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Title

Development and validation of selected biomarkers for the health status of the Nile crocodile *(Crocodylus niloticus)* and sharptooth catfish *(Clarias gariepinus)*

by

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A thesis submitted in fulfillment of the requirements for the degree Doctor of Philosophy (PhD)

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THE OLD SAILOR

There was once an old sailor may grandfather knew
Who had so many things which he wanted to do
That, whenever he thought it was time to begin,
He couldn't because of the state he was in.

He was shipwrecked, and lived on an island for weeks,
And he wanted a hat,
And he wanted some nets, or line and some hooks
For the turtles and things which you read of in books.

And, thinking of this, he remembered a thing
Which he wanted (for water) and that was a spring;
And he thought that to talk to he'd look for, and keep
(If he found it) a goat, or some chickens and sheep.

Then, because of the weather, he wanted a hut
With a door (to come in by) which opened and shut
(With a jerk, which was useful if snakes were about),
And a very strong lock to keep savages out.

He began on the fish-hooks and when he'd begun
He decided he couldn't because of the sun.
So he knew he ought to begin with, and that
Was to find, or to make, a large sun-stopping hat.

He was making the hat with some leaves from a tree,
When he thought, "I'm as hot as a body can be,
And I've nothing to take for my terrible thirst;
So I'll look for a spring, and I'll look for it first."

Then he thought as he started, “Oh, dear and oh, dear!
I’ll be lonely to-morrow with nobody here!”
So he made in his note-book a couple of notes:
“I must first find some chickens”

He had just seen a goat (which he knew by the shape)
When he thought, “But I must have a boat for escape.
But a boat means a sail, which means needles and thread;
So I’d better sit down and make needles instead.”

He began on a needle, but thought as he worked,
That, if this was an island where savages lurked,
Sitting safe in his hut he’d have nothing to fear,
Whereas now they might suddenly breathe in his ear!

So he thought of his hut….. and he thought of his boat,
And his hat and his breeks, and his chickens and goat,
And the hooks (for his food) and the spring (for his thirst)….
But he never could think which he ought to do first.

And so in the end he did nothing at all,
But basked on the shingle wrapped up in a shawl.
And I think it was dreadful the way he behaved –
He did nothing but basking until he was saved!

Unknown author

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27. Last but not the least, Leon Prozesky for never stopping to remind me about "*the PhD mountain*" in front of me, which must be crossed…! Leon, you are not only a nightmare, but also a genuine and concerned colleague! You kept my dream and nightmare alive!

Any errors or omissions are solely the responsibility of the author.

My hope is that the different research projects associated with this thesis would ignite numerous new crocodile and catfish research projects at the Faculty of Veterinary Science, in future.

May the few wild crocodiles of Southern Africa still stay wild in the years to come!

It would also help if South Africans try to appreciate them more ……

DEDICATION

This thesis is dedicated to:

Fritz Huchzermeyer, crocodile specialist, colleague and friend, who inspired me to pursue my childhood dream to work with crocodiles and African freshwater fish. Fritz passed away on 3 March 2014 at the age of 84.

My wife, Susan, who tolerated me spending long hours away from home working with crocodiles and catfish, or dreaming about them while at home. She was also willing to help with fieldwork when nobody else was available.

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Soli Deo gloria!

DECLARATION

I Jan Gabriël Myburgh declare that this Thesis entitled, *Development and validation of selected biomarkers for the health status of the Nile crocodile* (Crocodylus niloticus) *and sharptooth catfish* (Clarias gariepinus), which I herewith submit to the University of Pretoria for the degree Doctor of Philosophy is my own original work, and has never been submitted for any academic award to any other institution of higher learning.

…….. ……
Jan Myburgh **Date**

PUBLICATIONS THAT EMANATED, DIRECTLY OR INDIRECTLY, FROM THE AFRICAN SHARPTOOTH CATFISH AND NILE CROCODILE RESEARCH PROJECTS

Mdegela R, Myburgh J, Correia D, Braathen M, Ejobi F, Botha C, Sandvik M, Utne Skaare J, 2005. Evaluation of the gill filament-based EROD assay in African Sharptooth catfish (*Clarias gariepinus*) as a monitoring tool for waterborne PAH-type contaminants. Ecotoxicology, 15, 51-59.

Katsu Y, Myburgh J, Kohno S, Swan G E, Guillette L J, Iguchi T, 2006. Molecular cloning of estrogen receptor α of the Nile crocodile. Comparative Biochemistry and Physiology, Part A, 143, 340-346.

Naicker D, Myburgh J G, Botha C J, 2007. Establishment and validation of primary hepatocytes of the African sharptooth catfish (*Clarias gariepinus*). Chemosphere, 68, 69-77.

Myburgh J G, Botha C J, Booyse D G, Reyers F, 2008. Provisional clinical chemistry parameters in the African Sharptooth catfish. Journal of the South African Veterinary Association, 79, 127-132.

Braathen M, Mdegela R, Correia D, Rundberget T, Myburgh J, Botha C, Utne Skaare J, Sandvik M, 2009. Vitellogenin in African sharptooth catfish (*Clarias gariepinus*): Purification, characterization and ELISA development. Journal of Toxicology and Environmental Health, 72, 173-183.

Katsu Y, Taniguchi E, Urushitani H, Miyagawa S, Takase M, Kubokawa K, Tooi O, Oka T, Santo N, Myburgh J, Matsuno A, Iguchi T, 2010. Molecular cloning and characterization of ligand- and species-specificity of amphibian estrogen receptors. General and Comparative Endocrinology, 168, 220-230.

Myburgh J G, Huchzermeyer F W, Soley J T, Booyse D G, Groenewald H B, Bekker L C, Iguchi T, Guillette L J, 2012. Technique for the collection of clear urine from the Nile crocodile (*Crocodylus niloticus*). Journal of the South African Veterinary Association. http://doi:10.4102/jsava.v83i1.8

Myburgh J G, Kirberger R M, Steyl J C A, Huchzermeyer F W, Soley J T, Booyse D G, Lowers R H, Guillette Jr. L J, 2014. The post-occipital sinus of the spinal vein of the Nile crocodile (*Crocodylus niloticus*): Its anatomy and use for blood sample collection and intravenous infusions. Journal of the South African Veterinary Association. http://dx.doi.org/10.4102/jsava.v85i1.965

Myburgh J G, Whitehead M, Goddard A, Huchzermeyer F W, 2015. Captive Nile crocodile (*Crocodylus niloticus*) blood biochemistry survey: effects of two different housing systems. Journal of the South African Veterinary Association, *in preparation*.

Myburgh J G, Bekker L C, Schillack V R, Van Niekerk M M, Goddard A, Huchzermeyer F, 2015. Nile crocodile (*Crocodylus niloticus*) plasma and urine chemistry investigation. Journal of Veterinary Diagnostic Investigations, *in preparation*.

FORMAT OF THIS THESIS

Take note of the following points:

1. The following articles (listed alphabetically) were used for this thesis and all five of them have already been published:

   Mdegela R, Myburgh J, Correia D, Braathen M, Ejobi F, Botha C, Sandvik M, Utne Skaare J, 2005. Evaluation of the gill filament-based EROD assay in African Sharptooth catfish (*Clarias gariepinus*) as a monitoring tool for waterborne PAH-type contaminants. Ecotoxicology, 15, 51-59. **Chapter 6**.

   Myburgh J G, Botha C J, Booyse D G, Reyers F, 2008. Provisional clinical chemistry parameters in the African sharptooth catfish. Journal of the South African Veterinary Association, 79, 127-132. **Chapter 5**.

   Myburgh J G, Huchzermeyer F W, Soley J T, Booyse D G, Groenewald H B, Bekker L C, Iguchi T, Guillette L J, 2012. Technique for the collection of clear urine from the Nile crocodile (*Crocodylus niloticus*). Journal of the South African Veterinary Association. **Chapter 9**.
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   Myburgh J G, Kirberger R M, Steyl J C A, Huchzermeyer F W, Soley J T, Booyse D G, Lowers R H, Guillette Jr. L J, 2014. The post-occipital sinus of the spinal vein of the Nile crocodile (*Crocodylus niloticus*): Its anatomy and use for blood sample collection and intravenous infusions. Journal of the South African Veterinary Association. **Chapter 8**.
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   Naicker D, Myburgh J G, Botha C J, 2007. Establishment and validation of primary hepatocytes of the African sharptooth catfish (*Clarias gariepinus*). Chemosphere, 68, 69-77. **Chapter 7**.

2. The pdf reprints of the above-mentioned published articles were included in this dissertation as chapters 6, 5, 9, 8 and 7, respectively.

3. The specific references for the published articles, as it appeared in the different publications, were not listed again in Chapter 11 (References).

4. All the references used in the following chapters: Introduction (Chapter 1); Aims and objectives (Chapter 2); Literature review (Chapter 3); Justification (Chapter 4); and General discussion and conclusions (Chapter 10) are listed in Chapter 11.

5. All the figures for this dissertation (published articles and the rest of the thesis) are listed under: List of Figures. The Figures are listed per chapter.

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LIST OF ABREVIATIONS

AChE	acetylcholine esterase activity
AEC	animal ethics committee
AhR	aryl hydrocarbon receptor
AMD	acid mine drainage
B[*a*]P	benzo(*a*)pyrene
BB	brown bullhead catfish
BF-2	bluegill sunfish fibroblast-2
C1	atlas
C2	axis
CCM	complete culture media
CHO-K1	Chinese hamster ovary K1
CT	computed tomography
CYP1A	cytochrome P450
DDE	dichlorodiphenyldichloroethylene
DDT	dichlorodiphenyltrichloroethane
DHA	docosahexaenoic acid
DIFFS	Department of Ichthyology and Fisheries Science
DMSO	dimethyl sulfoxide
DO	dissolved oxygen
DTI	Department of Trade and Industry
EM	electron microscopy
ER	endoplasmic reticulum
ER	oestrogen receptor
EROD	ethoxyresorufin-*0*-deethylase
FAC	fluorescent aromatic compounds
FG	french gauge
FHM	fat head minnow
g	gram
G	gauge
GST	glutathione *S*-transferase
HaN	head and neck
Hep G2	human hepatocellular carcinoma

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hg	mercury
HOD	Head of the Department
HPLC	high-performance liquid chromatography
ISO	Integumentary sensory organ
Jr	junior
KNP	Kruger National Park
L or l	litre
m	metre
µg	microgram
ml	millilitre
mm	millimetre
MTPA	Mpumalanga Tourism and Park Agency
MTT	methyl thiazol tetrazolium
n	number
NUFU	Norwegian Council for Higher Education's Programme for Development, Research and Education
OVI	Onderstepoort Veterinary Institute
PAHs	polycyclic aromatic hydrocarbons
PBS	phosphate buffered saline
PLHC-1	*Poeciliopsis lucida* hepatocellular carcinoma cell line 1
POP	persistent organic pollutants
RBC	red blood cell
RTG-2	rainbow trout gonad-2
Rtl-W1	rainbow trout liver-W1
SADC	Southern African Development Community
SASS	South African Aquatic Scoring System
TAASA	Tilapia Aquaculture Association of South Africa
TEM	transmission electron microscopy
TDS	total dissolved solids
TL	total length
USA	United States of America
VPH	veterinary public health
VTG	vitellogenin

ABSTRACT

Aquatic species, such as the African sharptooth catfish (*Clarias gariepinus*) and Nile crocodile (*Crocodylus niloticus*), are considered to be valuable sentinels of ecosystem health. Although the Nile crocodile is still widely distributed in Africa, viable populations of wild crocodiles are nowadays only found in protected areas in South Africa, like the Kruger National Park and iSimangaliso Wetland Park. The sharptooth catfish, on the other hand, also has a wide distribution in Africa, but is still commonly found in nearly all waterbodies in South Africa. Biomarkers to determine the health status of the sharptooth catfish and Nile crocodile are, unfortunately, lacking. This PhD project can be divided into three focus areas, namely: (1) the development of blood and urine sampling techniques for the Nile crocodile; (2) determining of blood biochemical parameters in the African sharptooth catfish if a standard veterinary clinical pathology profile is used; and (3) the establishment of two *in vitro* methods (primary hepatocyte system and gill filament assay) for the African sharptooth catfish.

Although the Nile crocodile blood sample collection technique has been established, the clinical anatomy of the post-occipital spinal venous sinus collection site has not been thoroughly investigated. The anatomy of the cranial neck region was investigated macro- and microscopically, radiographically and by means of computed tomography. The spinal vein runs within the vertebral canal, dorsal to, and closely associated with the spinal cord and it changes into a venous sinus, cranially, in the post-occipital region. For blood collection the spinal venous sinus is accessed through the interarcuate space between the atlas and axis (cervical vertebrae: C_1 and C_2) by inserting a needle angled just off the perpendicular, in the midline through the craniodorsal cervical skin, just cranial to the cranial borders of the first cervical osteoderms. Hypodermic and spinal needles with short bevels are the most suitable to prevent complications.

Crocodilian urine is not often used as a diagnostic sample - most likely due to the fact that a practical urine collection technique has not been described before. However, urine is a very useful diagnostic sample in human and veterinary medicine, and can most probably be used for the same purpose in crocodilians. A simple urine

collection technique is described and illustrated. With this technique, it was possible to collect relatively clean urine samples from Nile crocodiles of different ages and sizes using canine urinary catheters. Based on the gross anatomical features of the cloaca of the Nile crocodile, it was confirmed that urine accumulates in a “urinary chamber” consisting of the urodeum and coprodeum. Faecal material is stored, temporarily, in the very short rectum, which is separated from the urinary chamber by the rectocoprodeal sphincter.

Use of *C. gariepinus* as a bioindicator species demands that its baseline clinical chemistry must be defined - there is unfortunately a paucity of data in this regard. Blood was collected from male and female catfish and a number of clinical chemistry parameters were determined. Plasma protein values, but particularly those of plasma albumin, were found to be very low, approximately half the value for dogs, but similar to the values in channel catfish (*Ictalurus punctatus*). Plasma urea values in sharptooth catfish were found to be much lower than in dogs, but only marginally lower than in channel catfish. Plasma creatinine in sharptooth catfish, however, was only a quarter of that of dogs and one third of that found in channel catfish. These findings may have implications for using urea and/or creatinine as an index of renal glomerular filtration, as is done in mammals. Plasma enzyme activity ranges were much lower in sharptooth catfish than in dogs, particularly for alkaline phosphatase (ALP) and alanine aminotransferase (ALT). By comparison, channel catfish have an even lower ALT activity range but an ALP range that is very similar to dogs. The very low plasma thyroxine (T_4) levels have important implications for laboratory personnel, who will have to set up calibration and standardization adaptations for the methods that are generally designed for human samples. However, the blood values obtained are a useful starting point for using *C. gariepinus* as a sentinel for aquatic ecosystem health.

The focus of the next study was the development and validation of a primary hepatocyte system for the African sharptooth catfish. The successful isolation of primary hepatocytes from *C. gariepinus* was achieved using an *in situ* surgical perfusion method developed specifically for this species. The primary hepatocytes responded to CYP1A induction, while a continuous Chinese hamster ovary (CHO-K1) cell line showed no activity when exposed to various concentrations of

benzo[*a*]pyrene (B[*a*]P). The primary catfish hepatocyte cell culture system, expressing CYP1A when exposed to B[*a*]P, could in future be used as a biomarker for aromatic hydrocarbon pollutants in aquatic ecosystems.

A gill filament-based EROD assay was also developed for the African sharptooth catfish as an ecosystem health monitoring tool. The ability of *C. gariepinus* in inducing cytochrome P-450 class 1A (CYP1A) and glutathione S-transferase (GST) biomarkers was determined in gill filaments and liver cells after four days of waterborne exposure to the polycyclic aromatic hydrocarbon, (B[*a*]P). The CYP1A activity was measured in hepatic microsomes and gill filaments. Benzo[*a*]pyrene strongly induced CYP1A activities in gill filaments. These findings suggest that the sharptooth catfish gill filament-based CYP1A assay can be used to monitor AhR agonist pollutants in aquatic ecosystems.

