed profiles show very promising information, but it is desirable to have a combination of the results to formulate a conclusion.

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All subjects: An interesting observation was the presence of cholesterol, mostly as dominant peak of the known/identified steroids/compounds, whereas the metabolite of corticosterone, 5β-pregnan-3α,11β,21-triol-20-one (THB), was not reported in crocodilian urine as yet. THB is usually identified and quantifiable in human urine samples.

Another factor to be considered is that not only steroids and their metabolites would show as peaks in the chromatograms. Normal dietary nutrients (proteins in chicken and fish) may be simultaneously extracted and may possess ketones that can react with the derivatization reagents. Analysis using high resolution mass spectrometric (HRMS) techniques would allow the empirical formulas to be determined from masses that are obtained

7.5. Conclusion

The fact that some of the peaks could not be identified shows the need for further investigation with more sophisticated instrumentation to facilitate structure elucidation of the unknown analytes detected in the various urine samples. In addition to identification, quantitative analysis of steroids and their metabolites in crocodilian urine may serve as a valuable diagnostic tool in the investigation of the ECD effects. The evaluation of urinary steroid profiles in crocodilians may aid in monitoring of the effects of steroid altering compounds in the environment. This investigation has established a platform for the evaluation of the significance of this method and matrix (urine) as a non-invasive diagnostic approach to investigate the health status of the Nile crocodile.

Possible future studies could include: the comparison of urinary steroid profiles of crocodilians of different ages and sizes; the investigation of seasonal differences in the excretion of steroid metabolites in the Nile crocodile; the comparison of profiles of crocodilians from clean and polluted environments; evaluation of clinical signs between healthy and sick crocodilians (from healthy and polluted ecosystems) and correlation with steroid profile results.

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7.6. Acknowledgements

The author was invited by prof. Louis J. Guillette Jr., Professor, Howard Hughes Medical Institute, to visit the University of Florida, Gainesville, FL, USA in the summer of 2009, to work on a collaborative project that was part of this PhD study.

7.7. Proposed publication from this chapter

Bekker L C, Cromarty A D, Botha C J, Myburgh J G. Urinary steroid profile of a reproductively active female Nile crocodile (*Crocodylus niloticus*). General and Comparative Endocrinology.

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CHAPTER 8: Adrenocorticotropic hormone study in Nile crocodiles

8.1. Introduction

During activation of the hypothalamic–pituitary–adrenal axis, glucocorticoids are secreted from the adrenal cortex following stimulation by adrenocorticotropic hormone (ACTH). In humans and most vertebrates, ACTH is produced and secreted by the anterior pituitary gland in response to corticotropin-releasing hormone (CRH) released by the hypothalamus (Guyton and Hall, 2006c). Confronted with stress situations (e.g. pollution of the environment for wild crocodiles and improper housing conditions in the case of captive crocodiles) this response in the animal is elevated and an increase in blood and urinary concentrations of glucocorticoids and glucocorticoid metabolites are expected. Various studies have reported an elevation in plasma glucocorticoids, specifically corticosterone, concentrations in crocodilians due to induced stress through manual handling, and stress as result of contaminated habitat, and elevation during reproduction (Guillette et al., 1995; Guillette et al., 1997; Moore and Jessop, 2002; Franklin et al., 2003). Investigation of urinary glucocorticoid metabolites and corticosterone concentrations could therefore give an indication of stress-levels in groups of animals from different environments (Ganswindt et al., 2014).

Aquatic pollution is an important global concern and crocodilians, as top predators in aquatic ecosystems, are considered to be excellent sentinels of ecosystem health (Myburgh, 2014). Most crocodilians are listed by CITES as endangered or threatened (http://www.iucncsg.org/pages/Conservation-Status.html), making it extremely difficult and in most cases inappropriate to kill them for research purposes. Many aquatic pollutants (e.g. pesticides and pharmaceuticals) are bio-transformed by the liver and excreted in the urine, making urine a very useful diagnostic sample for scientists using crocodilians as sentinels.

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Blood samples have been routinely used to evaluate the endocrine system of crocodilians (Guillette et al., 2000; Guillette et al., 2001; Hamlin et al., 2011). It is well known that aquatic pollutants (e.g. pesticides and endocrine disruptors) adversely affect species associated with the contaminated ecosystem, even if exposed to low pollutant concentrations (Colborn, et al., 1996; Colborn and Thayer, 2000; Guillette, et al., 2000). Many xenobiotic compounds have been confirmed to affect crocodilians, specifically the American alligators (*Alligator mississippiensis*) (Guillette et al., 2000; Guillette et al., 2001). Guillette et al. (1994) reported that alligators from Lake Apopka were affected regarding fertility and thyroid status amongst others. Crocodilians, as top predators in aquatic ecosystems, could therefore be used as sensitive sentinels of aquatic pollution status (Guillette et al., 2000; Colborn and Thayer, 2000).

The aim of this investigation was to assess adrenocortical activity in captive Nile crocodiles (*Crocodylus niloticus*) by measuring steroid (including glucocorticoid) metabolite concentrations in urine samples obtained after an intervention with ACTH. The objective was to observe whether an ACTH challenge would influence corticosterone concentrations in the urine of a Nile crocodile in a 6 h time period. This investigation was conducted as a sub-project of a study by Ganswindt (2014), performed with approval of the University of Pretoria Animal Use and Care Committee (Reference Vo45-10). As an extention to the work done in Chapters 6 and 7, GC/MS was the method of choice for assessing the glucocorticoid profile in the Nile crocodile urine.

8.2. Materials and methods

8.2.1. Study subjects, housing and nutrition

The experiment was conducted on 18 captive Nile crocodiles, 16 males and 2 females, between 1.8 and 2.2 m in total length, with body masses between 26 and 55 kg. The animals were temporarily housed in a separate enclosure at *Le Croc* (*Le Croc Crocodile Breeding Farm and Tannery*, South Africa) for five weeks, to ensure controlled conditions and easy frequent access to the animals. Housing differed for three groups of animals to accommodate possible housing stress. Six

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of the study animals (4 males and 2 females) were housed in individual pens, four in pairs (4 males) and two groups of four (8 males), as shown in Table 8.1.

Throughout the study period, feeding with minced chicken supplemented with vitamins and minerals, was scheduled five times per week. The crocodiles had permanent access to water for drinking and submerging/thermoregulation. Recording of environmental temperature (maximum and minimum) and humidity was performed daily, and the enclosures were cleaned six times per week.

Table 8.1. Housing of the Nile crocodiles in the assessment study.

Pen ID (control/exp.)	Subject ID	Pen ID (control/exp.)	Subject ID
Pen A5 (controls):	M441 M442 M443 (M225)	**Pen B5 (experimental):**	M551 M552 M553 (M226)
Pen A4 (controls):	M444 M446	**Pen B4 (experimental):**	M555 M556
Pen A3 (control):	M554	**Pen B3 (experimental)**	M550
Pen A2 (experimental):	M445	**Pen B2 (experimental):**	F222
Pen A1 (experimental):	M202	**Pen B1 (control)**	F224

Controls: injected with 1 mL saline after electrostunning

Experimental: injected with 0.5 IU/kg Synacthen Depot© after electrostunning

8.2.2. Experimental design

An ACTH stimulation test was performed halfway through the 5 week experimental period. Immobilization was achieved via electrostunning, followed by intramuscular injections of 10 animals with 0.5 IU/kg synthetic ACTH (Synacthen Depot©, Hoffman LaRoche AG), and 8 animals (controls) with 1 mL saline. (See Table 8.1).

Blood samples were drawn from all the animals at different intervals to monitor the effects of the ACTH challenge on circulating glucocorticoid concentrations. Initial blood samples were drawn at 3 min from electrostunning, another blood sample after one h, and a final blood sample after 5 h. These samples were collected from the spinal venous sinus of the crocodile, according to the technique described by Myburgh et al. (2014). Faeces samples in all the pens were collected 3 times a

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day for a period of 2.5 weeks after administration of ACTH/saline. Faeces samples were also collected for a period of 2.5 weeks prior to the challenge to determine baseline fecal glucocorticoid metabolite (FGM) concentrations. The faeces samples were frozen at -20°C, until analysis to determine FGM concentrations for the study of Ganswindt (2014).

In addition, a dataset of 24 crocodile urine samples was collected pre- and post-ACTH/saline injection for this study. Table 8.2 lists information on collection time and volumes collected. Despite only two of the 18 crocodiles providing urine at t=0 (baseline/pre-stimulation time), and that only 4 urine samples could be collected at t=1 (1 h post-stimulation), the study proceeded with sample preparation and GC/MS analysis of the limited number of samples collected.

8.2.3. Sample preparation

Sample preparation was performed as previously described in Chapter 6, involving an initial SPE extraction with C18 octadecyl cartridges, of the 24 urine samples. The extraction volume of each urine sample is shown in Table 8.2. A 20 mL aliquot of a 24 h control urine collection was included in the extraction batch. Calibration mixes of pure standards at concentrations 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, and 20.0 µg were prepared, dried and derivatized, to inject with the batch of samples.

The first extraction was followed by overnight enzymatic hydrolysis. Glusulase, a preparation from the intestinal juice of the Roman snail *Helix pomatia*, was selected as hydrolysis agent. This preparation, certified to contain specific activities of the enzymes β-glucuronidase and sulfatase, is relatively non-specific and therefore it was expected to be able to achieve deconjugation of additional conjugates (Myers and Northcote, 1958; Takeda et al., 1966; Crabbe et al., 2002).

Following the hydrolysis step, a second SPE extraction was performed with additional clean-up and water removal through NH2 cartridges to which anhydrous sodium sulphate was added. The eluents were dried and the residues were subjected to derivatization (MO-TMS) as described in Chapter 6.

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Table 8.2. Data set of urine samples collected from the study subjects pre- and post-ACTH/saline injections, and prepared for GC/MS analysis.