In conclusion, the focus of the Nile crocodile projects was on the establishment of sample collection techniques. However, the development of the blood and urine sample collection techniques not only confirmed the normal anatomy of the sample collection sites, but also created the potential for screening crocodile blood and urine samples for an array of chemicals and laboratory parameters. Screening of blood samples, collected from apparently healthy African sharptooth catfish, contributed to our database of reference ranges, as well as evaluating the potential diagnostic value of standard veterinary clinical pathology tests. The establishment of specific *in vitro* assays for the African sharptooth catfish contributed to the implementation of innovative methods that can be used to effectively monitor aquatic animal health.

CHAPTER 1: INTRODUCTION

An increase in the human population and the associated environmental impacts are threatening global freshwater resources (Dudgeon et al., 2006; Leveque et al., 2005; Pimentel et al., 2007). The freshwater ecosystem is not only the most important system for a sustainable future, but it is also the global ecosystem most severely threatened at this stage (Darwall et al., 2009; Dudgeon et al, 2010; Vörösmarty et al., 2010; Zimmerman et al., 2008). It is well-known that athropogenic changes to the world's freshwater ecosystems increased significantly during the last 50 years (Steffen et al., 2007; Zimmerman et al., 2008).

Fig 1.1: Acid mine drainage from the Klipspruit (clear water in small stream) mixing with the water of the Olifants River (green water flowing from left to right) (picture: Jan Myburgh)

In southern Africa, freshwater ecosystems are similarly imperiled and it is mainly due to the impacts of acid mine drainage (AMD) (Fig 1.1), sewage processing plant outflow, agricultural run-off, chemical pollution and general mismanagement (Darwall et al, 2009; Oberholster et al, 2010; Oberholster et al., 2012; Van Vuuren, 2009; Van

Vuuren, 2013a). Other threats, such as high volumes of water extraction, drainage of wetlands, deforestation leading to sedimentation, invasive aquatic species and global climate change, may also impact significantly on aquatic ecosystems (Darwall et al., 2009; Davies & Day, 1998; Van Vuuren, 2013b).

Driver et al (2005) concluded that South Africa's aquatic ecosystems are, in general, under more pressure than its terrestrial ecosystems. It is also accepted that the quality of freshwater (surface water) is slowly deteriorating in South Africa (Ashton, 2007; Davies & Day, 1998; De Villiers et al., 2009; Van Vuuren, 2009). Ashton (2007) reported ±7 years ago that only 30% of South Africa's main rivers were still intact and able to contribute to conservation of biodiversity. During the same year, Nel et al (2007) came to a similar conclusion that the state of the main rivers in South Africa, inside and outside protected areas, was dire, and that >80% off the ecosystems were threatened - with more than half of the threatened ecosystems, critically endangered.

South Africa is mostly a semi-arid country (<500 mm average rainfall per annum), especially the westerns parts, with the livelihoods of the poorest communities very often directly dependant on surface water (Ashton, 2007; Davies & Day, 1998; Van Vuuren, 2009). Unfortunately, developing countries very often do not have the capacity to protect or sustain water quality (Zimmerman et al., 2008). In South Africa, river systems are the primary source of freshwater for agricultural, domestic (Fig 1.2) and industrial uses, and supply more that 85% of all the water that is used, with groundwater systems providing the remainder (Ashton, 2007; Davies & Day, 1998).

Aside from the obvious local negative effects of poor water quality (living close to the source of pollution), the health of downstream river water-users (e.g. fish, livestock, wildlife and humans), far away from the source, may also be influenced (Heath et al., 2010; Liebel et al., 2013) (Figs 1.1 & 1.2). Therefore, upstream management of rivers is often a cause for concern for managers of downstream protected areas, like national parks, due to their inability to prevent pollutants entering their well-managed sanctuaries via flowing rivers (Ashton, 2010; Deacon, 1994; Russel, 2011) (Fig 1.3). The Kruger National Park (KNP) is one of the largest conservation areas in Africa and covers 19 485 km^2 on South Africa's eastern border with Mozambique (Joubert,

2007). Numerous rivers flow from west to east through South Africa and eventually into the KNP, and the most prominent river flowing through the KNP is the Olifants River (Ashton, 2010). All these rivers, flowing from west to east, potentially may accumulate pollutants as they flow eastwards through the provinces of Gauteng, Mpumalanga and Limpopo, and into the KNP (Ashton, 2010; Joubert, 2007) (Figs 1.3). For this simple reason, a pristine and well-managed conservation area, such as the KNP, is most often affected by aquatic pollutants originating from outside the park (Deacon, 1994; Joubert, 2007).

Fig 1.2: Person collecting water from a stream in the upper catchment of the Olifants River for domestic use (picture: Paul Oberholster)

Another factor that is very often not recognised is that aquatic ecosystems provide man with free “ecosystem services”; examples are the filtering and cleaning of water, provision of food and building material., recreation and sport, spiritual and cultural ceremonies, and the control of floods and erosion (Carpenter et al., 2011; Davies & Day, 1998; Van Vuuren, 2006). In Africa, freshwater fish (most wild-caught fish) provide 21% of the protein consumed by humans (Revenga, 1998). Unfortunately,

ecosystem services are most often also negatively affected by anthropogenic impacts (Baron et al., 2003; Carpenter et al., 2011; Myers et al., 2013). In the end, man is the biggest loser. Man is therefore, not only directly affected by the deteriorating water quality (most often caused by man), but also, indirectly, through ecosystem services that are failing (Myers et al., 2013) (Figs 1.1 & 1.2). The demand for good quality water and the increase in human numbers in southern Africa are creating a situation of an approaching catastrophe, especially if one takes into consideration that the volume of the available freshwater is constantly declining due to the on-going misuse of this finite resource (Ashton, 2007; Dallas & Day, 2004; Postel, 2000; Van Vuuren, 2009).

Fig 1.3: Water in the Wilge River with blue-green colour due to the accidental release of acid mine drainage by one of the coal mines in the upper catchment of the Olifants River during 2007 (picture: Jan Myburgh)

The diversity of species in freshwater habitats is disproportionately high if compared to other ecosystems (Balian, 2008; Dudgeon, 2010). Although freshwater habitats cover less than 1% of the earth's surface, it provides a home for ±7% (±126 000

species) of the estimated 1.8 million described species on earth, including a quarter of the ±60 000 vertebrates (Balian, 2008; Dudgeon, 2010; Gleick, 1996). There are roughly ±28 000 freshwater species of fish, molluscs, crabs, dragonflies and plants, and only an estimated 6 000 of these species have been studied globally (Balian, 2008). Unfortunately, freshwater species are extremely threatened, possibly more so than species from marine and terrestrial ecosystems (Darwall et al., 2009; Dudgeon et al., 2006; Lévêque et al., 2005) (Figs 1.4 & 3.1).

Fig 1.4: Dead mature, male Nile crocodile (*Crocodylus niloticus*) in the lower Olifants River (Olifants Gorge) of the Kruger National Park during the 2008 pansteatitis outbreak (picture: Jan Myburgh)

Freshwater animals are sensitive to aquatic ecosystem changes (Dallas & Day, 2004; Guillette & Edwards, 2007; Heath & Claassen, 1999; Irwin et al., 2006; Milnes & Guillette, 2008; Rowe, 2008; Van Vuuren, 2013; Woodward et al., 2011). Dudgeon et al (2006) reported that the main threats to the freshwater inhabitants were: over-exploitation, water pollution, flow modifications, destruction or degradation of habitat and the invasion of exotic species. Aside from sporadic die-offs of aquatic animals

(Fig 1.4; Fig 3.1), which usually cause significant publicity (Van Vuuren, 2013b), an even bigger concern to scientists is the insidious and slow deterioration of freshwater quality and the potential to negatively affect the health and reproduction of the inhabitants (Guillette & Edwards, 2007; Heath & Claassen, 1999; Huchzermeyer et al., 2011; Irwin et al., 2006; Liebel et al., 2013; Milnes & Guillette, 2008; Rowe, 2008; Woodward et al., 2011). It is often perceived by the general public that minor or less extreme changes to aquatic ecosystems would have zero or insignificant health impacts on aquatic animals. However, it is nowadays common-knowledge that even very low concentrations of pollutants can seriously affect the health of aquatic inhabitants and their offspring (poor reproduction, poor growth, endocrine disturbances, etc.) (Colborn et al., 1996; Vandenberg et al., 2012). To be able to monitor or diagnose these subtle health changes in aquatic animals - specialised techniques (histopathology) or tests (specific biomarkers) are needed (Bunn et al., 2010; Huchzermeyer et al., 2011; Liebel et al., 2013; Woodward et al., 2011).

Reports of poor health, die-offs or decline in freshwater animal numbers were regularly published during the last decade, examples are: dragonflies (Clausnitzer et al., 2009; Clausnitzer et al., 2012), crabs (Cumberlidge et al., 2009), amphibians (Beebee & Griffiths, 2005), terrapins (Grosse et al., 2009; Nasri et al., 2008), fish (Darwall et al., 2011; Dudgeon et al., 2006; Huchzermeyer et al., 2011; Russel, 2011), piscivorous birds (Neagari et al., 2011; Rattner & McGowan, 2007) and crocodilians (Botha et al., 2011, Combrink et al., 2011; Honeyfield et al., 2008; Irwin & Irwin, 2006; Schoeb et al., 2002; Whitaker et al., 2008) (Figs 1.4 & 3.1).

Aquatic animals can be used as sentinels of aquatic ecosystem health (Bunn et al., 2010; Colborn & Thayer, 2000; Guillette & Edwards, 2007; Milnes & Guillette, 2008; Sedeño-Díaz & López-López, 2012; Zhou et al., 2008). The American alligator (*Alligator mississippiensis*) was used as a sentinel species to study the effects of a DDT spill in Lake Apopka (Guillette & Edwards, 2007; Milnes & Guillette, 2008; Woodward et al., 2011). Similarly, different fish species are used as sentinels to study the effects of aquatic pollutants (Bunn et al., 2010; Sedeño-Díaz & López-López, 2012; Van der Oost et al., 2003).

Funding was received from the Norwegian Council for Higher Education's Programme for Development, Research and Education (NUFU) for collaborative research projects between some southern and eastern African countries and Norway (north-south collaboration). The NUFU veterinary network comprised of six partner institutions in eastern (Uganda and Tanzania) and southern Africa (Mozambique, South Africa, Zambia and Zimbabwe), and the Norwegian School of Veterinary Science and National Veterinary Institute in Oslo the northern partners. Within this partnership there were two research themes *viz*. zoonotic diseases (diseases transmitted from animals to humans) and freshwater ecotoxicology (Etox project). The Department of Paraclinical Sciences was fortunate to be an equal partner in this project and received funding for a 5 year period.

During the first year of the NUFU project, the African sharptooth catfish (*Clarias gariepinus*) was identified as the most suitable African freshwater fish species for the Etox project. The sharptooth catfish has a wide distribution in Africa and it occurs in all the participating Etox countries (South Africa, Tanzania, Uganda and Mozambique) (Skelton, 2000). It was decided to concentrate on different methods and biomarkers (different countries focused on different aspects) which could be used to evaluate the health of the sharptooth catfish, and thereby indirectly also the "health" of the relevant aquatic ecosystems. Fish, in general, are considered to be good sentinels of aquatic ecosystem health (Bunn et al., 2010; Van der Oost et al., 2003; Zhou et al., 2008). In addition, the sharptooth catfish is also commercially farmed (aquaculture) in all of the participating countries, making it convenient to obtain live catfish or specific tissue samples that were needed for the different research projects. Aquaculture fish are usually kept under ideal conditions and in water of good quality.

For the South African part of the Etox project, it was seen as a golden opportunity to also include the Nile crocodile (*Crocodylus niloticus*), in addition, to the sharptooth catfish. The Department of Paraclinical Sciences, at that stage, was already involved with several Nile crocodile research projects. The three most important motivating factors to focus on the Nile crocodile, as well, were: (1) the close proximity of several big commercial crocodile farms to the Faculty of Veterinary Science; (2) existing collaboration with international scientists who also focused on crocodilians (Louis

Guillette Jr. (USA), Taisen Iguchi (Japan), Woody Woodward (USA), Richard Ferguson (Zimbabwe), Fritz Huchzermeyer (RSA), etc.); and (3) the valuable sentinel roles that crocodilians played in other parts of the world. Nile crocodiles are slaughtered on a daily basis on commercial crocodile farms close to the Faculty of Veterinary Science, and this was seen as a valuable opportunity to investigate different clinical procedures and parameters using crocodile carcasses. In addition, large numbers of live and healthy crocodiles, from hatchling to breeder size, were also available for specific projects. The different techniques and tests developed to evaluate the health of crocodiles would be very valuable and helpful whenever Nile crocodiles must be examined in future.

As indicated it was decided to concentrate on two South African aquatic species, namely the African sharptooth catfish (scavenger and predator in natural ecosystems) and the Nile crocodile (recognised top predator in natural ecosystems). This PhD project can be divided into three focus areas, namely: (1) the development of blood (from the spinal venous sinus) and urine (from the cloaca) sampling techniques for the Nile crocodile; (2) determining of blood biochemical parameters in the African sharptooth catfish if a standard veterinary clinical pathology profile is used; and (3) the establishment of specific *in vitro* tissue culture assays (primary hepatocyte and gill filament assays) for the African sharptooth catfish.

CHAPTER 2: AIM AND OBJECTIVES

2.1 Aim

The aim of this project was to develop and validate selected methods and biomarkers for the evaluation of African sharptooth catfish and Nile crocodile health.

This project is part of ongoing research in the Department of Paraclinical Sciences to study the health of different aquatic vertebrate species (fish, terrapins and the Nile crocodile) in southen Africa.

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

- Development of safe and efficient urine and blood collection techniques for the Nile crocodile.
- Evaluation of blood biochemical markers in the African sharptooth catfish.
- Development, validation and evaluation of specific *in vitro* tissue culture methods for the sharptooth catfish.

2.2 Objectives

The objectives of the five sub-projects were:

1. To determine normal blood chemistry parameters of healthy African sharptooth catfish using standard clinical pathology tests.

2. To evaluate the gill filament-based EROD assay in the African sharptooth catfish as a monitoring tool for waterborne polycyclic aromatic hydrocarbon pollution in aquatic ecosystems.

3. To establish a primary hepatocyte culture system for the African sharptooth catfish in the Department of Paraclinical Sciences, and to validate this system as a method to assess polycyclic aromatic hydrocarbon pollution in aquatic ecosystems.

4. To develop a practical method for the collection of blood from the post-occipital spinal venous sinus of the Nile crocodile. The normal anatomy of the spinal venous sinus, and the surrounding vertebral bones in the neck region of the Nile crocodile, will be studied to improve the efficiency and safety of this sampling technique.

5. To develop a practical method for the collection of clean urine from the Nile crocodile. The associated anatomy of the cloaca will be investigated to improve the efficiency of urine sampling from the Nile crocodile.

CHAPTER 3: LITERATURE REVIEW

3.1 INTRODUCTION

3.1.1. Aquatic ecosystems - the South African situation

South Africa is facing a freshwater crisis, both from a quantitative and a qualitative perspective (Ashton, 2007; Holtzhausen, 2006b; Van Vuuren, 2009). It is generally accepted that freshwater ecosystems in South Africa are severely threatened - more so when compared to terrestrial ecosystems (Ashton, 2007) and that they are in urgent need of on-going, integrative, multi-disciplinary research and monitoring (Oberholster et al., 2013). Environmental research conducted in southern Africa in the past has mostly focussed on the management and conservation of terrestrial ecosystems (Joubert, 2007).