Time	Subject ID	Volume collected/ extracted (mL)	Time	Subject ID	Volume collected/ extracted (mL)
t=0	F224	37/32	t=6	M442	102/32
t=0	M445	34/30	t=6	M443	60/30/
t=1	M555	34/30	t=6	M444	6831
t=1	M552	36/31	t=6	M445	70/33
t=1	M444	33/29	t=6	M446	76/35
t=1	M443	35/34	t=6	M550	114/35
t=6	F222	88/39	t=6	M551	112/36
t=6	F224	42/37	t=6	M552	110/39
t=6	M202	121/37	t=6	M553	56/21
t=6	M225	118/37	t=6	M554	115/35
t=6	M226	116/37	t=6	M555	110/33
t=6	M441	96/29	t=6	M556	108/34

t=0: immediately following electrostunning, before Synacthen/saline injection

t=1: 1 h afer intramuscular injection of Synacthen/saline

t=6: 6 h afer intramuscular injection of Synacthen/saline

8.2.4. GC/MS analyses

The derivatized residues were shipped on ice to the VM-ATLS laboratories, where the standardized GC/MS method was used for urinary steroid profiling in the standard mixes, control and 24 Nile crocodile samples. A Thermo Scientific Trace-GC gas chromatograph, equipped with a Trace MS single quadrupole mass spectrometer (Thermo Scientific, Austin Tx, USA) was employed. Data collections were performed with Thermo Scientific Xcalibur software, and library searches were done with the NIST MS library.

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8.3. Results

Results were investigated quantitatively and qualitatively to assess the steroid profiles from the different time points following ACTH challenge.

The aim was to find urinary glucocorticoid metabolites or specifically THB (the tetrahydro metabolite of corticosterone) in the resulting chromatograms. Library searches were performed on all the peaks but none of the EI m/z spectra correlated with standard EI m/z spectra of the MO-TMS or TMS derivatives of either THB or corticosterone. Figure 8.1 shows the EI m/z spectrum of THB-MO-TMS as it featured in the peak at 21.39 min of the TIC of the 2.5 µg/mL steroid standard mix that was injected with the Nile crocodile urine samples (this spectrum is not available in the NIST library). Figure 8.2 shows the EI m/z spectrum of tetrahydrocorticosterone tris- TMS as derived from the NIST library, and Figure 8.3 shows the EI m/z spectrum of corticosterone methyloxime TMS in NIST.

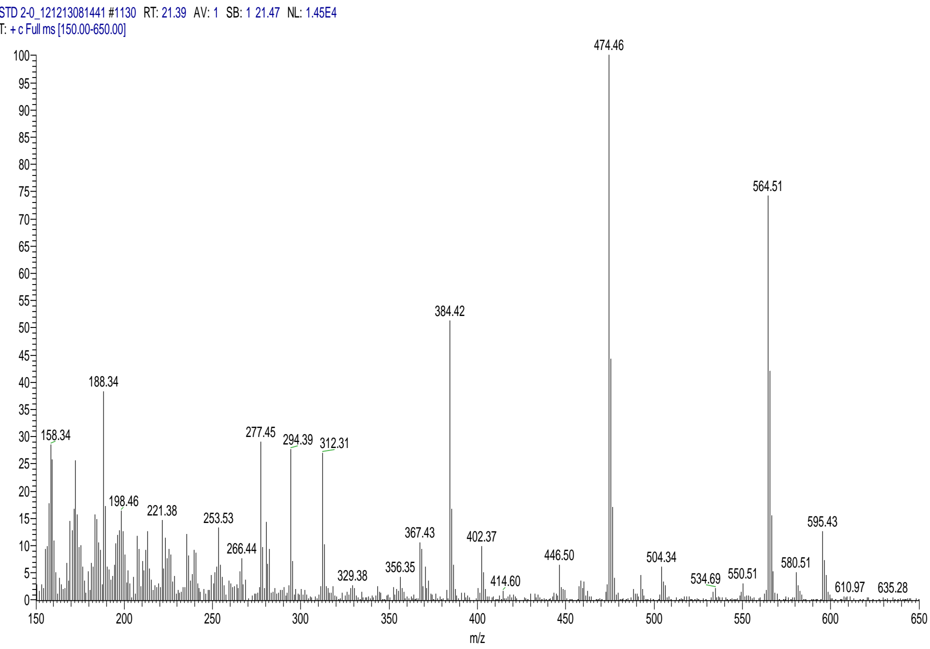

Figure 8.1. EI m/z spectrum of tetrahydrocorticosterone methyloxime TMS.

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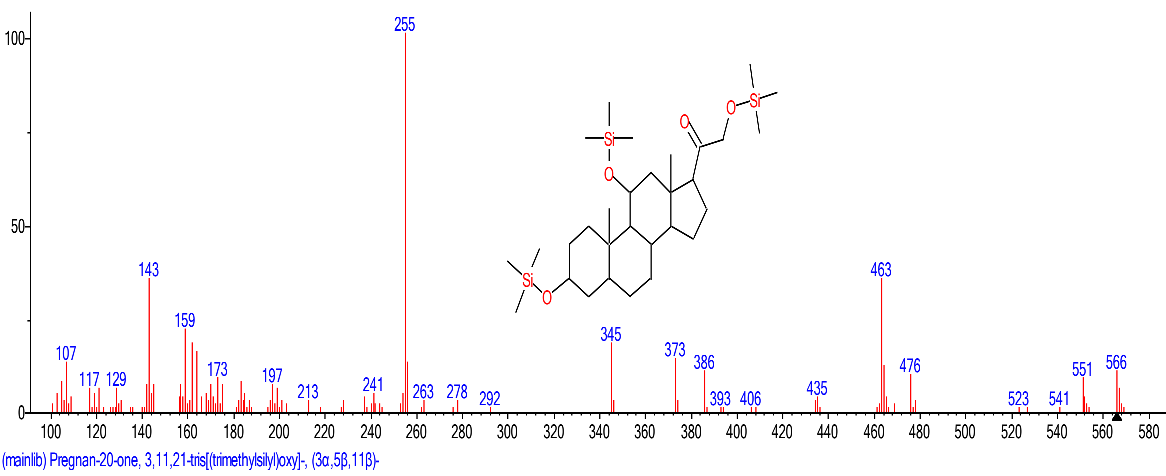

Figure 8.2. EI m/z spectrum of tetrahydrocorticosterone tris- TMS (NIST).

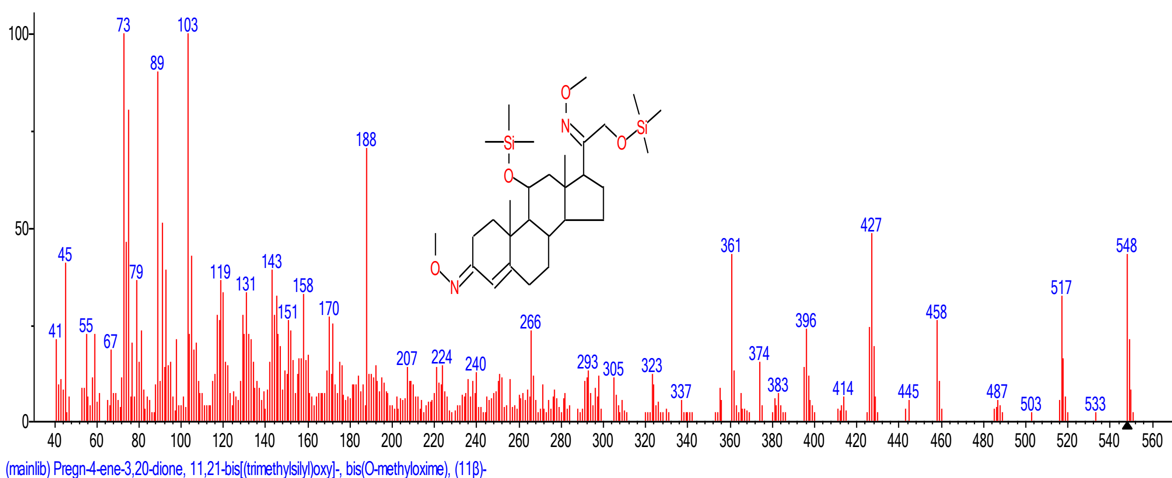

Figure 8.3. EI m/z spectrum of corticosterone methyloxime TMS (NIST).

From the chromatograms, the androgen metabolite etiocholanolone, could be identified, eluting at approximately 15.6 min in all of the samples. Cholesterol mono-TMS was also positively identified by NIST library search (Figure 8.4) in all the samples at approximate RT of 23.5 min.

None of the EI m/z spectra of tetrahydrocorticosterone or corticosterone were present in the chromatograms of the Nile crocodile samples. However, tetrahydrocorticosterone methyloxime TMS could be detected in the human control

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sample (21.42 min) as identified by matching RT and spectrum with the peak in the steroid standard mix.

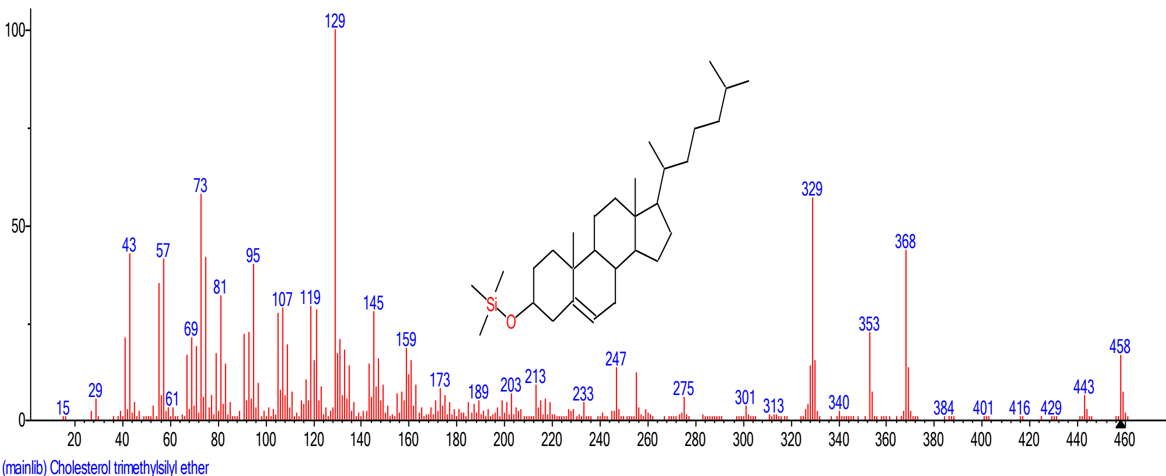

Figure 8.4. EI m/z spectrum of cholesterol mono-TMS (NIST).