South Africa is a water-scarce country with a rapidly expanding human population, and has, in general, a very poor history of freshwater conservation (Van As et al., 2012; Van Vuuren, 2009). Despite the attempts that were made to address the "*quantity*" problems in South Africa (e.g. trans-basin pumping of freshwater - Lesotho Highlands Water Project), it is not only the quantity of water that is a concern! The on-going deterioration of water "*quality*" is slowly becoming a major constraint for a healthy and a sustainable future in southern Africa (Holtzhausen, 2006b; Oberholster et al., 2013). Water of poor quality becomes nearly worthless to humans, animals and plants as it may affect their health negatively whenever the water is consumed or used (Heath et al., 2010; Holtzhausen, 2006b; Johnson et al., 2001) (Fig 3.1). Freshwater is unfortunately a finite resource and any waterbody that has been affected by anthropogenic impacts (e.g. by AMD), decreases even further the quantity of potable water available (Johnson et al., 2001; Postel, 2000; Sun et al., 2014).

In southern Africa the dilution capacity of aquatic ecosystems is slowly disappearing (Turton, 2014) and any additional pollutants entering a river or dam will most likely have significant ecotoxicological effects. Despite the deterioration of freshwater quality, and the loss of dilution capacity, some individuals, municipalities and

governmental institutions in South Africa still adhere to the out-dated idea of "…*the solution to pollution is dilution…*" (Holtzhausen, 2006b).

Fig 3.1: A multi-species fish die-off at the inflow to Loskop Dam during 2007 after acid mine drainage was accidently released in the upper catchment of the Olifants River (picture: Jan Myburgh)

Poor maintenance of sewage purification works by most municipalities (Fig 3.2), uncontrolled release of AMD (Figs 1.1; 1.3 & 3.1), industrial pollution and agricultural mismanagement of irrigation water are some of the major factors contributing to the freshwater quality crisis in South Africa (Holtzhausen, 2006b; Oberholster et al., 2013). In addition, the limited implementation of the South African National Water Act (Act 36 of 1998) to stop or prosecute polluters is also not supporting a sustainable solution to this looming water quality crisis in South Africa (personal communication, Adv. Marike van der Walt, 2013).

Fig 3.2: Sewage processing plant in the eMalahleni (Witbank) municipality not fully functional and discharging raw sewage in the upper catchment of the Olifants River (picture: Johan Eksteen)

In addition, human-induced global climate changes may also negatively influence the health of aquatic ecosystems, animals and humans (Holtzhausen, 2006a). In a recent document published by the National Wildlife Federation the effects of global climate change on fish are discussed (Staudt et al., 2013). Staudt et al (2013) predicted that changes in climate pose serious risks to freshwater fish, namely that the warming of water will cause habitat loss for cold-water species and the encroachment of species coming from warmer areas. Stressors such as habitat loss, pollution, invasive species, cyanobacterial blooms and diseases will also be promoted by global warming (Lübcker et al., 2014; Paerl & Huisman, 2008; Staudt et al., 2013). More extreme weather events, especially longer and more intense droughts, heat waves, veldfires and floods may increase the risk for fish die-offs (Staudt et al., 2013).

To be able to make a positive and significant contribution to the understanding and management of the slow deterioration of aquatic ecosystem health in South Africa, a strong multi-disciplinary approach is needed (Oberholster et al., 2013). Not only must

aquatic specialists become involved, but also veterinarians with interests in toxicology, veterinary public health (VPH), epidemiology and aquatic animal health. Veterinarians not only can contribute to better understand the health problems in waterbodies and its inhabitants, but they are also suitably trained to promote the idea of "*One Health*" (linking ecosystem-, animal- and human health) (Nielsen et al., 2012; Osborn et al., 2009). Unfortunately, the current focus of the One Health discipline is more on infectious diseases (Osborn et al., 2009). Aquatic toxicology will most likely, in future, become a bigger focus-point in VPH as water sources becoming more polluted, and the suitability of aquatic animals (e.g. fish, crabs, molluscs, etc.) for human consumption (public health issues) deteriorates (Aron & Patz, 2001).

3.1.2 Aquatic ecosystem health

Vallentyne & Munawar (1993) stated that for effective and sustainable management of the biosphere, it must be seen as one entity consisting of three integrated role-players, namely *society*, *economy* and the *environment*. Unfortunately, no-one of these role-players (or compartments) can be sacrificed for any other without a decrease in the overall quality of human life (Vallentyne & Munawar, 1993). Focusing only on human needs was completey acceptable ±50 years ago, but, nowadays, considered to be outdated and very anthropocentric (Steffen et al., 2007; Vallentyne & Munawar, 1993).

The concept of "*health*" has classically been used to describe the vitality of individual humans and animals. However, more recently it has been expanded to ecosystems as well (so-called ecosystem health) due to the overwhelming evidence that the "*health*" of ecosystems is also affected and slowly deteriorating (Rapport et al., 1998; Vallentyne & Munawar, 1993). Rapport and co-workers (1998) stated that: "*extending the notion of health to regional levels (ecosystems, catchment areas, basins and landscapes) provides new opportunities to integrate the social, natural and health sciences*".

According to Rapport (1998) ecosystem health, in general, is a metaphor used to describe the *condition* of an ecosystem. The condition or health of an ecosystem (terrestrial and aquatic) can vary as a result of mismanagement, pollution, mining,

invasive species, flooding, drought, climate change, logging, fire, extinctions and a host of other reasons (Rapport, 1998). Constanza and Mageau (1999) proposed that a healthy ecosystem is one that is sustainable, i.e. that it has the ability to maintain its *structure* (organization) and *function* (vigour) over time in the face of *external stressors* (resilience).

Aquatic ecosystem health is often defined in terms of the "*absence of signs of pathology*" in a waterbody (lake, dam, river or wetland), i.e. the absence of obvious signs of pollution, algal blooms, loss of aquatic species, sick aquatic animals, die-offs, etc. (Rapport, 1998). Focusing on health, *per se*, brings into play an entirely different set of criteria seeing that it is usually easier to describe "*pathology*" than to define good health. A healthy aquatic ecosystem could be defined as an ecosystem that is able to sustain its ecological structure and maintain biological functions, on the one hand, and the ability to achieve reasonable and sustainable human goals on the other (Depledge & Galloway, 2005; Rapport, 1998). Unfortunately, there is no universally accepted benchmark for a healthy aquatic ecosystem, as the apparent health status of an ecosystem can vary depending upon which health parameters are employed to evaluate it, and which societal aspirations are driving the assessment (Rapport, 1992). For example, an aquatic ecosystem that is supporting an above–average fish production due to prolific algal growth (at the expense of drinking water quality) may be classified as healthy by fishermen and as unhealthy by consumers that must drink the water (Rapport, 1992; Woodward et al., 2012; Zanchett et al., 2013).

It is widely accepted that large-scale anthropogenic changes to ecosystems will influence the health of humans and animals (Confalonieri & Effen, 2011; Corvalan et al., 2005; Depledge & Galloway, 2005; Guillette & Iguchi, 2012; Myers & Patz, 2009; Myers et al., 2013) (Fig 3.3). Degraded and "*sick*" ecosystems may be influenced the health of humans and animals via two routes, namely directly due to pollutants, toxins, etc. present in the system, and indirectly through the destruction of ecosystem services (Corvalan et al., 2005; Myers et al., 2013). Similarly, Depledge and Galloway (2005) stated that the survival of humans as a species is intimately linked to the well-being of ecosystems and the ecosystem services they provide. Examples of factors that may influence the health of aquatic ecosystems and

degrade ecosystem services are: water pollution (Schwarzenbach et al., 2010), metals (Schwarzenbach et al., 2010; Solomon, 2008), cyanobacterial blooms (Fig 3.3) and toxin production (Paerl & Huisman, 2008; Zanchett & Oliveira-Filho, 2013), invasive species and their parasites (Du Preez & Smit, 2013; Lymbery et al., 2014; McNeely, 2001), pharmaceuticals and personal care products in aquatic systems (Caliman & Gavrilescu, 2009; Kunkel & Radke, 2012), nutrients (Damásio et al., 2011; Schwarzenbach et al., 2010; Woodward et al., 2012), pesticides (Colborn & Carroll, 2007; Schwarzenbach et al., 2010) and endocrine disrupters (Colborn & Carroll, 2007; Colborn & Thayer, 2000; Guillette & Edwards, 2007).

Fig 3.3: Cyanobacterial bloom (*Microcystis aerigunosa*) in the inflow area of Loskop Dam (picture: Jan Myburgh)

The rapid deterioration of the world's aquatic ecosystems has encouraged the need to diagnose and monitor ecosystem health (healthy vs. unhealthy systems) through the use of effective surveillance procedures, tests and biomarkers (Constanza & Mageau, 1999; Depledge & Galloway, 2005; Rapport et al., 1998).

3.1.3 Aquatic ecosystem monitoring

In South Africa water quality is often routinely monitored for specific physico-chemical parameters (e.g. pH, total dissolved solids (TDS), etc.) by mines, municipalities, irrigation boards, farmer's organisations promoting export of goods, and researchers (Oberholster et al., 2013; Roux et al., 1993). However, the health of an aquatic ecosystem should preferably be assessed in a more holistic way. A more inclusive approach would, therefore, be to monitor the health of the aquatic inhabitants (preferably from different trophic levels), like invertebrates, fish and crocodilians over several seasons or years (Roux et al., 1993; Vidal., 2008).

A biomonitoring system, South African Aquatic Scoring System (SASS), was developed in South Africa to investigate the abundance or absence of specific invertebrates, as well as diatoms in shallow waterbodies (Davies & Day, 1998; De la Rey et al., 2008; Dickens & Graham, 2002). Unfortunately, this biomonitoring method is not suitable for the monitoring of dams or deep rivers (Davies & Day, 1998; Dickens & Graham, 2002).

It is generally acknowledged that aquatic vertebrates (e.g. fish and other animals higher up in the food chain) are valuable sentinels of aquatic ecosystems health (Kleynhans & Louw, 2007; Moiseenko et al., 2008; Vidal, 2008). Aquatic vertebrates are, however, not often used in Africa due to a lack of basic research (e.g. development and validation of biomarkers for African species), unavailability of specific laboratory tests in Africa and the shortage of suitably qualified specialists in Africa to interpret research findings (e.g. fish histopathology, interpretation of aquatic animal biomarker- and physiological results, etc.).

3.1.4 Aquatic animal health assessment

Aquatic animals are globally used as sentinels to assess the health of different ecosystems (Bourtis et al., 2014; Colborn & Thayer, 2000; Guillette & Edwards, 2007; Kleynhans & Louw, 2007; Moiseenko et al., 2008; Schmitt et al., 2005; Vidal., 2008). However, it is preferable that more than one species should be evaluated (Depledge & Galloway, 2005; Reynoldson & Metcalfe-Smith, 1992; Schmitt et al.,

2005) and that animals from different trophic levels be used (Myers et al., 2013; Schmitt et al., 2005).

Depledge & Galloway (2005) stated that "*by measuring the health status of a range of species representing different phylogenies and feeding types, we can use a weight of evidence approach to envisage the ecological consequences of pollutant exposure".* The approach adopted by many researchers has been to focus only on one particular group in an ecosystem since biota in an ecosystem is interdependent (Depledge & Galloway, 2005). However, this may result, for example, in non-significant findings if the focus was only on fish and the most important pollutant in the system is a herbicide. Some herbicides are endocrine disrupters (Colborn & Thayer, 2000) and therefore the most significant effects will be on aquatic plants (Depledge & Galloway, 2005). On the other hand, to investigate more than one species, simultaneously, and in sufficient detail is a laborious task, discouraging the use of multiple species by individual researchers, especially if animals must be collected from different points in an extensive river system or dam (Depledge & Galloway, 2005). However, if the objective is solely to investigate the effect of an ecosystem change (e.g. pollution by aluminium) on a specific species, then it is justifiable for the researcher to concentrate only on that one species (e.g. fish) (Oberholster et al., 2012).

Researchers may use different approaches to assess aquatic animal health, namely (1) measuring the concentrations of specific pollutants in tissue samples (exposure or bioaccumulation) collected from different species; (2) determining changes in physiological parameters and biomarkers (biochemical alterations) in animals exposed to pollutants; or (3) studying pathological lesions (cellular changes) in organ systems of affected inhabitants (Depledge & Galloway, 2005; Salamat & Zarie, 2012; Schmitt et al., 2005; Van der Oost et al., 2003; Zhou et al., 2008). However, some anthropogenic ecosystem changes and chemical pollutants may also affect the behaviour, fertility, thyroid activity and function of the immune system of aquatic animals and piscivorous birds (Colborn & Carroll, 2007; Colborn & Thayer, 2000; Guillette & Edwards, 2007; Woodward et al., 2011). Guillette & Iguchi (2012) stated that *in utero* exposure of human and animal foetuses to chemicals with endocrine disruption activity has the potential to alter gene expression profiles that may cause

obesity, abnormal behaviour, infertility and reproductive tract developmental problems later in life.

Concentrations of chemical pollutants in animal tissue samples have been used to confirm exposure to specific pollutants (Hinck et al., 2006; Hinck et al., 2008; Horai et al., 2014; Oberholster et al., 2012; Schmitt et al., 2005; Woodward et al., 2011). It has also been used to determine the bioconcentration of pollutants in organs of specific animals (Oberholster et al., 2012). Similarly, this approach has also been used to study biomagnification of persistent organic pollutants (POP) in animals from different trophic levels in the food chain (Woodward et al., 2012). A possible disadvantage of this approach, i.e. if only the presence of a specific pollutant is measured (exposure or bioaccumulation), is that complex physiological effects are not determined (Hanson, 2008; Van der Oost et al., 2003). It could be argued that the concentrations measured of specific pollutants in tissue samples (e.g. DDT in fat stores) are only confirming exposure.

The biomarker approach has gained some popularity (Rudneva et al., 2012; Van der Oost et al., 2003). Hanson (2008) stated that because the biomarker response to pollutants is assumed to occur at low levels of exposure, biomarkers are sensitive early warning diagnostic signals. Biomarkers also provide a mechanistic link between exposure and effects (Hanson, 2008). Abnormal biomarker results, as indicated by the measured enzyme concentrations or changes in physiological parameters, may give an indication of the magnitude of the physiological changes in the sentinel animal (Van der Oost et al., 2003). The advantages of this approach are that the researcher gains some indication of the significance of the effect and can compare the results from different sites or collecting points. Blood samples are most often used for these investigations (Hinck et al., 2006; Hinck et al., 2008; Van der Oost et al., 2003). Blood parameters and enzyme activities (e.g. enzymes to study oxidative stress) are monitored in blood samples that are usually collected from animals without the need to kill them (Hinck et al., 2006; Hinck et al., 2008; Rudneva et al., 2012). The non-lethal health assessment of aquatic animals must be promoted as a future priority (Bourtis et al., 2014).

Tissue samples can also be used to determine the changes in biomarkers whenever animals were exposed to different pollutants (Van der Oost et al., 2003). Unfortunately, animals must be sacrificed to obtain these samples. Examples are: brain tissue for acetylcholinesterase (AChE) activity to screen for exposure to carbamate or organophosphorous compounds, and gill or liver cells to determine ethoxyresorufin-*0*-deethylase induction (EROD) after exposure to polycyclic aromatic hydrocarbons (PAHs) (Hinck et al., 2006; Hinck et al., 2008; Moyo et al., 2013; Van der Oost et al., 2003).

The *in vitro* (tissue culture method) approach also has some advantages. Primary tissue cultures of gill cells and/or hepatocytes of fish are most often used to study and monitor aquatic pollution (Jönsson et al., 2002; Wood et al., 2002; Zhou et al., 2006). The two most important advantages of the tissue culture methods are: that fewer experimental animals need to be sacrificed, and a large number of replicates can be done on the cells (gill or liver cells) from the same animal (Wood et al., 2002; Zhou et al., 2006).

A very helpful and practical approach to investigate the effects of pollutants is to focus on cellular changes (pathological changes) in tissue samples collected from different organs (Huchzermeyer, 2012; Marchand et al., 2012; Moiseenko et al., 2008; Van Dyk et al., 2012). A necropsy or post mortem is needed to study gross pathology and to collect the samples for routine staining and light microscopy (Fig 3.4). Collected samples can also be used for electron microscopy (EM). Unfortunately, animals must be sacrificed to obtain these samples. In the study of any disease or health problem, pathological abnormalities are critically important to try and understand the pathogenesis of the disease or pollutant (Huchzermeyer, 2012; Kruger et al., 2013).

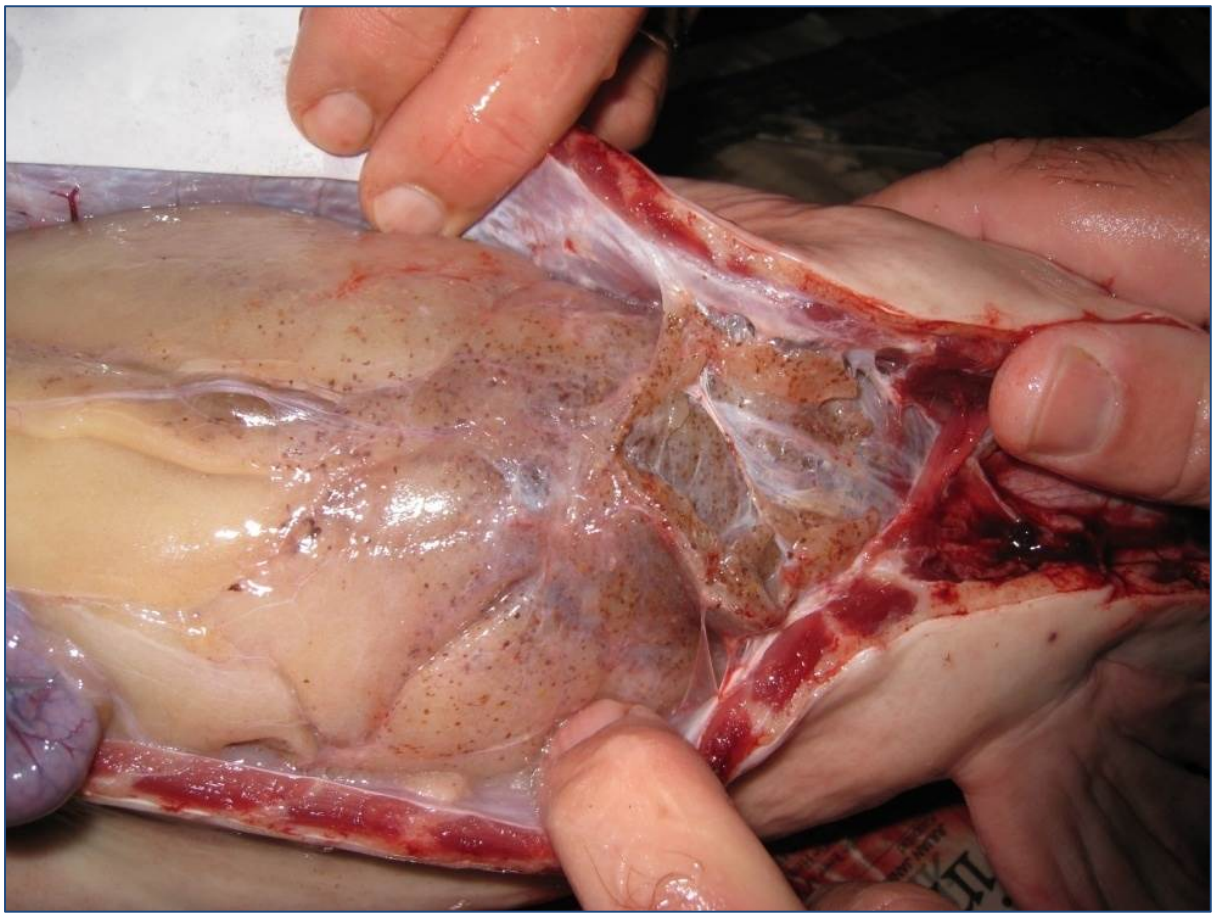

Fig 3.4: African sharptooth catfish (*Clarias gariepinus*) with steatitis lesions (brown spots) in abdominal fat (picture: Jan Myburgh)

3.2 AQUATIC SPECIES

3.2.1 Fish and crocodilians as indicators of ecosystem health

Sentinel animals are important role-players in the monitoring of aquatic ecosystems. Both fish (Bourtis et al., 2014; Edwards et al., 2006; Hamlin et al., 2008; Hanson, 2008; Solé et al., 2013) and crocodilians (Guillette & Edwards, 2007; Rowe, 2008; Vidal., 2008; Woodward et al., 2012) are considered to be good indicators of aquatic ecosystem health.

Hanson (2008) suggested that fish are very suitable sentinels for evaluating aquatic environments, seeing that they are found in most aquatic systems and play a significant role in aquatic food webs. Solé et al (2013) measured PAH, polychrorinated biphenyl (PCB), organochlorinated pesticide and polybrominated

diphenyl ester concentrations in sediment samples collected from seven sites in the northwest Mediterranean Sea. Simultaneously two benthic fish species, common sole (*Solea solea*) and Senegal sole (*Solea senegalensis*), were collected from the same sites. The *Solea* spp. seemed to be highly sensitive to chemical pollutants, particularly the PAHs (Solé et al., 2013). Schmitt et al (2005) collected, examined and analized 368 fish from diffrerent sites of the Rio Grande River in the United States of America (USA). The biomarker results of fish from the lower Rio Grande River were consistent with responses of fish exposed to contaminants - an interpretation supported by the chemical results (Schmitt et al., 2005). Hinck et al (2008) collected largemouth bass (*Micropterus salmoides*) and common carp (*Cyprinus carpio*) from several rivers (different sites) in the southeastern USA. In another survey, Hinck et al (2006) collected several fish species from the Columbia River (different sites) in the USA. Chemical concentrations, biomarker responses and histopathological changes were evaluated in both surveys, and degraded sites and emerging problems could be detected (Hinck et al., 2006; Hinck et al., 2007).

Fish blood biomarkers were used by Rudneva et al (2012) to evaluate the health of scorpion fish (*Scorpaena porcus*) collected from three locations in the Black Sea, Ukraine. Differences in the biomarker responses were detected in fish from polluted and non-polluted sites (Rudneva et al., 2012). Blazer et al (2014) collected fish from three river drainages (16 sites) in Pennsylvania, USA. Three species, namely smallmouth bass, *Micropterus dolomieu*; white sucker, *Catostomus commersonii* and redhorse sucker, *Moxostoma* sp., were evaluated for reproductive health biomarkers. Evidence of exposure to oestrogenic contaminants was observed in all three rivers drainages; measurable concentrations of plasma vitellogenin were detected in male bass and white sucker. Testicular oocytes were only observed in smallmouth bass from the Susquehanna drainage (Blazer et al., 2014). After the Nile crocodile mortality during 2008 in the KNP the health status of African sharptooth catfish from the Olifants Gorge, as well as from other localities in the KNP, was investigated by Huchzermeyer et al (2011 & 2012).

Crocodilians from all over the world, but more specifically alligators from the USA have been used very effectively as sentinels to investigate the effects of anthropogenic changes in their ecosystems (Guillette & Edwards, 2007; Woodward

et al., 2012). Milnes & Guillette (2008) stated that alligators are important sentinels of ecosystem health in the wetlands of the south-eastern USA. Over the last two decades, a series of studies have demonstrated that environmental exposure to a complex mixture of contaminants, from agricultural and municipal activities, alters the development and physiological function of alligators' reproductive and endocrine systems. Similarly, Guillette and Edwards (2007) confirmed that crocodilians are good models for understanding the impacts of aquatic contamination on fertility, because exposure doses are representative of actual mixtures and concentrations in the environment. Guillette et al (1999) reported that neonatal and juvenile alligators from the contaminated Lake Apopka, in central Florida, exhibited abnormal plasma sex steroids as well as morphological abnormalities of the gonads. Phallus size was significantly smaller in males from Lake Apopka when compared to males from Lake Woodruff (Guillette et al., 1999). Hamlin et al (2011) concluded that wildlife sentinels, including crocodilians, play a very important role to shed light on the physiological hazards of chronic exposure to chemical agents in the environment that can be maternally transferred to the embryo or foetus, and are associated with developmental abnormalities.

Large numbers of adult Nile crocodiles died from pansteatitis during the autumn and winter of 2008 in the lower Letaba and Olifants River Gorge in the KNP (Ferreira & Pienaar, 2011; Huchzermeyer, 2011; Myburgh & Botha, 2009). It is interesting to note that ecosystem problems where the end-point is mortality of flagship species, usually elicit a severe emotional response from the general public and the authorities. Unfortunately, subtle anthropogenic ecosystem changes (sub-clinical) could be more devastating, in the long run, to the survival of the inhabitants in a specific waterbody (Woodward et al., 2011). The on-going challenge is, therefore, for scientists to describe and quantify the sub-clinical problems. Thorough and in-depth, on-going research (sometimes taking decades) is needed to determine the impact on the health and fertility of these animals. A good example is the American alligators that were studied by a large number of scientists over several decades in Lake Apopka (Guillette & Edwards, 2007; Guillette et al., 1999; Milnes & Guillette, 2008; Woodward et al., 2011). The findings of all these scientific investigations not only significantly contributed to our understanding of the complexity of aquatic problems,

but also promoted better management of the affected aquatic ecosystems to the benefit of all the interested parties (Woodward et al., 2011).

For the NUFU funded research projects (this PhD project) it was decided to focus on two aquatic species occurring in South Africa, namely the sharptooth catfish (*Clarias gariepinus*) and Nile crocodile (*Crocodylus niloticus*).

3.2.2 African sharptooth catfish *(Clarias gariepinus)*

Clarias gariepinus is a typical catfish, with a gray to black skin colour on the dorsal side fading to a white belly underneath, and the skin is typically without scales (Skelton, 2000). It may easily grow to a total length (TL) of more than 1 m and can weigh up to 30 kg. The sharptooth catfish has a long slender body with flat bony head. The mouth is horisontally flat and broad with 4 pairs of barbels. Only the pectoral fins have spines (Skelton, 2000) (Fig 3.5).

Fig 3.5: The African sharptooth catfish (*Clarias gariepinus*) is a very popular angling fish. This catfish was caught with a fly in the Vaal River (picture: Jacques O'Dell)

Clarias gariepinus also has an accessory breathing organ, inside the cranium, enabling them to stay alive outside water for some time (Ahmed et al., 2008) (Fig 3.6). Furthermore, it is also able to survive in the sediment of partially dried up freshwater pools (moist mud) for protracted periods (Van der Waal., 1998).

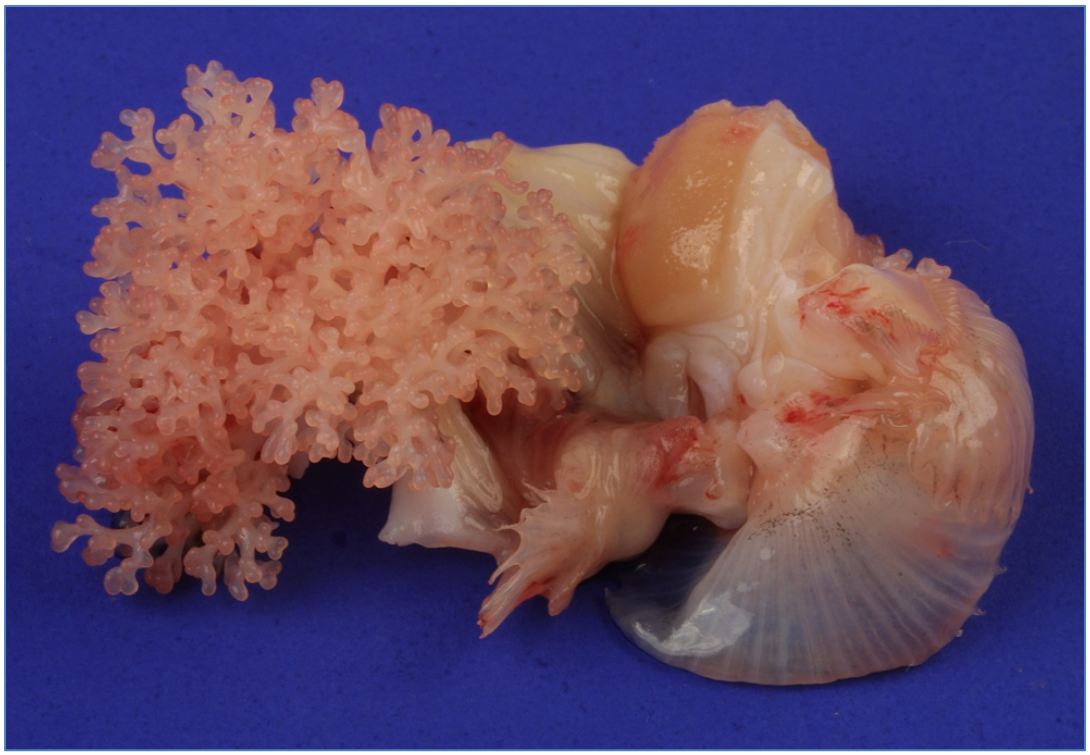

Fig 3.6: Acessary breathing organ removed from the cranium of an African sharptooth catfish (*Clarias gariepinus*). This organ enables the fish to breath outside the water (picture: Christo Botha)

The sharptooth catfish is a well-known freshwater fish in Africa, which has a wide distribution in South Africa, as well as in the rest of Africa and even extending into the Middle East and Turkey (Olaifa et al., 2004; Skelton, 2000; Yalçin et al., 2001). It is found in freshwater lakes, man-made dams, rivers and wetlands, as well as in urban sewage systems (Olaifa et al., 2004; Skelton, 2000) (Fig 3.7).

The sharptooth catfish is an opportunistic omnivorous feeder that will eat animal., as well as plant material. With the big wide mouth, it is able to swallow relatively large prey items (Skelton, 2000) (Fig 3.7).

Fig 3.7: African sharptooth catfish (*Clarias gariepinus*) hunting in a shallow stream in northern KwaZuluNatal (picture: Xander Combrink)

Wild caught freshwater fish (several species) contribute significantly to the daily protein intake of humans in Africa (WorldFish Center, 2009). Africa sharptooth catfish meat (flesh or muscle) is consumed by humans whenever available, and it is very popular in some African countries, like Nigeria (Olaifa et al., 2004). *Clarias gariepinus* is used for aquaculture in South Africa (e.g. Catfish Supreme Pty Ltd, Ventersdorp). However, people from Africa usually prefer tilapia - Mozambique tilapia (*Oreochromis mossambicus*) or Nile tilapia (*O. niloticus*) and it is, therefore, the species most often farmed with in Africa (personal communication, Frans Swanepoel, chairperson Tilapia Aquaculature Association of South Africa, 2014). Sharptooth catfish meat is occasionally exported to Nigeria from South Africa, whenever excess meat is locally available (Johan Kooij, Catfish Supreme Pty Ltd, personal communication, 2012).

The African sharptooth catfish has been the focus of numerous scientific investigations all over the world, from anatomical (Ahmed et al., 2008), physiological (Van Vuren et al., 1994), ecological (Van der Waal., 1998), pathological (Huchzermeyer, 2012; Marchand et al., 2008; van Dyk et al., 2012) to reproductive projects (Yalçin et al., 2001). It has also been intensively studied from an aquaculture viewpoint (Ndimele & Owodeinde, 2012; De Graaf & Janssen, 1996).

The biggest focus on the African sharptooth has been to study different physiological and pathological changes associated with exposure to aquatic stressors and chemical pollutants (Abalaka, 2013; Adeyemi et al., 2011; Ezemonye & Ikpesu, 2011; Farombi et al., 2007; Gbem et al., 2001; Hoyle et al., 2007). Studies that focused on the sharptooth catfish in South Africa are also numerous (Barnhoorn et al., 2004; Huchzermeyer et al., 2011; Huchzermeyer, 2012; Kruger et al., 2013; Marchand et al., 2008; Marchand et al., 2012; Van Dyk et al., 2009; Van Dyk et al., 2012; Van Vuren et al., 1994). All the above-mentioned investigations confirmed the value of the sharptooth catfish as a sentinel species in Africa, specifically, to assess aquatic ecosystem health.