To facilitate quantitation of any positively identified glucocorticoid metabolites, the urine samples were also analysed to determine creatinine concentrations with a standardised liquid chromatography/mass spectrometry (LC-MS/MS) method (Hou et al., 2012). Due to the conventional clinical method for measuring urinary creatinine concentrations having inadequate sensitivity for the low creatinine levels typically found in crocodile urine, a more sensitive analytical technique was used. The steroid concentrations would be reported as nmole steroid/µmole creatinine, as shown in Equation 6.2 (Chapter 6). However, no EI m/z spectra were found which would positively identify any of these metabolites in the Nile crocodile samples.

The identification of corticosterone was re-visited by analysing a pure corticosterone standard on the Shimadzu GC/MS at Onderstepoort Veterinary Faculty, using the experimental conditions described in Chapter 7 (Section 7.2.3.1). Two peaks feature on the TIC: Figure 8.5 shows the peaks eluting at approximate RT 21 and 24.5 min respectively, were confirmed by EI m/z spectra of cholesterol mono-TMS and corticosterone MO-TMS (see Figure 8.6). The peak at 21.142 min, identified as cholesterol mono-TMS, was the dominant peak in this chromatogram.

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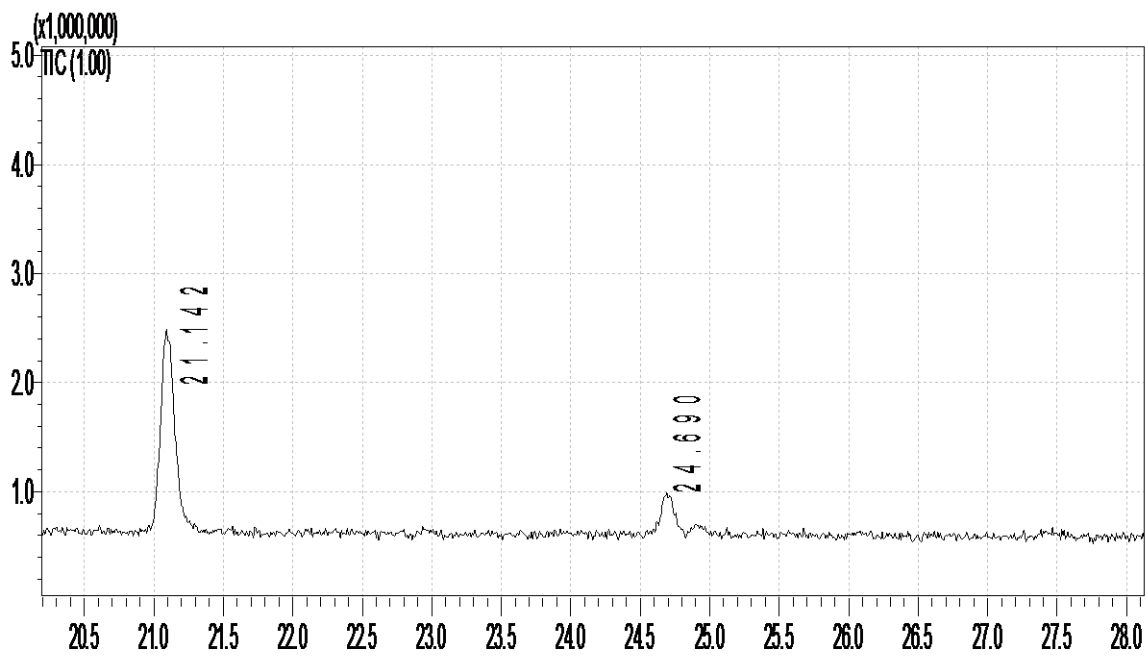

Figure 8.5. Peaks in the eluting segment of the TIC of the injection of the corticosterone standard, showing retention times at 21.142 and 24.690 min.

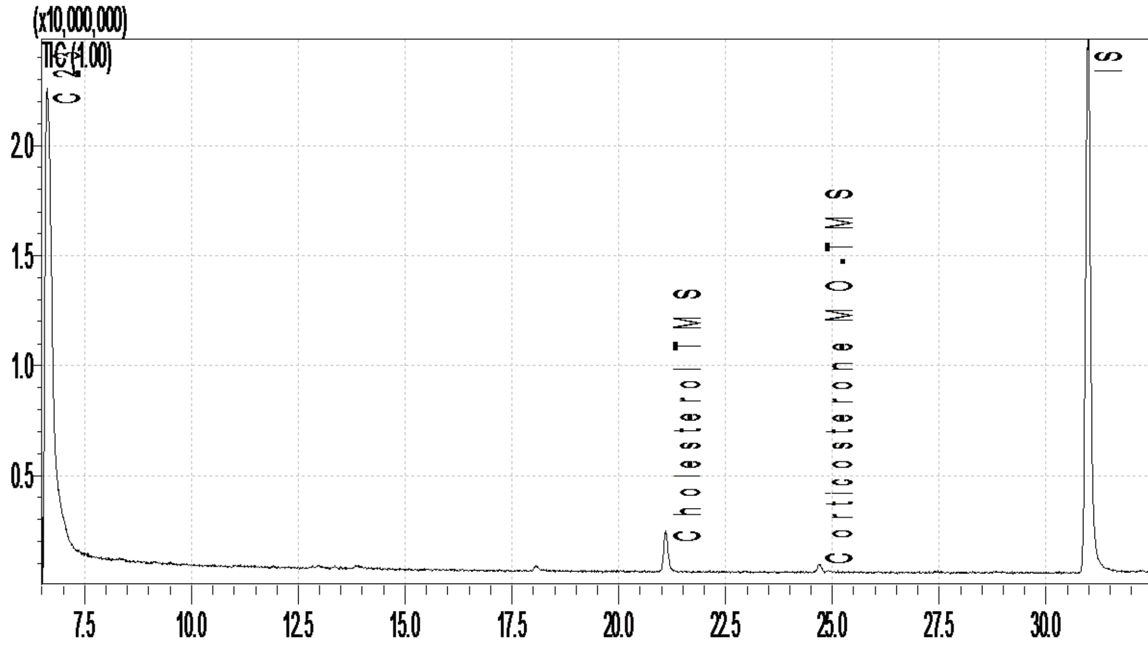

Figure 8.6. Peaks in the TIC of the injection of the corticosterone standard, showing the names as identified by EI m/z spectra.

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Figure 8.7 shows one of the Nile crocodile urine samples analysed on the Thermo Scientific GC/MS. This was the t=1 sample from subject M552, a male experimental subject (i.e. challenged with ACTH) The dominant peak on the TIC was identified as cholesterol mono-TMS by NIST library search. The difference in retention times between the two GC/MS systems is mainly due to a slight variation in column length.

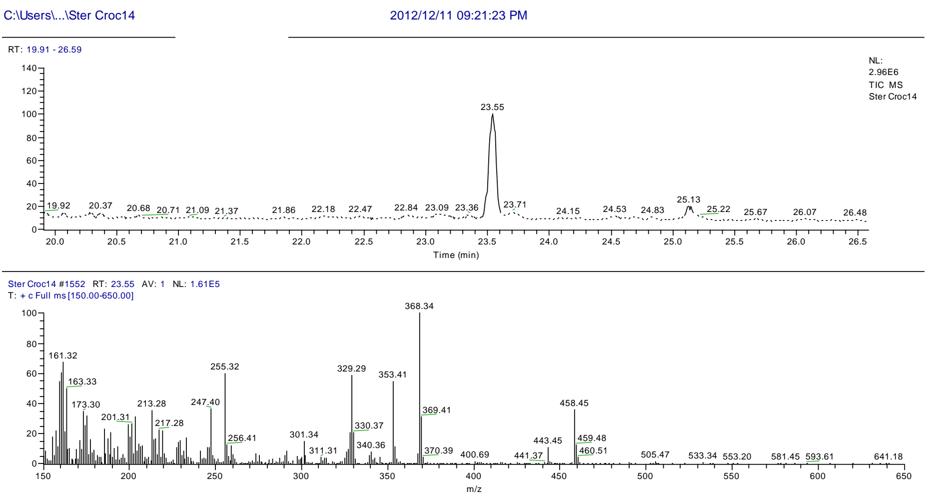

Figure 8.7. Peak and EI m/z spectrum obtained in the urine sample (t=1) of one of the experimental subjects that were challenged with ACTH.

In the TIC's of 5 of the t=6 samples (3 experimental, 2 control), the tetrahydro metabolite of cortisone (THE) was detected at approximately 22.4 min, with a relatively high abundance related to the cholesterol mono-TMS peak. Two of the experimental samples that showed THE, also showed a positive relation to the RT's and EI m/z spectra of tetrahydrocortisol (approximately 22.73 min) and allo tetrahydrocortisol (approximately 22.75 min), respectively. Unfortunately, these subjects did not have a t=0 sample for comparison. Figure 8.8 shows the TIC of subject M551, t=6, and Figure 8.9 the EI m/z spectrum of the peak at 22.37 min. Figure 8.10 shows the EI m/z spectrum of the peak at 22.73 min.