Barnhoorn et al (2004) reported histological evidence of intersex in sharptooth catfish inhabiting the Marais and Rietvlei dams in the Rietvlei Nature Reserve, South Africa. Twenty percent of the gonads of male catfish, collected from both the dams, had histological evidence of primary oocytes scattered in testicular tissue. The most likely cause is exposure to oestrogenic pollutants (Barnhoorn et al., 2004). Nearly ten years later, Kruger et al (2013) examined sharptooth catfish collected from the same dams. They reported urogenital papillae abnormalities, as well as histological changes in the gonads of male catfish, similar to what Barnhoorn et al reported in 2004; reconfirming again the potential exposure to oestrogenic pollutants (Kruger et al., 2013).

The African sharptooth catfish has also been the focus of several histological studies. Marchand et al (2009) evaluated the histopathological alterations in the livers of *Clarias gariepinus* from the Marais and Rietvlei dams. Assessment of the liver tissue samples revealed marked histological alterations. Later, Marchand et al (2012) compared the histopathological changes in two freshwater fish species, namely *Clarias gariepinus* and *Oreochromis mossambicus*, inhabiting the hypertrophic Roodeplaat Dam near Pretoria. Histopathological changes were observed in the target organs (gills, liver, ovaries, testes, kidney, and heart) of both fish species, with the most significant pathological changes being noted in the livers.

A semi-quantitative histological assessment was used by Van Dyk et al (2009) to investigate histological alterations in the gills of *Clarias gariepinus* from the Marais and Rietvlei dams, which receive effluent from sewage treatment plants and industries, as well as runoff from informal settlements. Results were compared to those of a control group. Histological alterations were prevalent in fish from both polluted dams, including circulatory disturbances such as telangiectasia and epithelial cell lifting, hyperplasia of mucous cells and epithelial cells between secondary lamellae, structural alterations in the form of fusion and branching of primary and secondary lamellae, and regressive changes in the form of intracellular alterations within gill epithelial cells. After investigating whether histopathological changes in the liver of the sharptooth catfish would differ between, and/or reflect the pollution status of, different freshwater aquatic ecosystems in South Africa, Van Dyk et al (2012) concluded that liver alterations could be a useful biomarker of freshwater pollution.

The health status of the sharptooth catfish from the Olifants Gorge, as well as from other localities in the KNP was investigated (Fig 3.8). Pansteatitis lesions were confirmed in the sharptooth catfish from three main locations, namely the Olifants River Gorge, Engelhard Dam on the Letaba River and from the Sabie River in the Sabiepoort (Huchzermeyer et al., 2011; Huchzermeyer, 2012). An increasing prevalence of pansteatitis was observed in sharptooth catfish during repeated samplings from the Olifants Gorge (2009 - 2011) and co-existence of old and recent lesions indicated on-going incitement of pansteatitis (Huchzermeyer et al., 2011; Huchzermeyer, 2012). Only a low prevalence of pansteatitis was observed in catfish sampled from the Olifants River, upstream of the Gorge, in the KNP and no pansteatitis was observed in catfish sampled from a rain-filled dam not connected to the Olifants River. Compared with other sites, analysis of stomach contents of sharptooth catfish from the Olifants Gorge and the Sabiepoort strongly suggested that consumption of a predominantly fish diet was associated with the development of pansteatitis in these fish (Huchzermeyer et al., 2011; Huchzermeyer, 2012).

Fig 3.8: Veterinarians studying the gross pathological lesions in an African sharptooth catfish (*Clarias gariepinus*) from the Kruger National Park (picture: David Huchzermeyer)

3.2.3 Nile crocodile *(Crocodylus niloticus)*

The Nile crocodile is the largest of the three African crocodiles - the other two species are the African slender-snouted crocodile (*Crocodylus cataphractus*) and the dwarf crocodile (*Osteolaemus tetraspis*) (Eaton et al., 2009; Hekkala et al., 2011; Huchzermeyer, 2003). The Nile crocodile is the only species present in South Africa (Freely, 2010; Huchzermeyer, 2003; Pooley, 1982). Hekkala et al (2011) genetically screened a large number of Nile crocodile tissue samples, including samples collected from 2 000 year old mummified crocodile carcasses. In conclusion, they proposed that two Nile crocodile species should be recognised, namely *C. niloticus* and *C. suchus* (Hekkala et al., 2011).

Crocodilians are anatomically well adapted to their semi-aquatic environment and it is, therefore, justifiable to refer to them as the "*super predators of aquatic*

ecosystems" (Grigg & Kirsher, 2015). Despite their large size, crocodilians are remarkably stealthy, and are well camouflaged in the water (Erickson et al., 2012; Pooley, 1982). Juveniles are grey, dark olive or brown with darker cross-bands on the tail and body. Their skin colour becomes progressively duller and darker with maturity and the cross-bands fade, especially those on the body (Pooley, 1982).

Crocodlians have incredibily powerful jaws, with 64-68 razor sharp conical teeth (Erickson et al., 2012; Huchzermeyer, 2003). Critical to the long-term success of crocodilians was the development (evolution) of a strong bite-force (Erickson et al., 2012). A powerful tail and webbed hind feet are used to propell them through the water - up to speeds of 35 km/h (Huchzermeyer, 2003).

Crocodilians have excellent eyesight and can see well during both day and night (Huchzermeyer, 2003). Their eyes are laterally and on top of a slightly flattened head, and coveniently protrude above the water when the animal swims or floats just below the surface of the water. Pupils are in a vertically slit during daylight, very much like those of a cat, and expand to become round at night when they are most active (Huchzermeyer, 2003). Ear openings are relatively small and in the form of cranio-caudal longitudinal slits, positioned just behind the eyes. The ear cavities and the sensitive tempanic membranes are covered by protective flaps that close thightly whenever they submerge (Bierman et al., 2014; Huchzermeyer, 2003). Seeing that the head is flattened and long, the eyes, ears and nostrils are all simultaneously above the water whenever the animal is floating or slowly swimming just underneath the water's surface (Huchzermeyer, 2003).

Integumentary sensory organs (ISOs) are located in dermal scales and are densly distributed over the heads and bodies of crocodiles (Jackson & Brooks, 2007; Leitch & Catania, 2012). Leitch and Catania (2012) concluded that the ISOs have diverse functions, including detection of water movements, indicating when to bite based on direct contact of persued prey, and fine tactile discrimination of items held in the mouth. In addition, Jackson and Brooks (2007) found that the integumentary sensory organs can also detect differences in water osmotic pressure (fresh vs. sea water).

The Nile crocodile used to have a relatively wide distribution over most of the eastern parts of South Africa, stretching as far south as the Eastern Cape (Freely, 2010; Jacobsen, 1984). Wild crocodile numbers have unfortunately been declining in South Africa over the last 3 to 4 decades, especially outside protected areas (Botha et al., 2011; Combrink et al., 2013) and similar trends were also observed in other parts of the world with other crocodilian species (Irwin & Irwin, 2006; Martin, 2008; McGregor, 2004). In contrast, Zisadza-Gandiwa et al (2013) reported that the Nile crocodile numbers in the Gonarezhou National Park in Zimbabwe were stable over the period 2008 to 2011.

Fig 3.9: Big male Nile crocodile (*Crocodylus niloticus*) returning to the water of Lake St Lucia, iSimangaliso Wetland Park, KwaZuluNatal after it was caught by Xander Combrink and Jonathan Warner (picture: Xander Combrink)

Currently (2013) viable populations of wild Nile crocodiles are present in big conservation areas in South Africa, such as the KNP and iSimangaliso Wetland Park (Fig 3.9) (Combrink et al., 2013). In South Africa, crocodile health problems mostly caused by anthropogenic activities, and population declines were reported during the last decade from: Lake Sibaya in KZN (Combrink et al., 2011), Loskop Dam in the Mpumalanga Province (Botha et al., 2011; Heath et al., 2010) and recently the

Olifants Gorge in the KNP (Ashton, 2010; Ferreira & Pienaar, 2011; Lane et al., 2013; Van Vuuren, 2013b) (Fig 3.7& Fig 3.8). It is important to emphasize that it is only the wild populations that are threatened. Large numbers of Nile crocodiles, fortunately, are kept on commercial crocodile farms in South Africa – most probably more than 1 million in total (personal communication, Stefan van As, 2014).

In Africa the Nile crocodile is not only recognised as: a spiritual animal; a flagship species; top predator; tourist attraction and a generator of income through sustainable egg harvesting from nests (e.g. Zimbabwe), but also as a killer of humans and livestock (Combrink et al., 2013; Musambachime, 1987; Wallace et al., 2011; Wallace et al., 2012). Conflict between Nile crocodiles and humans has been going on for eons in southern Africa, and anthropogenic killing of Nile crocodiles is still a significant cause of the decline of their numbers in the wild, especially outside conservation areas (Aust et al., 2009; Combrink et al., 2011; Combrink et al., 2013; Pooley, 2013; Wallace et al., 2011). In addition, Nile crocodiles are sometimes actively hunted or targeted by fishermen (e.g. Massingir Dam), rural livestock owners, modern trophy hunters, and traditional healers or sangomas (crocodile blood, fat, liver, brain and gall bladder are collected for the *muthi* market) (Coleman, 2014; Combrink et al., 2013; McGregor, 2004). Habitat destruction and the increase in human numbers are, furthermore, also contributing to the increased risk of contact and conflict between humans and crocodiles outside conservation areas in southern Africa.

During the last decade mass crocodilian mortality (outbreaks) has been reported from other parts of the world (Honeyfield et al., 2008; Irwin & Irwin, 2006; Schoeb et al., 2002; Whitaker et al., 2008). Crocodilian die-offs usually cause the more educated and wealthy members of society to immediately take note of the crisis (emotions very often playing a role) due to the publicity received via the electronic and printed media.

The large-scale mortality of Nile crocodiles in the lower Letaba and Olifants rivers, and in the Olifants Gorge of the KNP (Figs 3.10 & 3.11) during the autumn and winter of 2008 (Ferreira & Pienaar, 2011; Huchzermeyer, 2011; Myburgh & Botha, 2009) prompted numerous scientists to start with research projects focusing on the

Nile crocodile in southern Africa. Huchzermeyer quoted by Van Vuuren (2013) suggested that the crocodile die-off in the Olifants Gorge in the KNP was most likely triggered by silver carp (*Hypophthalmichthys molitrix*) that moved upstream into the Olifants Gorge from the Massingir Dam during May 2008. A defective sluice gate in the wall of the Massingir Dam was fixed during 2007 and the dam became 100% full during the summer of 2007/2008. Silver carp thrive on phytoplankton blooms and the fat in their adipose tissue reflects a high omega 3 to omega 6 fatty acid ratio (Van Vuuren, 2013). The unusually high intake of omega 3 fatty acids by crocodiles and catfish due to over-consumption of the silver carp, which moved into the Olifants Gorge during the summer of 2008 when the Massingir Dam was 100% full and the Olifants Gorge flooded, resulted in oxidative stress that overwhelmed the antioxidant protective mechanisms (e.g. α-tocopherol) in large numbers of crocodiles and catfish in the Gorge (Van Vuuren, 2013).

Fig 3.10: Nile crocodile (*Crocodylus niloticus*) unable to move during the 2008 panteatitis outbreak in the lower Olifants River (Olifants Gorge), Kruger National Park (picture: Danie Pienaar)

Further support of this hypothesis is that the fatty acid composition of fat samples collected from pansteatitis positive sharptooth catfish and Nile crocodiles was similar in the affected catfish and crocodiles (ratio of omega 3 to omega 6 fatty acids), but differed significantly from healthy catfish and crocodiles (Huchzermeyer et al., 2013; Van Vuuren, 2013). Pansteatitis affected catfish and crocodiles had particularly high levels of docosahexaenoic acid (DHA) in their fat depots (Huchzermeyer et al., 2013). Huchzermeyer quoted by Van Vuuren (2013) stated that this polyunsaturated fatty acid (DHA) is an essential nutrient that cannot be synthesised by catfish and crocodiles and therefore reflects what they were feeding on.

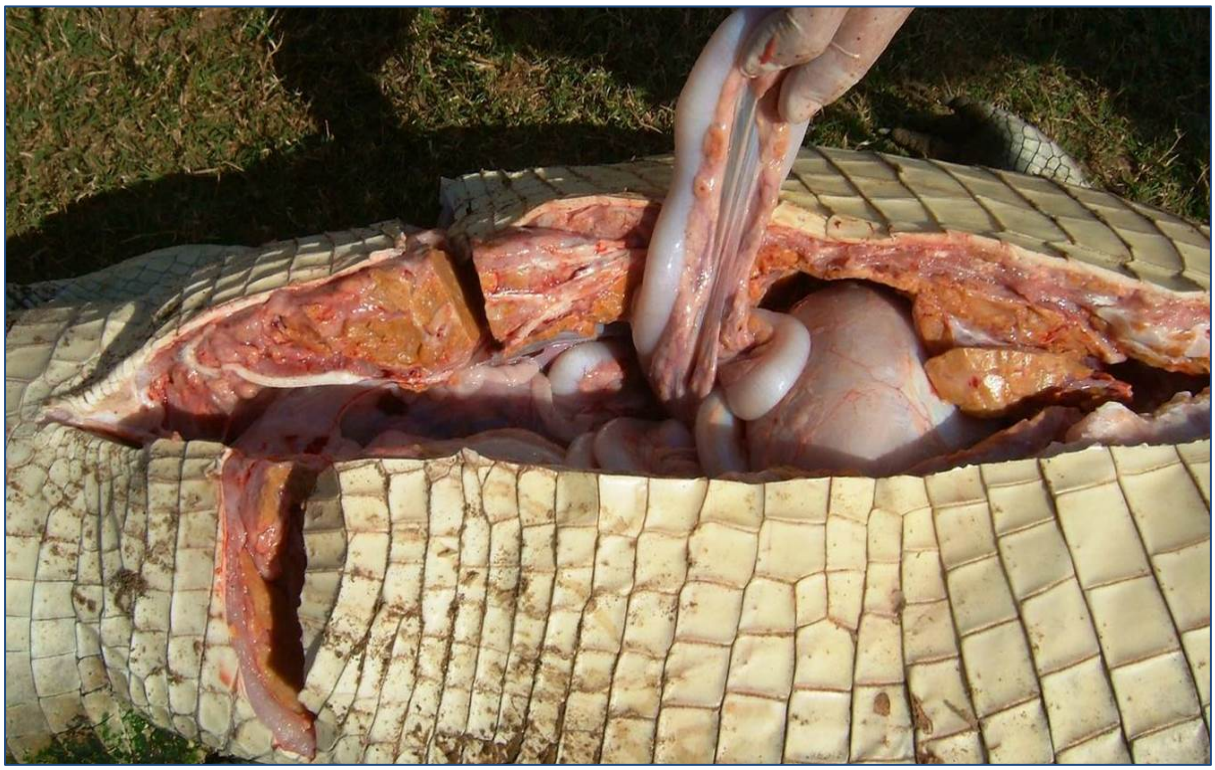

Fig 3.11: Yellow / brown fat in a Nile crocodile (*Crocodylus niloticus*) from the Olifants Gorge, Kruger National Park, photographed during the pansteatitis outbreak of 2008 (picture: Jan Myburgh)

The trigger for the development of pansteatitis in the Nile crocodiles that died at Loskop Dam, during 2007, was most likely also fish-related (Myburgh & Botha, 2009). However, in this case the crocodile mortality was closely associated with a preceeding fish die-off due to AMD that was released by an upstream coal mine (Myburgh & Botha, 2009) (Figs 1.3 & 3.12). During this specific outbreak a diagnosis of pansteatitis was also confirmed in serrated hinged terrapins (*Pelusios sinuatus*) that died. The simultaneous development of pathology (pansteatitis) in Nile

crocodiles and serrated hinged terrapins, immediately after the fish die-off (Fig 3.1), is a strong indication that the intake of rancid fish material by the crocodiles and terrapins was most probably the trigger for the pathological changes in their fat depots (unpublished data, Myburgh, 2007). Nile crocodile numbers have been declining for years in Lake Loskop and pansteatitis most likely played a significant contributing role in the decline, seeing that regular fish die-offs have been reported over the years in the inflow area of the Loskop Dam (Botha et al., 2011; Jacobsen, 1984).

Fig 3.12: Nile crocodile (*Crocodylus niloticus*) found on the shoreline of Loskop Dam after an acid mine drainage spill and subsequent fish die-off during 2007 (picture: Jan Myburgh)

It is estimated that South Africa has more than 1 million Nile crocodiles in captivity on commercial farms (personal communication, Stefan van As, 2014). The main focus of these farms is the export of raw or/and processed crocodile skins. In South Africa no eggs are harvested from the wild as it is illegal, and all the hatchlings are from breeders kept on commercial farms (Fig 3.13). To the crocodile researcher, the large

number of Nile crocodiles on commercial farms is a readily accessible source of animals and tissue samples.

Wild and captive crocodile behaviour is another research field that is in need of attention. Tony Pooley published his pioneer-work on crocodile behaviour in his book, *The Discoveries of a Crocodile Man*, during 1982 (Pooley, 1982). It is worth noting that this publication probably "*opened the eyes*" of many international crocodilian biologists and behaviour specialists, and that this research was done in South Africa and on the Nile crocodile (Pooley, 1982). Pooley's findings were described as: "…*astonishing discoveries concerning maternal care and behaviour have helped change the image among laymen and scientists of these survivors from the age of dinosaurs…*" (Pooley, 1982). Interestingly, more than thirthy years later, Vladimir Dinets still discovered "new" crocodilian behaviour. His behavioural observations of most of the extant crocodilian species are described and discussed in his book, *Dragon Songs* (Dinets, 2013).

3.3 CONCLUSION

The sharptooth catfish and Nile crocodile can be considered to be keystone species for aquatic ecosystem conservation in South Africa, wherever they occur in South Africa. Unfortunately, basic research on both species is desperately needed.

In addition, both the sharptooth catfish and Nile crocodile would be ideal sentinels of aquatic ecosystem health in South Africa. However, to be able to monitor the health of these animals, specific techniques, tests and biomarkers will have to be developed and validated.

Fig 3.13: Mature Nile crocodiles (*Crocodylus niloticus*) in a typical breeder dam set-up on a modern commercial crocodile farm (picture: Fritz Huchzermeyer)

CHAPTER 4: JUSTIFICATION

South Africa is facing a freshwater crisis, both from a quantitative and qualitative perspective. Most of the environmental research in southern Africa has been focussed on the terrestrial ecosystems and the conservation of terrestrial animals. It is generally accepted that freshwater ecosystems in South Africa are severely threatened (more than terrestrial ecosystems) and are in urgent need of more scientific attention (e.g. ecotoxicology).

South Africa is a water scarce (quantity) country with a rapidly expanding human population, and a very poor track record of freshwater conservation. Attempts are made to address the *quantity* problem by trans-basin pumping of freshwater (e.g. from Sterkfontein Dam to the Vaal River to supply Gauteng Province). Unfortunately, it is not only the quantity of water that is a problem; the on-going deterioration of water *quality* is slowly becoming a major constraint for a healthy and a sustainable future. Water of poor quality becomes useless to humans, animals and plants and may also affect their health. Freshwater is a finite resource, any water source that has become useless (due to pollution), decreases the quantity of potable water, even further.

"*We never know the worth of water until the well runs dry*...."

Thomas Fuller

The poor maintenance of sewage purification works by most municipalities, uncontrolled release of acid mine drainage, industrial pollution and agricultural mismanagement of irrigation water are some of the most important contributors to freshwater quality deterioration. In addition, the non-enforcement of the South African National Water Act (Act 36 of 1998) to stop or prosecute polluters is also not contributing to a sustainable solution for this looming crisis.

"*….and the river flows short of life.*"

Mike Cuyugan

I was entrusted with the responsibility to develop the ecotoxicology research programme in the Department of Paraclinical Sciences. Thus, the programme was initiated by asking how we can we start with on-going research projects that will contribute to a better understanding of the current freshwater quality dilemma, stimulate research initiatives, and establish aquatic animal diagnostic capabilities at the Faculty of Veterinary Science.

"….*I was looking at a river bed. And the story it told of a river that flowed made me sad to think it was dead*."

From the song: *A horse with no name* by Dewey Bunnell

4.1 The potential benefits of this PhD project are:

1. Stimulation and establishment of basic aquatic animal research at the Faculty of Veterinary Science. The basic anatomical research projects (Nile crocodile cloaca and spinal venous sinus) will be to support safe and efficient sample collection. Considering that most crocodilians are listed as endangered or threatened, it is nearly impossible to obtain ethical permission to kill any wild crocodilians for research purposes. However, the large numbers of Nile crocodiles on commercial farms in South Africa afforded us the opportunity to do basic research on slaughtered animals without influencing wild animals. Samples will be collected by using the established techniques for biochemical laboratory investigations.

2. Samples collected from the sharptooth catfish will be used to test specific blood biochemical parameters. Some of the biochemical parameters have never been evaluated in the sharptooth catfish before, and others only occasionally for clinical diagnostic purposes (no survey). The clinical chemistry results would not only give us some insight into the physiological functioning of these aquatic species (stimulate new research questions and future research projects), but will also serve as normal reference values for the specific species and parameters.

3. Development and validation of specific tissue culture techniques for the African sharptooth catfish. These *in vitro* tissue culture techniques are innovative (never been established for the sharptooth catfish before) with numerous diagnostic and research advantages.

4. On-going monitoring of aquatic animal health could become a reality by applying the established techniques and methods.

5. All of the planned research projects will involve a number of national (mostly from the University of Pretoria) and international collaborators. Through these different research projects the Department of Paraclinical Sciences will build capacity and also strengthen its international network (United States of America, Japan, Norway, Tanzania and Mozambique). In addition, the collaboration with all the international researchers could potentially stimulate new research questions for future projects, as well as the exchange of expertise and post graduate students.

6. The development and validation of sampling techniques and selected biomarkers will open the door for future research projects for postgraduate students in our Department, and eventually also more scientific publications.

CHAPTER 5: Blood chemistry parameters in the African sharptooth catfish (*Clarias gariepinus*)

5.1 Preface

Blood is a very convenient sample for routine health status monitoring of animals and humans. Although the collection method is minimally invasive, it is not a very difficult procedure, and samples can be collected without being harmfull to the patient. Blood samples are, unfortunately, not as often used for diagnostic purposes in fish, especially not in the case of the African sharptooth catfish, if compared to other animals and humans.

The head of the sharptooth catfish is flattened horizontally and the elongated body is flattened dorso-ventrally, making it easy to work with the catfish lying on its side (in lateral recumbency) (Fig 5.1). Although the method of blood collection from the sharptooth catfish is relatively well known, the anatomy of the caudal vein, running just below the vertebral column, has not been described in any detail for the sharptooth catfish. No detailed blood collection technique investigation was deemed necessary, since this clinical procedure has been used successfully before by the main author to sample large numbers of African sharptooth catfish.

A hypodermic needle is inserted perpendicular over the lateral line, in the region behind the cloacal opening (Fig 5.1), until the tip of the needle touches a vertebral bone. The lateral line is directly over the vertebral column. As the sharptooth catfish has no scales, it is much easier to find the lateral line on the side of the body of the catfish, if compared with scaled fish. The bloodvessel is directly underneath the vertebral bones; therefore, after touching a verterbral bone, the tip of the needle is adjusted slightly ventrally to access the area just underneath the vertebral column. Regular aspiration is needed to detect if the tip of the needle has entered the bloodvessel. The caudal vein was used for the collection of the blood samples.

It was decided to collect blood samples from apparently healthy looking African sharptooth catfish to determine reference ranges for blood parameters, and to investigate if a standard clinical pathology profile, usually used for companion

animals and livestock, would be of any diagnostic value (see: aim and objectives). For this investigation, blood was collected directly from the caudal vein using a hypodermic needle and syringe (Fig 5.1). The inside surfaces of all the syringes were pre-coated with heparin and the collected blood samples were immediately transferred to heparin containing blood tubes to prevent coagulation before being centrifuged. Plasma samples were submitted to the laboratory for testing. During this investigation larger internal diameter hypodermic needles (20G; Terumo Corporation, Tokyo, Japan), were also tried to reduce unnecessary red blood cell (RBC) damage due to turbulence when the blood was aspirated through the needle (Fig 5.1).

Fig 5.1: Blood collection from the African sharptooth cartfish (*Clarias gariepinus*) using a hypodermic needle and syringe. Blood is collected from the caudal vein (caudal sub-vertebral bloodvessel) (picture: Christo Botha)

It is also important to take note that the mucous layer on the skin of the African sharptooth catfish is easily disturbed when a catfish is handled - predisposing the fish to a fungal infection (e.g. *Saprolegnia spp.*) of the skin afterwards. As far as possible, wet hands or wet gloves must be used, as well as wet towels for the handling of the catfish so that the mucous layer is not unnecessary disturbed or

removed. A *Saprolegnia* skin infection would seriously affect the survival of the catfish that was handled, as well as other fish in the same closed aquatic system.

5.2 Aim

Blood samples collected from catfish were screened by the Clinical Pathology Laboratory at the Faculty of Veterinary Science of the University of Pretoria. Standard parameters were used to determine possible biomarkers of health

5.3 Objective(s)

1. To analyse blood samples, collected from male and female sharptooth catfish, for several clinical chemistry parameters
2. To determine blood plasma enzyme (ALT, ALP, AST, CK, GGT, LD, HBD, GLD) activities
3. To determine plasma protein (TPP, albumin, globulin, A/G ration) concentrations
4. To determine total bilirubin, urea and creatinine concentrations
5. To determine thyroxine (T_4) concentrations
6. To define baseline blood chemistry parameters and propose provisional reference ranges

5.4 Collaborators (alphabetical), affiliation and their respective contributions

1. Booyse D G. Department of Anatomy and Physiology, Faculty of Veterinary Science, University of Pretoria, Onderstepoort 0110, South Africa. Handling of the catfish and collection of the blood samples.
2. Botha C J. Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, Onderstepoort 0110, South Africa. Planning of the project and preparation of the manuscript.

3. Myburgh J G. Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, Onderstepoort 0110, South Africa. Planning of the project, handling of the catfish, collection of the blood samples, interpretation of results and preparation of the manuscript.
4. Reyers F. Digital Veterinary Diagnostics, Garsfontein East 0060, South Africa. Interpretation of results and preparation of the manuscript.

5.5 Publication

Myburgh J G, Botha C J, Booyse D G, Reyers F, 2008. Provisional clinical chemistry parameters in the African sharptooth catfish. Journal of the South African Veterinary Association, 79, 156-160.

Chapter 5 Clarias blood.pdf

[5 pages for article]

CHAPTER 6: Evaluation of the gill filament-based EROD assay in the African sharptooth catfish (*Clarias gariepinus*) as a monitoring tool for waterborne PAH-type contaminants

6.1 Preface

Gills are important organ systems in fish, and it does not only have important homeostatic functions to perform in the body, but are also constantly in contact with the water wherein the fish lives.

As part of the NUFU funded research project (see: aim and objectives) an African sharptooth catfish primay gill filament system was investigated (Fig 6.1). Induction of cytochrome P-450 (CYP1A) enzyme activity was determined in gill filaments and liver tissue in the laboratory after 4 days of water-borne exposure of sharptooth catfish to benzo[a]pyrene (B[a]P). 7-ethoxyresorufin-*0*-deethylase (EROD) activity was used as a measurement of CYP1A induction, and B[a]P strongly induced EROD activity in the gill filaments in both sexes of the sharptooth catfish exposed.

The results confirmed that a sharptooth catfish gill filament-based EROD assay could be a valuable monitoring tool valuable monitoring tool for the detection of polycyclic aromatic hydrocarbon (PAHs) pollutants in aquatic ecosystems. Mining of coal in the Mpumalanga Province is not only one of the most serious causes of freshwater pollution, but also a very important source of PAHs for streams and rivers in this Province.

This gill filament assay that was developed and validated at the Faculty of Veterinary Science, Onderstepoort for the sharptooth catfish was used in follow-up studies at the Sokoine University of Agriculture, Morogoro, Tanzania.

Fig 6.1: Drs Correia (Mozambique), Mdegela (Tanzania) and Myburgh (South Africa) (from left to right) during the development of the sharptooth catfish gill filament assay in the Department of Paraclinical Sciences, Faculty of Veterinary Science, Onderstepoort (picture: Christo Botha)

6.2 Aim

Evaluation of gill filament and liver EROD assays in the African sharptooth catfish

6.3 Objective(s)

1. To determine if the sharptooth catfish gill filament-based EROD assay can be used to monitor pollution of PAH-type pollutants
2. To determine CYP1A enzyme induction in gill filaments after 4 days of waterborne exposure to B[a]P
3. To determine hepatic CYP1A enzyme induction after 4 days of waterborne exposure to B[a]P

4. To determine the induction of hepatic gluthathione-s-transferase (GST) activity after 4 days of waterborne exposure to B[a]P
5. To measure the concentrations of B[a]P-type fluorescent compounds in bile after 4 days of waterborne exposure to B[a]P

6.4 Collaborators (alphabetical), affiliation and their respective contributions

1. Botha C. Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, Onderstepoort 0110, South Africa. Planning of the project, collection of samples and preparation of the manuscript.
2. Braathen M. Department of Pharmacology, Microbiology and Food Hygiene, Norwegian School of Veterinary Science, Oslo, Norway. Planning of the project, collection of samples and preparation of the manuscript.
3. Correia D. Veterinary Faculty, University of Eduardo Mondlane, Maputo, Mozambique. Planning of the project, collection of samples and preparation of the manuscript.
4. Ejobi F. Department of Veterinary Public Health and Preventative Medicine, Faculty of Veterinary Medicine, Makerere University, Kampala, Uganda. Planning of the project, collection of samples and preparation of the manuscript.
5. Mdegela R. Department of Veterinary Medicine and Public Health, Faculty of Veterinary Medicine, Sokoine University of Agriculture, Morogoro, Tanzania. Planning of the project, collection of samples, interpretation of results and preparation of the manuscript.
6. Myburgh J. Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, Onderstepoort 0110, South Africa. Planning of the project, obtaining AEC approval from the University of Pretoria, sharptooth catfish and aquarium management, collection of samples, interpretation of results, and preparation and editing of the manuscript.
7. Sandvik M. Norwegian Veterinary Institute, Oslo, Norway. Planning of the project, collection of samples, interpretation of results and preparation of the manuscript.

8. Utne Skaare J. Norwegian Veterinary Institute, Oslo, Norway. Planning of the project, collection of samples and preparation of the manuscript

6.5 Publication

Mdegela R, Myburgh J, Correia D, Braathen M, Ejobi F, Botha C, Sandvik M, Utne Skaare J, 2005. Evaluation of the gill filament-based EROD assay in African sharptooth catfish (*Clarias gariepinus*) as a monitoring tool for waterborne PAH-type contaminants. Ecotoxicology, 15, 51-59.
DOI:10.1007/s10646-005-0041-5

Chapter 6 Gill filament.pdf

[9 pages for article]

CHAPTER 7: Establishment and validation of a primary hepatocyte system for the African sharptooth catfish (*Clarias gariepinus*)

7.1 Preface

Aquatic pollution is a world-wide problem, but going hand-in-hand with pollution is the dilemma of how to practically monitor and study the pollutants. During the last decade or two, *in vitro* tissue culture systems have gained in popularity, mostly due to the decreased number of experimental animals required to be sacrificed and the large number of replicates that can be performed with the cells obtained from one animal. In general., fish primary hepatocyte systems received a lot of attention in the past, and brown trout and Nile tilapia are two of the fish species most often used.