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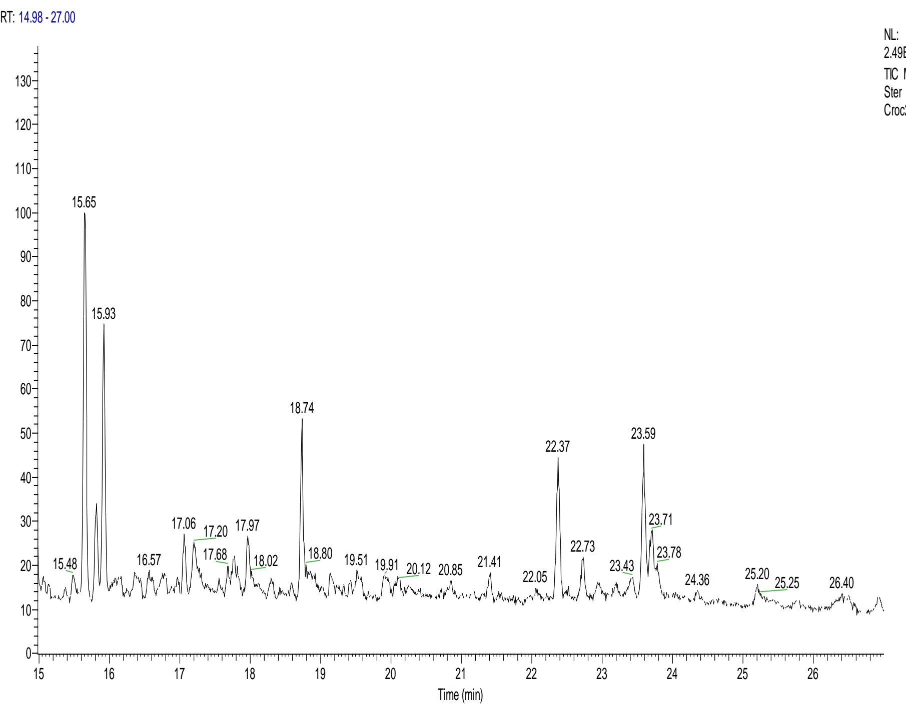

Figure 8.8. TIC of subject M551, t=6.

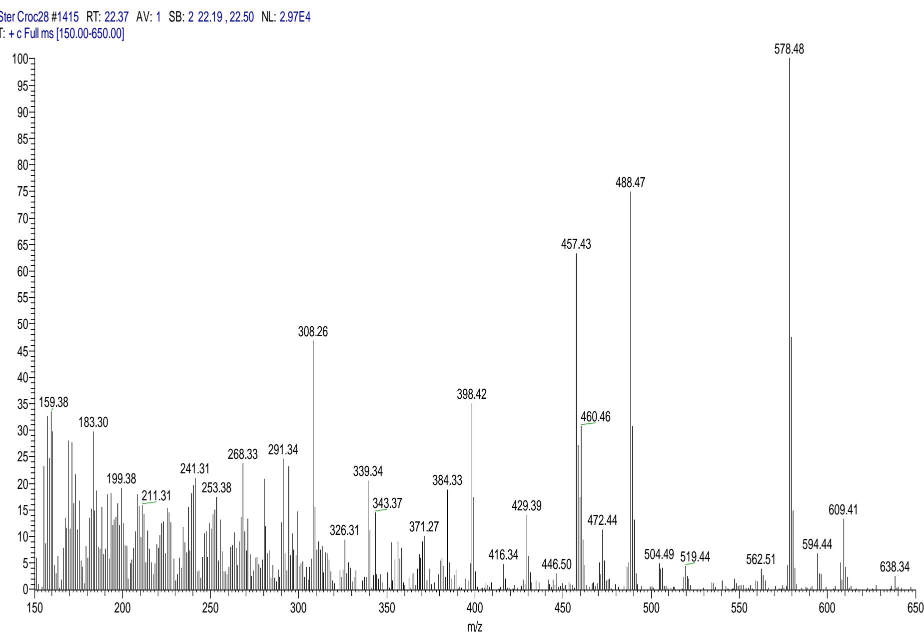

Figure 8.9. EI m/z spectrum of the peak at 22.37 min.

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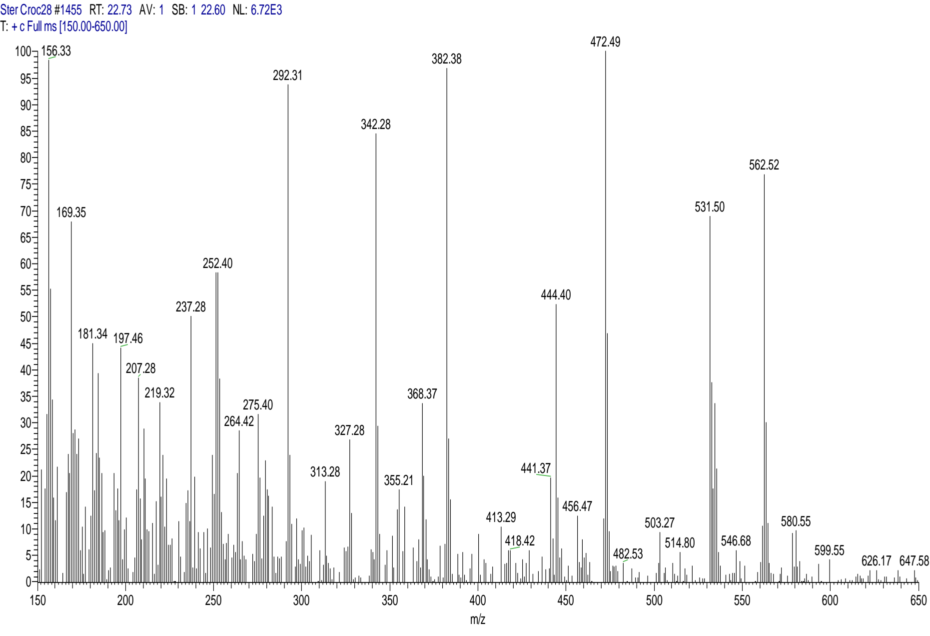

Figure 8.10. EI m/z spectrum of the peak at 22.73 min.

8.4. Discussion

Five of the 18 urine samples collected at t=6 showed peaks in the TIC that corresponded with retention time and EI m/z spectrum of the MO-TMS derivative of tetrahydrocortisone (THE). Two of the three samples from experimental subjects that showed THE, were from control subjects (injected with saline) and the other three were from experimental animals (injected with Synachten/ACTH). Two of the three samples from experimental subjects also showed peaks that correlated with the MO-TMS derivatives of tetrahydrocortisol (THF) and allo-tetrahydrocortisol (α-THF), respectively. Unfortunately, none of the subjects in which THE was positively identified in t=6 urine samples, had urine samples at t=0. Quantitation of baseline concentrations vs post-ACTH challenge concentrations of urinary glucocorticoid metabolites may have provided information on the stress response in these subjects.

In all the other samples analysed, neither corticosterone, THB or any other glucocorticoid metabolites could be identified in the TIC's of the samples. Hence, no evidence could be found of differences between the baseline and 6 h urine

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samples. Therefore, our findings could not confirm that a urine sample can be used to monitor expected corticosterone increments in the Nile crocodile.

The results in the study of Ganswindt et al. (2014), showed a significant increase in serum corticosterone concentrations following ACTH administration, with an overall baseline increase of 121% 1 h after treatment, and 272% 5 h after treatment. The serum corticosterone concentrations of the saline treated animals showed less significant differences. Faecal corticosterone concentrations did not peak before 5 to 10 days after administration of ACTH.

The resulting outcome of this sub-study can be due to various factors, metabolic or experimental. Two options that have been considered are:

It could be a kinetic problem; there may not have been enough time for the conversion and excretion of the glucocorticoid metabolites into the urine of the Nile crocodile. It is a known fact that cold-blooded animals (including reptiles) show a reduced metabolic rate compared to mammalian animals. Sample timing of this experiment was based on information of studies on other species and may have not been accurate for this species. In humans the stress response in an ACTH challenge can show in the first 30 min post-administration. In Nile crocodiles, the timing of this response, and specifically the metabolism and excretion of the glucocorticoid metabolites is still unknown. Considering the time when faecal corticosterone concentrations peaked in the study of Ganswindt et al. (2014), it can be speculated that sampling of urine at a longer interval may have shown detectable glucocorticoid metabolites.

The other reason for failure to detect a stress response, could be that the conjugate that is formed and exists in the urine of the crocodile, is not a glucoronate or a sulfate and therefore the conjugated corticosterone metabolite is not hydrolysed (e.g. in free form) after sample preparation. Detection of the total amount of steroid by the GC/MS method would in this case not be possible. It would be possible to find small amounts of e.g. products formed that are not hydrolysed, thus library searches of unknown peaks may not reveal all derivatives

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of compounds that are uncommon in GC/MS analysis. The enzyme-immunoassays employed to measure corticosterone in the serum and faecal samples in the study of Ganswindt et al. (2014) are capable of measuring corticosterone in bound or free form.

Another question that can be raised, is whether ACTH stimulation really is the mechanism to support stress in crocodiles. Is there not an alternative stimulant than ACTH to consider? It would not automatically be the same mechanisms in crocodilians as in humans and mammals. Why would ACTH be its main stress initiator?

A factor to also consider with stress studies in crocodilians, is that stress levels can increase in various situations, e.g. increased stress when away from water as in amphibians, saltwater vs. freshwater environment, and manual handling of the animal. This study included blood sampling from baseline, and therefore may have had an influence.

8.5. Conclusion

Crocodilians confronted with stressful situations (e.g. pollution of the environment for wild crocodiles, improper housing conditions for captive crocodiles) are expected to excrete more corticosterone in their urine. Another factor that should be taken into consideration, is that invasive sampling techniques (eg collection of blood samples) have the disadvantage of a possible self-induced stress, whereas urine sample collection is an almost non-invasive sampling technique, and according to Myburgh et al. (2012), is relatively simple and atraumatic.

The outcome of this study leads to a few unanswered questions: Is 6 h the correct time for post-ACTH administration urine sampling; is ACTH the most suitable stimulant to test stress response in crocodiles; are the analytes missing because of different conjugates that are formed in crocodilians vs mammals? Future investigations are required clarify this.

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CHAPTER 9: General discussion and conclusions

The survival of the Nile crocodile (*Crocodylus niloticus*) is a concern, especially for those populations residing in polluted anthropogenically-changed aquatic ecosystems (Combrink et al., 2011; Botha et al., 2011). In addition, the routine health monitoring of the Nile crocodiles in captivity (commercial crocodile farms) has also become an essential component of intensive farming. Monitoring of their health status has traditionally been done via the evaluation of blood parameters, although documentation on reference intervals for most biomarkers are still far from comprehensive.