It was decided, as part of the NUFU programme, to develop a primary hepatocyte system for the African sharptooth catfish. This warmwater fish species, with its wide distribution in Africa, was seen as ideal to focus on, mostly due to the advantage that if this system was found to be working well (developed and validated) that it could be implemented immediately in any other country interested in using this model, and where this fish occurs. The aim and objectives of this project was to establish a primary hepatocyte system for the African sharptooth catfish at the Faculty of Veterinary Science (see: aim and objectives). Due to a lack of experience with *in vitro* tissue culture laboratory work, it was decided to closely collaborate with the Toxicology laboratory (Dharma Naicker) at the Onderstepoort Veterinary Institute (OVI).

The technique to harvest the sharptooth catfish liver cells (Fig 7.1) was based on the technique of *in situ* canulization of the portal vein that was developed for brown trout. However, it was realized that the technique could be improved, as the butterfly needles that were proposed and initially used caused numerous problems (e.g. lacerating the fragile portal vein wall). The canulization technique was improved by using a 20G Jelco catheter (20G Jelco intravenous catheter, Medex Medical Ltd., Rossendale, Great Britain) that was introduced into the portal vein and secured in

the bloodvessel with fine suture material (Fig 7.2). A pump was attached to the Jelco, using a sterile drip infusion set tube, and the different solutions were pumped through the liver at a slow rate while the Jelco was kept in position by the surgeon (Fig 7.1). The heart of the catfish was severed as soon as it was seen that the liver was under pressure (becoming swollen). The liver, under pressure with fluid, was also used to assess if the Jelco was securily sutured in the portal vein and not leaking. The cutting of the heart allowed the fluid to freely flow through the liver and out through the severed heart.

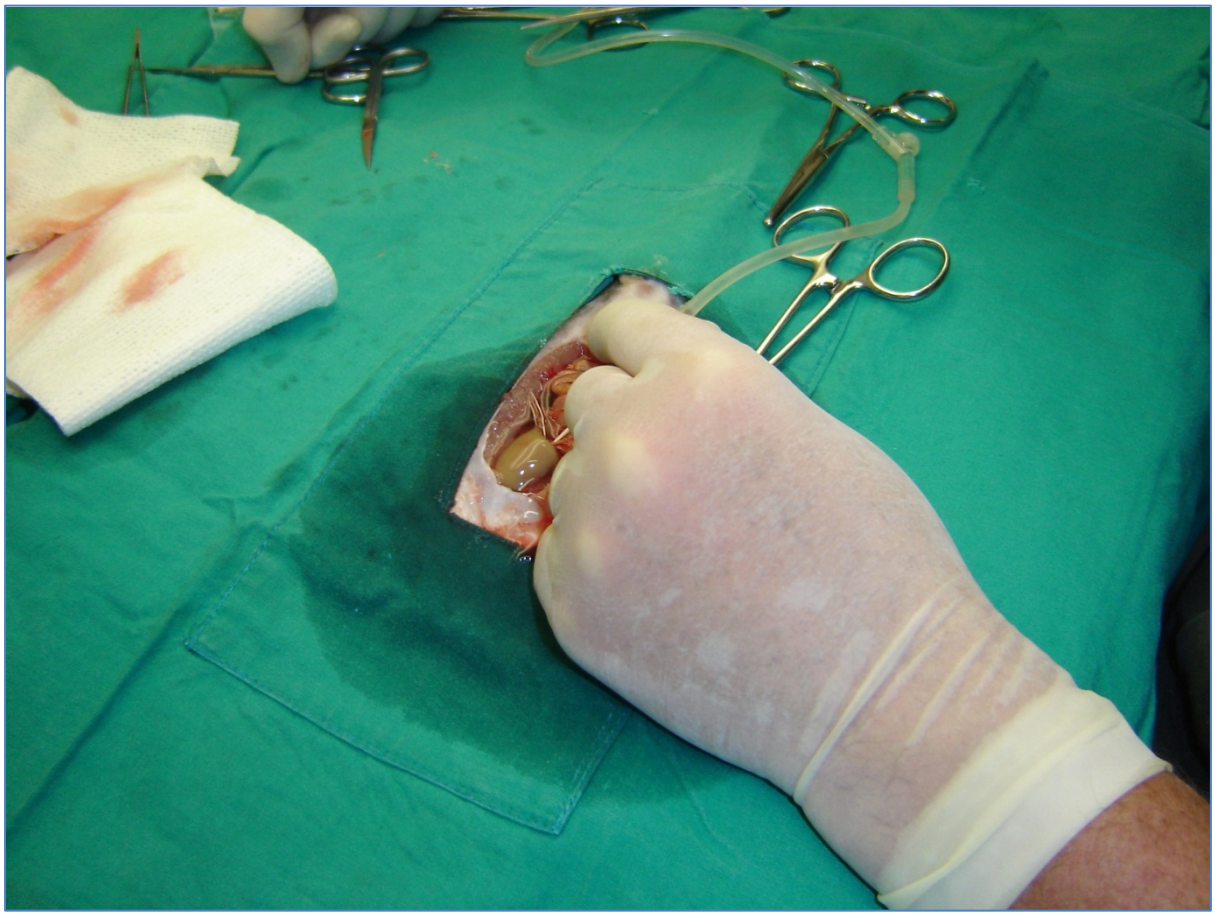

Fig 7.1: Jelco catheter (20G) in the portal vein of an African sharptooth catfish. The catheter is kept dead still and in position by hand during the perfusion process. Liver is left of the surgeon's ring finger. There is blanching (paleness) of the as all the blood was washed out of the liver with the large volume of saline pumped through it. The tube is attached to a peristaltic pump or an ordinary 20 ml syringe (used in the beginning) (picture: Annette Venter)

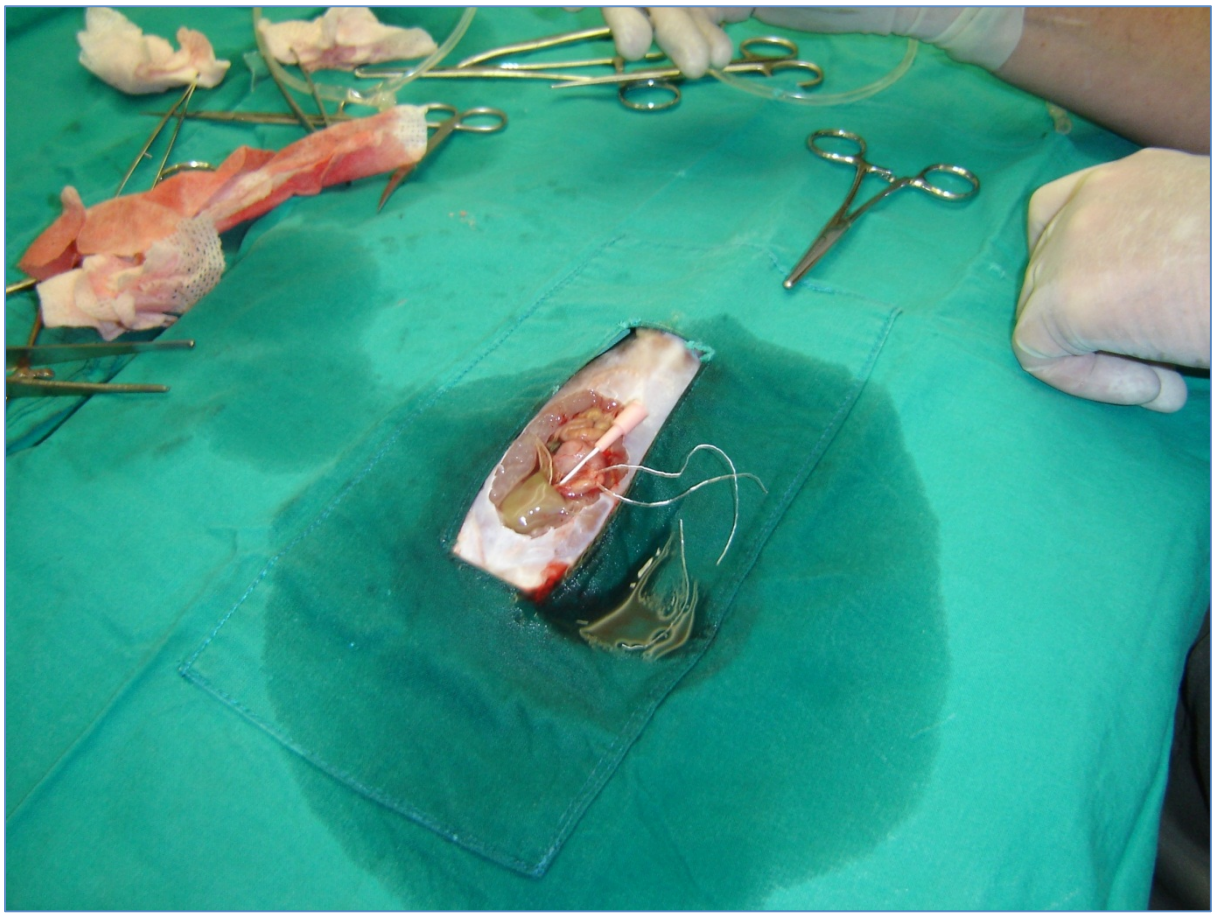

Fig 7.2: After the liver of the sharptooth catfish was perfused with all the solutions. The pale liver is an indication that the perfusion went well. The liver also looks soft (after collagenase was used – last step). Jelco catheter (20G) still in the portal vein of the liver, but the tube from pump already removed (picture: Annette Venter)

In conclusion, a primary African sharptooth catfish primary hepatocyte system was developed and validated and this established *in vitro* system can now be used to monitor some aquatic pollutants in freshwater ecosystems. This primary heptocyte system has already been used for other diagnostic projects; an example is the Rooiwal Sewage processing plant survey (data not published yet) that was done in collaboration with scientists from Tanzania.

7.2 Aim

Establishment of a primary hepatocyte system for the African sharptooth catfish

7.3 Objective(s)

1. To develop and validate a sharptooth catfish primary hepatocyte system for aquatic pollution monitoring
2. To develop a method for the cannulisation of the portal vein and flushing of the liver *in situ*
3. To evaluate primary hepatocyte cell activity *in vitro* by using CYP1A induction after benzo[a]pyrene exposure as confirmation
4. To compare the activity of sharptooth catfish primary hepatocytes with a continuous cell line (Chinese hamster ovary)
5. To evaluate cytotoxicity using the MTT and trypan blue exclusion assays

7.4 Collaborators (alphabetical), affiliation and their respective contributions

1. Botha C J. Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, Onderstepoort 0110, South Africa. Planning of the project, interpretation of results and preparation of the manuscript.
2. Myburgh J G. Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, Onderstepoort 0110, South Africa. Planning of project, obtaining AEC approval from the University of Pretoria, catfish surgery, development and improvement of the liver perfusion method, collection of hepatocytes, interpretation of results and preparation of the manuscript.
3. Naicker D. Division of Toxicology, Onderstepoort Veterinary Institute, Agricultural Research Council, Onderstepoort 0110, South Africa. Planning of the project, laboratory method development, interpretation of results and preparation of the manuscript.

7.5 Publication

Naicker D, Myburgh J G, Botha C J, 2007. Establishment and validation of primary hepatocytes of the African sharptooth catfish (*Clarias gariepinus*). Chemosphere, 68, 69-77. DOI:10.1016/j.chemosphere.2006.12.037

Chapter 7 Primary hepatocytes.pdf

[9 pages for aricle]

CHAPTER 8: The post-occipital sinus of the spinal vein of the Nile crocodile (*Crocodylus niloticus*): anatomy and its use for blood sample collection and intravenous infusions

8.1 Preface

Blood is probably the sample most often collected from crocodilians. It is, unfortunately, not very easy to evaluate the health of an individual crocodile with a clinical examination. Therefore, a follow-up laboratory investigation is usually needed, and most often a blood sample is collected. The spinal vein, in the post-occipital region, is a practical and accessable bloodvessel for the collection of blood samples from crocodilians (Fig 8.1). Blood collection is unfortunately invasive, but it is not a very difficult clinical procedure, even in the Nile crocodile, and a blood sample can usually be collected without harming the animal. However, a basic knowledge of the anatomy of the post-occipital neck region, as well as the blood vessel is essential to support efficient and safe sample collection.

Although this sampling method is well established and has been used for several crocodilian species over the years, the technique and associated anatomy has not been investigated in the Nile crocodile. The clinically relevant anatomy of the post-occipital spinal venous sinus was studied. This included the description of practical external landmarks to guide the blood collector to locate this bloodvessel (see: aim and objectives). In addition, an attempt was made to investigate and clinically evaluate the procedures of blood sampling and intravenous infusion in the Nile crocodile (see: aim and objectives). This blood sampling technique opens the door for safer and more efficient sample collection, and will be used in future, to study normal blood chemistry and hormone concentrations in the Nile crocodile. Similarly this technique of blood sampling and intravenous infusions can be used for pharmacokinetic studies.

This method of blood collection, unfortunately, also has some disadvantages. The most significant complication is trauma to the wall of the spinal venous sinus by the

tip of the needle inserted perpendicular into the bloodvessel. Crocodile clinicians and researchers must guard against unnecessary movement of the patient or the needle during the blood sampling procedure, seeing that this is often the cause of the bloodvessel wall laceration by the tip of the needle.

Fig 8.1: American alligator (*Alligator mississippiensis*) after a blood sample was collected from the spinal venous sinus at Kennedy Space Center, Cape Canaveral in the United States of America. From left to right: Jan Myburgh, Lou Guillette and Hannes Botha (picture: Russell Lowers)

8.2 Aim

Nile crocodile spinal venous sinus anatomy and the adjoining anatomical structures

8.3 Objective(s)

1. To investigate the anatomy of the spinal venous sinus of the Nile crocodile

2. To investigate the microanatomy of the spinal venous sinus
3. To investigate the spinal venous sinus using diagnostic imaging
4. To evaluate the blood sampling technique and intravenous infusion
5. To report the blood sampling technique and to describe the external landmarks
6. To apply the established method to collect blood samples from Nile crocodiles for a biochemical investigation

8.4 Collaborators (alphabetical), affiliation and their respective contributions

1. Booyse D G. Department of Anatomy and Physiology, Faculty of Veterinary Science, University of Pretoria, Onderstepoort 0110, South Africa. Planning of the project, processing of the head and necks (HaNs) and handling of the animals.
2. Guillette L J. Department of Obstetrics and Gynecology, Marine Biomedicine and Environmental Sciences Center, Medical University of South Carolina, and Hollings Marine Laboratory, Charleston, South Carolina 29425-6190, United States of America. Demonstration of the technique, planning of the project and preparation of the manuscript.
3. Huchzermeyer F W. Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, Onderstepoort 0110, South Africa. Planning of the project, interpretation of results and preparation of the manuscript.
4. Kirberger R M. Department of Companion Animal Clinical Studies, Faculty of Veterinary Science, University of Pretoria, Onderstepoort 0110, South Africa. Planning of the project, diagnostic imaging, interpretation of the findings and preparation of the manuscript.
5. Lowers R H. InoMedic Health Applications, Kennedy Space Center, Florida 32899, United States of America. Demonstration of the technique, preparation of the manuscript.
6. Myburgh J G. Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, Onderstepoort 0110, South Africa. Planning of

the project, processing of the HaNs, handling of the animals, collection of data, interpretation of the findings and preparation of the manuscript.