Urine as a diagnostic sample in crocodilians may become a critically important role-player in future. Determination of biochemical and hormonal parameters in urine have a potential to provide clinical information regarding the health and nutritional status of the Nile crocodile. Cloacal urine is relatively easy to collect through a non-invasive and atraumatic process (Myburgh et al., 2012) and can be a very important biomatrix for diagnostic purposes.

The aim of this project was to evaluate urine from the Nile crocodile as the sample for biochemical and hormonal analyses and to be able to achieve the aim of this research project the following objectives were undertaken:

- Determination of urine biochemical variables in juvenile Nile crocodiles by means of a standard veterinary clinical pathology profile, and establishment of the relationship between the urine- and the blood biochemical variables (Chapter 5).
- Validation of the sample preparation and gas chromatographic mass spectrometric (GC/MS) technique by optimizing the sample preparation procedure and the instrument parameters, to detect steroid metabolites in crocodilian urine (Chapter 6).
- Application of this validated analytical method to perform qualitative and quantitative measurement of steroids and steroid metabolites in the urine of a cohort of individual crocodilians (Chapter 7).

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- Investigation if a 6 h period was adequate to confirm a significant increase of adrenal steroid metabolites in the urine of Nile crocodiles after an ACTH stimulation test (Chapter 8).

Urine and plasma biochemical variables were determined in samples from juvenile Nile crocodiles, by means of a standard veterinary clinical pathology profile. The relationships between the urine- and the blood biochemical variables in 101 corresponding samples were established (Chapter 5). Establishment of baseline concentrations for biochemical variables in urine and plasma of the Nile crocodile may be considered a major breakthrough to aid in the evaluation of the health status of these reptiles. Correlation of the blood and urine biochemical variables in corresponding samples of farmed juvenile Nile crocodiles, showed positive relations in two variables (Cl and U/A), therefore the urine concentrations of these two variables may serve as proxy for plasma concentrations. From this study, plasma and urinary biochemical concentrations may contribute to the understanding of the normal concentrations (reference intervals) for crocodilians.The animals used in this study were not selected or exposed to any specific conditions. The results for these parameters in young Nile crocodiles. may serve as baseline data for future urine examinations in the Nile crocodile.

The sample preparation and GC-MS technique was validated regarding optimization of sample preparation and the instrument parameters, to detect steroid metabolites in crocodilian urine (Chapter 6). Qualitative and quantitative investigation of steroids in crocodiles from clean and polluted areas, may serve as a diagnostic tool to investigate similar problems in crocodilians and as a sentinel of water quality in areas where potential endocrine disruptor pollution would be difficult to trace.

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This validated analytical method was applied to perform qualitative and quantitative measurement of steroids and steroid metabolites in the urine of a cohort of individual crocodilians (Chapter 7). Urinary steroid profiling could become a routine investigation in future, to provide information on the reproductive and adrenal endocrinology in crocodilians. This investigation has established a platform for the evaluation of the significance of this method and matrix (urine) as a non-invasive diagnostic approach to investigate the health status of the Nile crocodile.

This GC/MS method was employed to investigate whether a 6 h period was adequate to confirm a significant increase of adrenal glucocorticoid metabolites in the urine of Nile crocodiles after an ACTH stimulation test (Chapter 8). The value of this study is that it serves as a guideline for future studies regarding the timing of urine sampling after ACTH administration, evaluating the stress response in Nile crocodiles.

The results of the various sub-projects (Chapter 5 to 8) of this study provided a significant contribution in terms of biochemical and hormonal studies in the Nile crocodile. In this study, the following was achieved:

- Establishment of baseline concentrations for biochemical variables in urine and plasma of the Nile crocodile may be considered a major breakthrough to aid in the evaluation of the health status of these animals.
- Correlation of the blood and urine biochemical variables in corresponding samples of farmed juvenile Nile crocodiles, showed positive relations in two variables (Cl and U/A), therefore the urine concentrations of these two variables may serve as proxy for plasma concentrations.
- Urinary steroid profiling may become a routine investigation in future, to provide information on the reproductive and adrenal endocrinology in crocodilians.

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In conclusion, the results of the various sub-projects of this study provided a significant contribution in terms of biochemical and hormonal studies in the Nile crocodile. The advantage of using urine instead of blood in the evaluation of the health status of the Nile crocodile is evident due to the non-invasive sample collection.

Future projects to follow up the outcome of this study is required:

- The use of urine samples as a routine diagnostic sample in crocodiles will require further investigation regarding the clinical usefulness. Cl and U/A are the variables with the most potential for future use, and therefore, a follow-up study on the value of Cl and U/A excretion as indicator of homeostasis would be valuable These two urinary variables were the only parameters of those investigated, to show a positive correlation with plasma concentrations (Chapter 5). Studying of post-renal modification of urine in the urinary chamber of Nile crocodiles should also be considered
- To achieve structure elucidation of unknown steroids, chromatographic separation combined with detection with high resolution accurate mass instrumentation should ideally be performed. For this purpose, GC-TOF-MS and GC-Orbitrap MS are the most appropriate technologies to be selected (Chapters 6 and 7).
- Re-conducting an ACTH stimulation test should be considered, with sampling of urine at longer intervals extending to days may show whether the stress response in the Nile crocodile is triggered in the adrenals and measurable by rising concentrations of glucocorticoid metabolites (Chapter 8).

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APPENDICES

Appendix 5.1. Urine results of all the subjects in the biochemistry study.

Crocodile urine biochemistry												
Croc ID	Na mmol/L	K mmol/L	Cl mmol/L	Urea mmol/L	Creat umol/L	Ca mmol/L	Mg mmol/L	PO4 mmol/L	UricA mmol/L	Osmol mosm/kg	NH4+ mg/L	NH4+/UA ratio
Croc#1	4.1	13.76	14.5	45	112	0.45	0.08	8.75	0.16	175	1800	11250.00
Croc#2	0.5	16.1	7.5	25	368	0.21	0.17	18.37	0.43	228	2000	4651.16
Croc#3	1	9.37	16.8	47.4	5	0.65	0.06	10.6	0.13	282	6000	46153.85
Croc#4	1	16.7	11.7	25.8	16	1.2	0.27	3.11	0.32	169	1500	4687.50
Croc#5	5.1	24.09	20.1	42.4	25	0.6	0.21	3.59	0.23	231	2000	8695.65
Croc#6	4.5	44.21	6.4	42	1398	0.38	2.24	42.56	4.5	274	1500	333.33
Croc#7	9.7	21.91	17.4	26.3	116	0.54	0.21	6.28	0.27	252	2400	8888.89
Croc#8	1.4	14.8	10.3	18	26	0.89	0.26	1.45	0.4	247	2000	5000.00
Croc#9	2.1	14.09	11.1	36.9	32	0.69	0.11	6.84	0.19	284	5000	26315.79
Croc#10	1.8	24.5	21.1	58.3	21	0.59	0.12	14.24	0.16	243	4000	25000.00
Croc#11	0.9	14.89	19.3	55.1	82	0.82	0.24	9.2	0.11	275	4000	36363.64
Croc#12	3.6	19.71	11.7	18.8	50	0.5	0.15	4.19	0.32	203	3600	11250.00
Croc#17	1.3	21.92	17.4	50.7	6	1.67	0.22	3.29	0.13	217	3800	29230.77
Croc#18	5.7	12.22	23.3	42.2	57	0.9	0.06	7.63	0.16	249	7000	43750.00
Croc#19	0.9	16.02	10.9	21.9	1	0.57	0.2	4.54	0.19	181	3600	18947.37
Croc#20	3.8	22.97	27.5	64.2	120	0.19	0.03	20.93	0.12	252	4000	33333.33
Croc#21	1.7	21.89	11.9	36.5	191	0.11	0.21	8.43	0.26	192	3000	11538.46
Croc#22	3.7	32.49	5.3	53.5	250	0.73	1.39	36.5	1.74	212	2000	1149.43
Croc#23	4.4	23.05	12.5	45	173	0.2	0.08	11.6	0.17	230	3500	20588.24
Croc#24	6.4	14.33	21.3	47.6	59	0.49	0.11	13.93	0.19	238	4200	22105.26
Croc#25	1.3	22.96	18.9	47.6	63	1.62	0.28	0.67	0.19	237	3600	18947.37
Croc#26	2.2	35.1	19.8	43.5	78	0.46	0.12	8.44	0.25	237	3800	15200.00
Croc#27	0.7	19.29	19.4	67.1	127	0.27	0.07	25.45	0.13	253	4000	30769.23
Croc#28	12.5	31.44	36.3	47.2	0	0.86	0.07	9.94	0.28	253	3600	12857.14
Croc#29	0.4	18.43	17.6	47.4	0	0.38	0.12	6.82	0.13	239	4800	36923.08
Croc#30	11	26.25	20.8	41.1	22	0.87	1.57	0.07	0.26	188	3600	13846.15
Croc#31	11	58.95	15.3	35.9	243	0.2	0.18	15.77	0.64	209	2200	3437.50
Croc#33	11.8	45.6	14.5	17.1	16	0.02	0.12	6.55	0.71	173	1800	2535.21
Croc#34	8.2	18.21	20.9	57.7	0	0.31	0.05	14.26	0.22	208	4600	20909.09
Croc#36	2.2	34.75	23.8	44.8	52	1.02	0.13	7.56	0.19	237	8800	46315.79
Croc#37	4.3	57.37	22.6	30.6	156	0.13	0.4	2.92	0.46	236	2200	4782.61
Croc#38	5.9	15.99	20.3	44.1	15	0.66	0.11	9	0.32	238	4000	12500.00
Croc#39	2.8	59.86	10	60.1	239	0.34	0.13	52.45	0.22	227	3800	17272.73
Croc#41	1.9	19.54	22.1	61.2	55	0.48	0.14	12.67	0.13	245	7000	53846.15
Croc#42	30.4	46.06	19.5	15.6	58	0.01	0.23	10.4	1.17	204	2000	1709.40
Croc#43	1.5	28.69	17.6	37.4	49	0.74	0.09	2.55	0.27	226	4400	16296.30
Croc#44	1.8	19.75	23.6	81.9	116	0.13	0.06	30.06	0.08	263	7600	95000.00
Croc#45	1.2	26.04	11.3	19.7	20	0.71	0.37	1.16	0.4	132	2200	5500.00
Croc#46	2.4	37	8.1	29.3	104	0.26	0.17	9.01	0.68	153	2000	2941.18
Croc#47	1.3	33.19	21.5	45.9	0	0.37	0.11	9.43	0.15	252	3200	21333.33
Croc#49	1.6	28.83	19.1	41.3	14	0.92	0.18	5.67	0.21	223	3600	17142.86
Croc#50	9.9	29.26	14.1	26.7	55	0.06	0.2	7.4	0.4	186	2400	6000.00
Croc#52	40.1	84.48	19.8	32.6	228	0.02	0.22	38.61	2.91	204	1400	481.10
Croc#53	13.4	36.8	39	32.1	0	0.42	0.24	4.72	0.31	249	3000	9677.42
Croc#54	2.65	8.97	22.4	52.3	32	0.04	0.06	8.01	0.34	90	220	647.06
Croc#55	2	16.7	21.6	58.8	127	0.16	0.15	16.71	0.25	215	300	1200.00
Croc#56	2.9	32.8	10.3	38.5	395	0.03	0.08	31.05	0.44	233	180	409.09
Croc#57	2.7	20.19	29.4	69.2	32	0.31	0.01	17.84	0.03	213	3500	116666.67
Croc#58	9.7	21.69	18.1	13.1	60	0.42	0.07	3.53	0.12	142	3200	26666.67
Croc#59	11.2	18.67	22.8	31.8	19	0.59	0.01	5.97	0.08	176	2800	35000.00
Croc#60	1.9	16.74	29.1	51	3	0.68	0.02	9.58	0.05	241	3000	60000.00
Croc#61	15.8	10.71	37.9	54.4	17	0.72	0	8.46	0.07	249	2700	38571.43

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Crocodile urine biochemistry (continued)												
Croc ID	Na mmol/L	K mmol/L	Cl mmol/L	Urea mmol/L	Creat umol/L	Ca mmol/L	Mg mmol/L	PO4 mmol/L	UricA mmol/L	Osmol mosm/kg	NH4+ mg/L	NH4+/UA ratio
Croc#62	3.6	24.51	29.5	63.4	12	0.31	0	14	0.05	238	3100	62000.00
Croc#63	9.8	17.23	23.2	51	27	0.41	0	11.85	0.05	237	5000	100000.00
Croc#64	12.5	19.71	20.8	23.7	3	0.65	0.04	4.28	0.12	140	2300	19166.67
Croc#65	8	29.39	35.2	62.2	10	0.62	0.01	9.73	0.04	200	3200	80000.00
Croc#66	6	29..45	29	68.6	2	0.34	0	18.61	0.12	246	3000	25000.00
Croc#67	39.7	25.2	43.4	44	2	0.67	0.02	6.84	0.16	256	3100	19375.00
Croc#68	6.6	32.07	30	47.7	7	0.85	0	7.44	0.14	199	2200	15714.29
Croc#69	1.9	12.8	21.8	42.4	35	0.36	0	13.83	0.06	184	3700	61666.67
Croc#70	11.7	18.77	22.8	49.3	10	0.23	0	18.44	0.08	195	2200	27500.00
Croc#71	2.6	12.33	25.6	64.3	10	0.33	0.02	16.18	0.06	198	3900	65000.00
Croc#72	10.1	17.05	15.1	22.1	0	0.5	0	4.07	0.23	108	1900	8260.87
Croc#73	29.3	16.14	18.2	19.8	54	0.75	0	2.9	0.49	128	2100	4285.71
Croc#74	8.5	25.03	20.5	30.2	70	0.59	0	6.88	0.15	150	2000	13333.33
Croc#75	9.1	21.01	22.2	38.7	0	0.82	0.06	5.85	0.15	132	2600	17333.33
Croc#76	33.6	8.69	9.5	25.8	11	1	0	3.69	0.15	147	2300	15333.33
Croc#77	6	26.08	27.4	66.2	11	0.56	0	11.03	0.19	182	3100	16315.79
Croc#78	2.9	22.41	21.9	45.2	50	0.92	0	4.5	0.04	176	3900	97500.00
Croc#79	14.8	21.83	15.4	22.6	5	0.53	0	4.38	0.21	123	2000	9523.81
Croc#80	11	19.88	17	23.9	34	0.79	0	4.79	0.08	143	2200	27500.00
Croc#81	12.9	13.41	18.2	31.3	56	0.81	0	3.37	0.07	121	2000	28571.43
Croc#82	25.2	6.76	14.9	19.8	32	0.61	0.04	3.26	0.19	125	2500	13157.89
Croc#83	11.1	18.92	22.3	34.8	55	0.77	0	5.73	0.05	193	3300	66000.00
Croc#84	9.6	6.17	9.2	20	0	0.39	0	2.91	0.12	102	2100	17500.00
Croc#85	10.5	18.61	20.7	47	10	0.15	0	9.32	0.06	186	2900	48333.33
Croc#86	21	26.66	25.4	35	0	0.64	0	4.8	0.07	190	2500	35714.29
Croc#87	6.3	30.59	24.9	36.9	0	0.19	0	5.91	0.06	168	2700	45000.00
Croc#88	5.7	29.44	21.9	34.6	15	0.55	0	7.19	1.05	164	3000	2857.14
Croc#89	5.4	29.37	43.5	73.1	10	1.6	0.07	6.68	0.16	266	2500	15625.00
Croc#90	3.3	37.36	28.1	45.9	115	1.01	0.04	5.67	0.16	221	2300	14375.00
Croc#91	1.2	7.67	25.6	63.7	0	0.88	0.1	2.95	0.09	211	3500	38888.89
Croc#92	3.2	43.51	31.4	55.8	42	0.1	0	13.68	0.1	239	3000	30000.00
Croc#93	3.5	42.75	37.3	47.8	35	1.13	0.02	5.93	0.11	228	3000	27272.73
Croc#94	3.2	39.85	26.5	56.4	0	0.6	0.01	10.17	0.08	230	2100	26250.00
Croc#95	6.2	35.22	26.3	51	42	0.91	0	6.25	0.09	238	3400	37777.78
Croc#96	2.5	37.15	31.3	48.5	2	0.59	0.04	7.99	0.09	215	1800	20000.00
Croc#97	15.1	39.8	42.5	39.6	0	0.68	0	7.74	0.17	241	2200	12941.18
Croc#98	3.5	34.84	33.8	55.5	8	0.44	0.02	9.61	0.12	205	2000	16666.67
Croc#99	2.3	16.16	19.4	31.2	0	0.69	0.03	2.95	0.11	335	3800	34545.45
Croc#100	1.4	13.71	19.4	38.9	0	0.75	0.05	1.82	0.14	168	1900	13571.43
Croc#101	1.5	7.96	11.6	25.8	11	0.12	0	3.39	0.11	103	3000	27272.73
Croc#102	6.7	22.42	34.4	31.9	0	0.57	0.05	3.91	0.14	270	1800	12857.14
Croc#103	17	28.74	37.3	27.5	0	0.98	0.05	4.7	0.24	138	3200	13333.33
Croc#104	5	36.92	40.2	47.3	10	1.04	0.03	6.59	0.09	209	3000	33333.33
Croc#105	5.8	20.34	24.9	41.2	55	0.67	0.04	6.75	0.17	199	3300	19411.76
Croc#106	3.5	49.5	32.1	44.6	9	0.18	0.01	10.66	0.1	203	3000	30000.00
Croc#107	1.4	9.01	11.2	31.8	38	0.47	0.05	5	0.18	121	1800	10000.00
Croc#108	36.3	30.54	71.4	45	0	1.64	0.08	2.76	0.35	255	1900	5428.57
Croc#109	6.4	34.25	33.6	56.9	0	1.08	0	8.97	0.23	250	2000	8695.65
Croc#110	2.5	34.2	31.7	54.8	2	0.72	0	7.12	0.1	237	1800	18000.00

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Appendix 5.2. Plasma results of all the subjects in the biochemistry study.

Crocodile plasma biochemistry													
Croc ID	TSP g/L	Gluc mmol/L	Na mmol/L	K mmol/L	Ca(T) mmol/L	Ca2+ mmol/L	Mg mmol/L	SIP mmol/L	Urea mmol/L	Creat umol/L	Cl mmol/L	Osmol mosm/kg	UricA mmol/L
Croc#1	40.5	6.4	121.8	2.27	2.05	1.29	0.99	1.09	0.2	23	105	279	0.12
Croc#2	52	6.6	117.3	5.16	2.54	1.26	1.18	1.56	0.3	27	96	276	0.17
Croc#3	67.1	5.9	143.2	3.56	2.94	1.29	1.11	2.27	0.4	30	120	338	0.11
Croc#4	69.7	8.4	130.6	4.4	2.97	1.34	1.38	1.4	0.2	25	98	298	0.25
Croc#5	59.1	8.4	137.7	3.51	2.7	1.32	1.09	1.12	0.2	26	112	261	0.17
Croc#6	73.3	6.8	127.8	4.38	2.78	1.42	1.76	2.08	0.2	53	101	278	0.86
Croc#7	59.1	6.8	141.3	3.91	2.59	1.32	1.43	2.21	0.1	32	115	307	0.36
Croc#8	64.3	5.8	136.2	4.35	2.62	1.27	1.39	2.27	0.2	30	107	294	0.19
Croc#9	55.7	5.8	137.3	4.99	2.54	1.26	1.28	2.34	0.2	27	115	469	0.19
Croc#10	56.3	5.6	130.5	3.67	2.53	1.25	1.15	1.17	0.2	21	114	305	0.11
Croc#11	52.7	6.2	133.9	3.66	2.52	1.29	1.23	1.73	0.2	28	110	297	0.09
Croc#12	66.4	5.4	131.9	4.69	2.76	1.31	1.56	2.37	0.2	37	102	288	0.45
Croc#17	76.3	8.2	126.1	5.78	2.46	1.25	1.53	1.29	0.2	33	93	347	0.32
Croc#18	61.1	6.5	144.4	3.95	2.66	1.33	1.28	1.68	0.2	31	117	305	0.15
Croc#19	63.7	7.7	133.8	4.9	2.54	1.33	1.39	1.75	0.1	31	106	297	0.19
Croc#20	59.5	8.5	140.7	3.88	2.63	1.3	1.26	1.62	0.2	28	116	309	0.08
Croc#21	80	5.9	124.9	5.37	2.92	1.31	1.5	2.26	0.2	35	103	291	0.36
Croc#22	81.9	6.5	127	5.12	2.69	1.36	1.83	2.43	0.4	39	87	288	0.63
Croc#23	70.7	8.1	136.7	4.91	2.7	1.25	1.48	1.38	0.3	35	108	312	0.29
Croc#24	67.6	4.6	141.8	3.67	2.64	1.34	1.19	1.96	0.2	34	122	320	0.15
Croc#25	59.8	7.7	140.2	3.61	2.52	1.35	1.25	1.9	0.2	21	118	309	0.13
Croc#26	60.6	3.7	136.2	5.27	2.17	1.24	1.39	1.46	0.2	25	109	302	0.29
Croc#27	61.1	3.9	137.4	3.99	2.38	1.16	1.22	1.35	0.2	29	121	356	0.22
Croc#28	46.7	6.7	133.7	4.96	2.51	1.31	1.34	1.69	0.2	23	122	302	0.18
Croc#29	59.7	5.7	140.3	3.83	2.81	1.27	1.36	1.38	0.2	25	125	277	0.15
Croc#30	69.7	6.3	138.6	3.7	2.92	1.29	1.41	1.43	0.2	32	107	276	0.43
Croc#31	63.9	5.9	146.1	3.95	2.75	1.32	1.57	1.18	0.6	25	119	289	0.41
Croc#33	56.8	5.4	125.8	3.96	2.48	1.33	1.3	1.49	0.2	30	111	289	0.43
Croc#34	67.4	6.8	140.3	4.26	2.73	1.45	1.27	1.58	0.4	20	130	322	0.18
Croc#36	51.1	5.9	128.3	4.35	2.35	1.3	1.22	1.74	0.4	21	118	296	0.22
Croc#37	59.9	4.4	127.4	3.61	2.74	1.39	1.37	2.09	0.9	28	111	303	0.64
Croc#38	63	5.7	146.3	3.41	2.85	1.38	1.31	1.91	0.5	24	123	318	0.12
Croc#39	71.5	4.8	139.8	4.56	2.44	1.26	1.28	1.63	0.5	33	113	293	0.3
Croc#41	41.7	5.3	151.9	3.8	2.98	1.39	1.27	1.16	0.2	28	121	294	0.19
Croc#42	67	8.2	126.9	4.15	2.9	1.4	1.52	0.98	0.8	19	108	296	0.45
Croc#43	65.4	5	122.8	5.71	2.68	1.17	1.74	1.31	0.8	21	102	291	0.46
Croc#44	63.1	6.1	152.6	3.85	2.92	1.36	1.43	1	0.5	26	124	346	0.16
Croc#45	79.6	7.5	111.4	4.94	3.02	1.23	2.3	1.08	0.7	28	86	271	0.35
Croc#46	76.8	8.8	112.2	5.05	2.84	1.23	1.59	0.99	0.7	23	78	233	0.58
Croc#47	52.7	6.9	137.6	3.19	2.49	1.25	1.14	1.09	0.2	18	122	279	0.13
Croc#49	76.1	7.3	127.4	3.86	2.67	1.33	0.16	0.98	0.6	23	109	298	0.19
Croc#50	69.8	6.6	129.1	3.37	2.74	1.31	1.42	0.84	0.7	31	102	287	0.5
Croc#52	75.6	8.1	129.9	3.9	2.99	1.36	0.89	1.17	1.1	28	103	288	1.21
Croc#53	41.8	6.2	138	4.01	2.35	1.28	1.21	1.07	0.2	19	121	296	0.19
Croc#54	61.7	6.4	149	4.63	3.15	1.42	1.08	1.26	0.9	35	119.9	314	0.45
Croc#55	61.5	4.2	146	4.38	2.9	1.36	0.99	1.64	0.9	32	118.9	324	0.34
Croc#56	48	3.3	149	4.57	3.04	1.38	1	1.6	1.1	26	121.6	316	0.38
Croc#57	50.1	10.8	142	3.43	2.74	1.43	1.14	1.15	0.1	23	118.9	332	0.26
Croc#58	39.7	12	144	4	2.47	1.35	1.12	1.13	0	21	125.1	339	0.3
Croc#59	41.1	9.9	147	4.93	2.63	1.48	1.07	1.12	0	19	127	349	0.07
Croc#60	37.1	6.7	134	3.35	2.37	1.38	0.95	0.73	0.9	22	114.2	299	0.09
Croc#61	55.6	12.1	142	2.99	2.79	1.44	1.02	1.05	0	13	115.4	336	0.31

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Crocodile plasma biochemistry (continued)													
Croc	TSP	Gluc	Na	K	Ca(T)	Ca2+	Mg	SIP	Urea	Creat	Cl	Osmol	UricA
ID	g/L	mmol/L	mmol/L	mmol/L	mmol/L	mmol/L	mmol/L	mmol/L	mmol/L	umol/L	mmol/L	mosm/kg	mmol/L
Croc#62	49	9.1	148	4.85	2.75	1.56	1.04	1.05	0.2	16	117.1	344	0.23
Croc#63	46.8	6.9	143	3.48	2.57	1.38	1.07	1.02	0.3	20	119.8	332	0.17
Croc#64	43	11.9	131	3.03	2.41	1.21	0.99	1.12	0.1	24	123.1	334	0.1
Croc#65	50.1	12.6	135	3.22	2.79	1.26	1.35	1.51	0	33	118.8	334	0.25
Croc#66	46.8	9.7	142	4.11	2.75	1.4	1.44	1.29	0	14	124.7	335	0.09
Croc#67	44.3	11.7	137	3.42	2.59	1.28	1.39	1.23	0	19	117.7	334	0.17
Croc#68	61.7	12.2	136	3.54	2.85	1.32	1.41	1.32	0	32	122.1	337	0.25
Croc#69	67.9	6.6	144	4.58	3.22	1.42	1.43	1.77	0	8	112.8	332	0.16
Croc#70	30	9.2	145	3.84	2.4	1.43	1.32	0.91	0.5	17	125.3	339	0.04
Croc#71	45.7	11.8	144	3.86	2.64	1.47	1.69	1.01	0.8	17	122.4	299	0.15
Croc#72	51.3	15.3	140	4.35	2.75	1.49	1.64	1.46	0.3	18	112.9	330	0.27
Croc#73	49.9	12.6	143	3.43	3.08	1.6	1.48	1.13	0.6	22	113.1	325	0.14
Croc#74	45	13.4	140	2.75	2.69	1.35	1.45	1.18	0.9	16	110.7	327	0.14
Croc#75	51.9	15.6	145	4.72	3.16	1.58	1.65	1.53	0.6	20	115.5	343	0.16
Croc#76	41.3	10.3	145	4.13	2.7	1.33	1.7	1.52	0	23	114.9	343	0.32
Croc#77	58.5	8.4	145	3.27	3.23	1.41	1.64	1.97	0.3	6	103.6	334	0.19
Croc#78	56.6	7.6	147	3.21	2.92	1.4	1.53	1.86	0	11	110.3	257	0.26
Croc#79	46.7	13.9	139	3.58	2.94	1.41	1.5	1.44	0.7	27	103.9	290	0.2
Croc#80	46.1	12.9	140	4.04	2.86	1.44	1.64	1.31	1.1	23	111.9	331	0.42
Croc#81	48.9	12.6	141	4.34	2.78	1.39	1.96	1.65	0.8	16	112.4	330	0.26
Croc#82	45.1	11.7	141	3.67	2.65	1.36	1.58	1.38	0.3	21	114	324	0.21
Croc#83	32.2	11.5	140	3.16	2.52	1.32	1.47	1.29	1.1	15	115.6	320	0.25
Croc#84	45.1	17	141	3.19	2.92	1.44	1.79	1.44	0.4	23	112.1	336	0.19
Croc#85	45.8	13.9	138	4.19	2.74	1.32	1.63	1.53	0.3	16	112.8	329	0.36
Croc#86	37.1	11.9	140	4.43	2.56	1.3	1.88	1.63	0.3	16	116..4	325	0.5
Croc#87	51.4	7.4	146	4.58	3.05	1.49	1.81	1.65	0.1	33	115.3	301	0.37
Croc#88	47.1	7.4	146	3.96	2.91	1.49	1.58	1.45	1.2	20	120.4	231	0.17
Croc#89	54.9	3.8	156	4.75	2.98	1.58	1.21	1.76	0	30	149.7	336	0.16
Croc#90	47.2	3.3	146	3.88	2.74	1.46	1.04	1.43	0	23	123.1	310	0.1
Croc#91	64.6	2.4	158	6.14	3.32	1.61	1.29	2.17	0	35	132.7	331	0.28
Croc#92	49.8	4.8	153	4.32	2.93	1.49	1.04	1.89	0.2	33	129.7	314	0.07
Croc#93	58.8	4.6	146	4.4	3.13	1.65	1.03	1.46	0.3	29	124.7	306	0.09
Croc#94	56.7	3.6	157	4.67	3.22	1.65	1.1	2.62	0.6	32	132	321	0.14
Croc#95	53.8	12.3	154	3.8	2.96	1.53	1.1	1.59	0	38	129.5	340	0.1
Croc#96	59.8	3.5	145	4.03	3.03	1.6	1.04	1.56	0.4	38	122.7	302	0.08
Croc#97	55.1	3.5	142	4.15	3.02	1.51	1.5	1.74	0	31	118.3	295	0.13
Croc#98	41.8	3.2	151	3.79	2.7	1.45	1.88	1.65	1	38	128.7	315	0.18
Croc#99	47.2	4.9	144	3.74	2.83	1.48	1.4	1.34	0	26	122.8	312	0.09
Croc#100	57	6.6	149	4.02	2.89	1.56	1.57	1.58	0	28	127.3	313	0.17
Croc#101	42.4	5	146	3.63	2.99	1.54	1.44	1.74	0	30	120.6	308	0.13
Croc#102	55.9	5	158	4.99	3.21	1.69	1.62	2.01	0.4	34	132.4	331	0.08
Croc#103	33	3.2	147	3.05	2.53	1.45	1.51	1.08	0	24	125	307	0.06
Croc#104	48.1	3.2	143	4.58	2.79	1.51	1.12	1.77	0	28	126.6	308	0.1
Croc#105	29.7	3.7	201	4.76	2.5	2.02	1.1	1.22	0.3	16	129.8	301	0.04
Croc#106	40.7	3.6	152	3.27	2.71	1.4	1.14	1.6	0.4	17	129.6	319	0.08
Croc#107	51.1	5.6	140	3.77	2.67	1.47	1.05	1.48	0.5	27	118.2	315	0.13
Croc#108	62.9	3.7	143	3.79	3.01	1.54	1.13	1.7	0	30	119.8	314	0.14
Croc#109	40.5	3.5	145	3.66	2.62	1.37	1.1	1.5	0	27	131.6	327	0.08
Croc#110	51.1	5.1	145	4.22	2.93	1.56	1.12	2.12	0	32	119.1	319	0.08

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Appendix 7.1. Data on samples prepared for GC/MS analysis.

Sample name	Sample ID	Collection date	Amount extracted
test 08/09/10	Steroid L1/L2/L3	7/29/2009	1/2/10 ug each *
test 11	S1 ID	7/29/2009	various **
test 12	S2 ID	7/29/2009	various ***
test 13	Blank	7/29/2009	0
test 14	NASA5143	4/18/2008	10 mL
test 15	NASA3092	4/15/2009	32 mL
test 16	NASA3108	4/20/2009	37 mL
test 17	NASA3083	4/13/2009	39 ml
test 18	NASA5310	11/13/2008	11 mL
test 19	NASA5152R	5/16/2008	10 mL
not analyzed	NASA5087	1/29/2008	10 mL
test20	NASA5345	12/30/2008	10 mL
test21	NASA5008R	1/20/2009	10 mL
test22	NASA5316	11/21/2008	10 mL
test23	NASA5106	2/22/2008	22 mL
test24	Stigmasterol	7/29/2009	20 ug
test25	NASA5234	7/18/2008	10 mL
test26	NASA5314	11/21/2008	9 mL
test27	NASA5053	4/18/2008	11 mL
test28	NASA5319	11/21/2008	10 mL
test29	NASA5155	4/30/2008	11 mL
test30	NASA3121	4/9/2009	36 mL
test31	NASA5058	12/01/2007	8.5 ml
test32	NASA5055	10/24/2007	10 mL
test33	NASA5053	10/18/2007	10 mL
test34	NASA5182	5/23/2008	9 mL
test35	NASA5150	4/25/2008	9 mL
test36	NASA5365R	no date	19 mL
test37	NASA5365R	no date	19 mL
test38	NASA5147	4/23/2008	8.5 mL
test39/40/41	KtrlZa/b/c	7/29/2009	14 mL
test42	NASA5067(B)	no date	11 mL
test43	NASA5062 (C)	no date	9 mL
test44	NASA5185	5/28/2008	6 mL
test45	NASA5290	10/14/2008	4 mL
test46	TestosE2 L3	7/29/2009	20 ug each

* An, DHEA, Corticosterone
** prepared for identification only: Et, KAn, HAn, Pd, a-THF
*** prepared for identification only: THS, Pt, THB, THE, THF

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Appendix 7.2. Poster presented at Faculty of Veterinary Science, University of Pretoria; Faculty Day, 6 September 2012.

STEROID PROFILING IN CROCODILIAN URINE BY GAS CHROMATOGRAPHY/MASS SPECTROMETRY

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BACKGROUND

Aquatic pollution is an important global concern and crocodilians, as top predators in aquatic ecosystems, are considered to be excellent sentinels of ecosystem health.

Many aquatic pollutants e.g. pesticides and pharmaceuticals are bio-transformed by the liver and excreted in the urine, making urine a very useful diagnostic sample for scientists.

Likewise, the evaluation of a urine sample, from individual human or animal patients, is a well-established diagnostic approach to obtain clinical data for evaluation of health and confirmation of specific diseases e.g. endocrine system abnormalities.

AIM

The focus of this investigation is primarily on endogenous steroid hormones and their metabolites. Steroid profiling has been employed for extensive studies of urinary steroid metabolites in humans.

The investigation of urinary hormones could provide valuable information on the endocrine axis of the crocodilians, and aid in the evaluation of the significance of this method and matrix (urine) as an indicator of endocrine disruption (ECD).

MATERIALS & METHOD

Urine samples from 22 American alligators (*Alligator mississippiensis*), collected and stored at the department of Zoology, University of Florida for analysis:

- 7 from males, who showed elevated plasma testosterone levels
- 7 from females with high plasma progesterone, and
- 8 from females with abnormal plasma oestradiol levels

The sample preparation and analyses were performed at the College of Medicine, University of Florida, Gainesville, FL, USA.
Preparation of urine samples for gas-chromatographic/mass spectrometric analysis was achieved with a five-step discipline, involving solid phase extraction (SPE), before and after enzymatic hydrolysis, and derivatization of the free steroids.
Separation was achieved with a TRACE TR5-MS capillary column (30 m X 0.25 mm id, 0.1 µm film) and detection was performed with a Thermo Polaris Q GC-MS (Thermo Scientific, Austin, TX, USA). Data collection and qualtitative reporting was done using Xcalibur software.

RESULTS

Steroid metabolites detected:

Steroid Metabolite	Trivial Name/Abbrevation	RT (min)
5α-androstan-3α-ol-17-one	Androsterone/An *	13.86
5β-androstan-3α-ol-17-one	Etiocholanolone/Et	14.44
5-androsten-3β-ol-17-one	Dehydroepiandrosterone/DHEA*	15.68
5α-androstan-3α,11β-diol-17-one	11-OH Androsteron /HAn	18.36
5β-androstan-3α,11β-diol-17-one	11-OH Etiocholanolone /HEt	19.24
5β-pregnan-3α,17α,20α-triol	Pregnanetriol /Pt*	20.23
5β-pregnan-3α,17α,21-triol-20-one	11-Deoxytetrahydrocortisol/THS*	22.48

*trace

Androsterone → TMSO

Structural modification of a steroid metabolite subjected to MO-TMS derivatization.

TMSO, TMSO

Structure of HEt (MO-TMS)

Urinary steroid profiles of male alligators with elevated plasma testosterone levels.
Chromatograms show prominent peaks in the region of the known human androgen metabolites:

Chromatogram and m/z spectrum of peak at 14.04 min.

Chromatogram and m/z spectrum of peak at 19.24 min, identified as HEt (MO-TMS).

Chromatograms showing peaks at 18.13 and 18.38 min. Retention times and m/z spectra correspond to doublet of testosterone (MO-TMS) standard.

CONCLUSION

In addition to identification, quantitative analysis of steroids and their metabolites in crocodilian urine may serve as a valuable diagnostic tool in the investigation of ECD effects. Establishing reference values for healthy animals from non-polluted environments will also aid in future monitoring of pollution.

ACKNOWLEDGEMENTS

- The staff of the University of Florida, Department of Zoology for their cooperation, in particular Satomi Khono, who provided lodging to me as student.
- Dr. Timothy Garrett for his support and assistance in the GC-MS laboratory of the Howard Hughes Medical Institute, University of Florida.
- Thermo Fisher Scientific (Jhb) (Pty) Ltd., for facilitating travel to USA.
- IUCN-SSC Crocodile Specialist Group for financial assistance.

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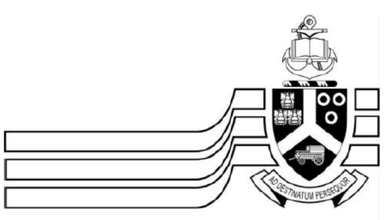
Appendix 11.1. AUCC approval of Protocol V007/08

V007-08



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Animal Use and Care Committee
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26 February 2008

Dr JG Myburgh
Paraclinical Sciences
Faculty of Veterinary Sciences
(jan.myburgh@up.ac.za)

Dear Dr. Myburgh

V007-08 - Establishment and validation of gas-chromatographic mass spectrometric method for the detection and identification of urinary steroid metabolites in the Nile Crocodile

The abovementioned protocol was approved by the Animal Use and Care Committee at its meeting held on 25 February 2008.

Kindly note that the protocol has to be approved by the Research Committee as well, before you may commence with the project.

Please contact this office should you have any questions.

Best regards

Elmarie Mostert
AUCC Contact Person

Copy: Lizette Bekker (lizette.bekker@up.ac.za)
Mrs. N Tromp (Niesje.tromp@up.ac.za)

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Appendix 11.2. Research Committee approval of Protocol V007/08

Ref: V007/08

31 March 2008

FACULTY OF VETERINARY SCIENCE
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Tel: 012-529 8043
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Dr JG Myburgh
Department Paraclinical Sciences
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Dear Dr Myburgh

PROTOCOL V007/08: ESTABLISHMENT AND VALIDATION OF A GAS-CHROMATOGRAPHIC/MASS SPECTROMETRIC METHOD FOR THE DETECTION AND IDENTIFICATION OF URINARY STEROID METABOLITES IN THE NILE CROCODILE (*CROCODYLUSNICOLTICUS*) – *Ms LC Bekker*

I am pleased to inform you that the abovementioned protocol was approved and signed by the **Research Committee Chairman**, and that you may now commence with your project.

Please take note of the attached document.

Kind regards

NIESJE TROMP
SECRETARY: RESEARCH COMMITTEE

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