7. Soley J T. Department of Anatomy and Physiology, Faculty of Veterinary Science, University of Pretoria, Onderstepoort 0110, South Africa. Planning of the project, interpretation of the anatomical findings and preparation of the manuscript.
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8.5 Publication

Myburgh J G, Kirberger R M, Steyl J C A, Soley J T, Booyse D G, Huchzermeyer F W, Lowers R H, Guillette L J, 2014. The post-occipital spinal venous sinus of the Nile crocodile (*Crocodylus niloticus*): Its anatomy and use for blood sample collection and intravenous infusions. Journal of the South African Veterinary Association, 85, article # 965. http://dx.doi.org/10.4102/jsava.v85i1.965

Chapter 8 Blood collection.pdf

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CHAPTER 9: Technique for the collection of clear urine from the Nile crocodile (*Crocodylus niloticus*)

9.1 Preface

Urine, as a diagnostic sample, has largely been ignored in the past, until Louis Guillette jr. (collaborator on this project) started to routinely collect urine samples from American alligators (*Alligator mississippiensis*). Urine has been collected before, but the focus was on the osmoregulatory capabilities of crocodilians. The urine samples collected for the osmoregulatory studies were obtained using crude methods, e.g. forcing a probe through the cloacal opening, into the cloaca, thereby opening the sphincter and allowing urine to flow out. The anatomy of the cloaca of crocodilians, including the Nile crocodile, was not studied during these investigations. It is well-known that crocodilians are not ideal patients for clinical examinations and the clinician or researcher, therefore, strongly depends on additionally collected samples for laboratory tests to evaluate the health status of individual crocodiles. In this regard, crocodile urine may become a very helpful diagnostic sample in future.

The aim of this project was to investigate the anatomy of the cloaca of the Nile crocodile and to develop a simple technique for the collection of clean urine using a dog urinary catheter (see: aim and objectives). The anatomy of the cloaca was studied to determine how and where the urine is stored in the cloaca, seeing that crocodilians do not have bladders. The urine accumulates in a urinary chamber consisting of the urodeum and coprodeum, and that the urine is “trapped” in this chamber by the uroproctodeal and rectocoprodeal sphincters. The anatomy of the cloaca of the Nile crocodile was also compared to that of the American alligator, Australian saltwater crocodile (*Crocodylus porosus*) and African ostrich (*Struthio camelus*) during this investigation.

A urine sample can easily be collected from the urinary chamber of the Nile crocodile with a catheter by gently pushing it through the uroproctodeal sphincter (Fig 9.1). The three most important advantages of this technique are: that it is completely

atraumatic; the procedure is easy to perform (not a complicated or tricky technique); and a relatively clean urine sample is collected.

Although the technique for the collection of a clean urine sample from the Nile crocodile has been established, additional basic research is still needed to determine the real diagnostic value of a Nile crocodile urine sample. Crocodilian urine samples might be of help with the following: investigation of toxicological problems (e.g. exposure to lead), conducting pharmacological studies (e.g. pharmacokinetic excretion studies), determining physiological parameters (e.g. concentrations of stress hormones) and studying pathological conditions. After this urine collection project, more than 100 urine samples from healthy, pre-slaughter, Nile crocodiles were collected for a chemical investigation to determine reference concentrations. This article is currently *in preparation*.

Fig 9.1: Collection of urine from a juvenile Nile crocodile from the Blyderivierspoort Dam in the Mpumalanga Province. Most of the wild crocodile work is done at night and from a boat (picture: Hannes Botha)

9.2 Aim

Establish a practical urine collection technique for the Nile crocodile

9.3 Objective(s)

1. To develop a simple and non-traumatic technique for the collection of clean urine from the Nile crocodile
2. To evaluate the suitability of different urinary catheters and small diameter stomach tubes
3. To investigate the gross morphology of the cloaca of the Nile crocodile
4. To document the clinical procedure of urine collection
5. To use the established method to collect urine samples from Nile crocodiles for a biochemical investigation

9.4 Collaborators (alphabetical), affiliation and their respective contributions

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8. Soley J T. Department of Anatomy and Physiology, Faculty of Veterinary Science, University of Pretoria, Onderstepoort 0110, South Africa. Planning of the project, interpretation of the anatomical findings and preparation of the manuscript.

9.5 Publication

Myburgh J G, Huchzermeyer F W, Soley J T, Booyse D G, Groenewald H B, Bekker L C, Iguchi T, Guillette L J, 2012.Technique for the collection of clear urine from the Nile crocodile (*Crocodylus niloticus*). Journal of the South African Veterinary Association. http://doi:10.4102/jsava.v83i1.8

Chapter 9 Urine collection.pdf

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CHAPTER 10: GENERAL DISCUSSION AND CONCLUSIONS

The Department of Paraclinical Sciences was an equal partner in a collaborative research project on environmental toxicology (Ecotox project) and zoonotic diseases funded by the Norwegian Council for Higher Education's Programme for Development, Research and Education (NUFU) for two 5-year periods. The NUFU Veterinary Network comprised of six partner institutions in eastern (Uganda and Tanzania) and southern Africa (Mozambique, South Africa, Zambia and Zimbabwe), and the Norwegian School of Veterinary Science and National Veterinary Institute in Oslo as the northern partners. Within this partnership there were two research themes *viz*. zoonotic diseases (diseases transmitted from animals to humans ~ Zoo group) and environmental toxicology (Ecotox group), and the acronym "ZooTox" was coined to refer to this NUFU partnership.

The NUFU-funded Ecotox project was in many ways a first for the Department of Paraclinical Sciences, as well as for the Faculty of Veterinary Science. The Ecotox project not only stimulated new research ideas, but also created numerous ongoing initiatives in the Department. Awareness was created for the different threats affecting the health of freshwater ecosystems in South Africa, as well as the urgent need for more focused research and on-going monitoring of these ecosystems and their inhabitants. The African sharptooth catfish was identified by the Ecotox group as the fish species all the participating countries in Africa will focus on. However, South Africa received special permission from the Ecotox group members and NUFU to use the Nile crocodile as an additional aquatic species.

One of the most important successes of the NUFU-funded programme was capacity building at the different collaborating veterinary faculties in Africa through post-graduate training of staff members and in-service training of other researchers. The ten years of collaborative research have resulted in a strong network between the veterinary faculties. Annual or bi-annual meetings rotated between the participating countries and this allowed all the participants to visit the different countries, and exposed group members to specific difficulties and problems that are being encountered in the respective host country. The deans of veterinary faculties in southern and eastern Africa were always invited to the annual., later bi-annual.,

NUFU group meetings. One of the major achievements was that these meetings created unique opportunities for the deans to establish direct contact (this never happened before in eastern and southern Africa) and discuss various aspects of mutual interest and cooperation. This has led to the establishment of a Regional Deans Group and has paved the way for a united voice from eastern and southern Africa at international veterinary meetings, such as at the World Organization for Animal Health (OIE), where issues such as harmonization of veterinary education and training are discussed. In addition, the NUFU-driven collaborative projects also stimulated other academic agreements and memoranda of understanding between African veterinary faculties and schools. On a more individual level, the regular NUFU group meetings also allowed participants to brain-storm and plan new projects. Flowing from this, research consortia were formed and grant applications were submitted together (e.g. Tanzania and South Africa; Mozambique and South Africa) to different funding bodies. Above and beyond academic collaboration and research projects, lasting friendships have also been formed across national borders.

The five research projects collated for this PhD contributed significantly to our clinical skills, and diagnostic abilities to evaluate the health of the sharptooth catfish and Nile crocodile. Although these projects did not address all the health monitoring needs, it contributed significantly as a biomonitoring platform to build on in future. The Ecotox initiated cell culture projects that were started in our Department, stimulated several other *in vitro* investigations, most likely due to the exposure to, and the experience gained with these techniques during the NUFU programme. An ultra-modern cell culture laboratory was established in the Department of Paraclinical Sciences after the sharptooth catfish cell culture projects were completed. The Nile crocodile blood and urine collection methods, as well as the in-depth study of the associated anatomy, created opportunities for the efficient collection and evaluation of blood and urine samples for biomonitoring purposes and other specific research projects (e.g. blood and urine biochemistry). The sharptooth catfish blood chemistry survey evaluated the potential of using a standard veterinary clinical pathology profile (most veterinary hospitals can test at least some of the parameters) to evaluate potential health abnormalities.

The African sharptooth catfish turned out to be an excellent fish species to keep in captivity and to work with. Although we had problems with a *Saprolegnia* outbreak in the beginning, it was controlled by strict hygiene, improved handling and intensive water treatment (daily replacement of some of the water, cleaning of the filters and water medication). This fish is an indigenous African species that certainly has a lot of potential for future controlled research projects, as well as using them as sentinels of aquatic ecosystem health.

Our sharptooth catfish projects contributed significantly to the following in the Department of Paraclincal Sciences: establishment of a dedicated aquarium room with enough glass tanks, holding dams and pumps for future fish research projects; gaining of practical experience by Departmental members in the handling and feeding of catfish; and the initiation of cell culture technology. Indirectly, it also played a role with the following: it made the Departmental members more aware of freshwater pollution and the potential effects of pollutants on fish health; stimulated an interest in aquaculture and aquaponics; a close collaboration with the Tilapia Aquaculture Association of South Africa (TAASA) was started; and collaboration with David Huchzermeyer (was appointed as an extraordinary lecturer in the Department during 2014) and the Department of Ichthyology and Fisheries Science (DIFFS), Rhodes University was established. In addition, the Pathology Section of the Department of Paraclinical Sciences also experienced an increase in the number of dead or sick fish submitted by aquaculture farmers and ornamental fish owners for necropsies. Similarly, fish carcasses collected during fish die-offs (e.g. Loskop Dam fish mortality) were brought in for diagnostic examinations. This prompted a re-evaluation of the fish diagnostic services in the Department of Paraclinical Sciences, and stimulated negotiations for the creation of a new post (if enough funding is received from Agriseta) for a pathologist that will concentrate on fish diseases and diagnostics. Dr Johan Steyl was afforded the opportunity to go and work with a fish pathologist in Norway - the NUFU project and collaborative network with the Norwegian Institutions (Norwegian School of Veterinary Science and National Veterinary Institute in Oslo) facilitated his visit.

In the case of the African sharptooth catfish, one blood chemistry (Chapter 5) and two *in vitro* cell culture (Chapters 6 and 7) projects were completed. For the *in vitro*

projects, a gill filament assay and a primary hepatocyte system were developed and validated. Both methods were successfully established and have potential for future monitoring of aquatic ecosystems.

For *C. gariepinus* to effectively serve as a bioindicator species, baseline clinical chemistry parameters must be defined. Unfortunately, only a paucity of clinical pathology data is currently available and to address this, blood samples were collected from apparently healthy male and female catfish and a number of parameters were determined (Chapter 5). Plasma protein concentrations, but particularly albumin, were very low, approximately half the value for dogs, but similar to the concentrations of channel catfish (*Ictalurus punctatus*) (Ellsaesser & Clem, 1987).

The plasma urea concentrations in the sharptooth catfish were much lower than in dogs, but only marginally lower than in channel catfish. Plasma creatinine in sharptooth catfish was only a quarter of that of dogs and one third of that reported for channel catfish (Ellsaesser & Clem, 1987). These findings may have implications for using urea and/or creatinine as an index of renal glomerular filtration, as is done in mammals.

Another finding of this investigation was that the plasma enzyme activity ranges were much lower in sharptooth catfish than in dogs (Latimer, 2011) - particularly in the case of alkaline phosphatase (ALP) and alanine aminotransferase (ALT). By comparison, channel catfish have an even lower ALT activity range but an ALP range that is very similar to dogs (Ellsaesser & Clem, 1987; Latimer, 2011). Unfortunately, the implications for using these enzymes as markers for liver disease are not clear from our data, as factors such as plasma half-life and tissue distribution remain to be determined. In addition, the very low plasma thyroxine (T_4) concentrations in sharptooth catfish have important implications for laboratory personnel, who will have to set up specific calibration and standardization adaptations for the methods that are generally designed for human samples.

Although our sample size was too small for reliable comparisons, it appeared that there was little difference in the parameters measured between male and female

catfish. The clinical pathology blood reference ranges obtained during this investigation are valuable when using *C. gariepinus* as a sentinel of aquatic ecosystem health.

In the second sharptooth catfish study, a gill filament-based assay was developed for the African sharptooth catfish, and evaluated as a monitoring tool for waterborne polycyclic aromatic hydrocarbon pollutants (PAH) (Chapter 6). PAH pollution of streams and rivers, associated with coal mining and burning, is a serious concern in the upper catchment of the Olifants River (Mujuru, 2009).

The ability of African sharptooth catfish (*Clarias gariepinus*) in inducing cytochrome P-450 class 1A (CYP1A) and glutathione S-transferase (GST) enzymes was determined in liver and gill filaments after 4 days of waterborne exposure to the PAH, benzo(a)pyrene (B[*a*]P). The 7-ethoxyresurufin-*0*-deethylase (EROD) activity was measured in hepatic microsomes and gill filaments, with B[a]P strongly inducing CYP1A activity in gill filaments of both male and female sharptooth catfish. Our findings indicated that the levels of fluorescent aromatic compounds (FACs) per ml of bile were 17-fold higher in exposed fish compared to the controls. Correlations between induction of EROD activities in gill filaments and liver, and between induction of EROD activities in gill filaments and levels of biliary FACs metabolites were strong.

Our findings indicated a greater induction of EROD activity in the gills, if compared to the liver, following the exposure to waterborne B[a]P. This suggested that the absorbed B[a]P was largely metabolised in the gills and, therefore, only a small fraction of unmetabolised B[a]P reached the liver. Similar finding were reported in previous studies (Levine & Oris, 1999; Jönsson et al., 2002; Jönsson et al., 2003). Therefore, even a relatively low metabolic capacity in the gills may contribute significantly to prevent accumulation of pollutants in organs such as the liver (Carlsson et al., 1999; Levine & Oris, 1999). The gills thus fulfil a vital role in the protection of fish against harmful chemical pollutants by lowering the total intake (Chapter 6).

This is the first study determining EROD activities in gill filaments and hepatic tissue, FACs in bile, and GST in hepatic tissues of the shaprtooth catfish after waterborne exposure to B[a]P. This filament assay has proven to be a robust, straightforward and a sensitive method for determination of basal and induced EROD activities. Furthermore, this method is relatively low cost and time saving if compared to traditional microsome-based techniques. Our study confirmed that the sharptooth catfish gill filament-based CYP1A assay can be used to monitor PAH-type pollutants in aquatic ecosystems, particularly in countries where advanced laboratory facilities are not always available.

The focus of the third African sharptooth catfish study was to develop and validate a primary hepatocyte system for this species. Worldwide, *in vitro* systems such as primary cells and continuous cell lines are gaining momentum in aquatic ecosystem toxicological studies, as well as tests using specific endpoints such as CYP1A induction (Segner, 1998; Van der Oost et al., 2003; Zhou et al., 2006).

Harvesting of primary hepatocytes was successfully achieved using an *in situ* surgical liver perfusion technique, developed specifically for the sharptooth catfish in our Department. Our modified perfusion method using collagenase and microsieve membrane filters proved to be very effective in isolating the catfish liver cells. This was determined by electron microscope, as the isolated catfish hepatocytes retain their structural integrity, with the organelles intact and virtually no swelling or rupturing (Chapter 7).

During the validation process, the primary hepatocytes expressed clear CYP1A induction *in vitro* when exposed to B[*a*]P. However, a continuous Chinese hamster ovary (CHO-K1) cell line did not show activity when exposed to various concentrations of B[*a*]P. This confirmed, what is well-known, that healthy primary cells behave very much like the original cells (Segner, 1998; Van der Oost et al., 2003) - CYP1A induction was observed with the primary hepatocytes while the CHO-K1 continuous cell line did not show any induction.

Cytotoxicity, as measured by the methyl thiazol tetrazolium (MTT) assay, was not observed following a 72 h exposure of the primary hepatocytes and the CHO-K1 cell

line to different B[a]P concentrations. However, the hepatocytes were damaged at higher concentrations of B[a]P (>10^{-6}M) as observed by a transmission electron microscopy investigation. We also confirmed the cytotoxicity effects using the trypan blue exclusion assay (TD_{50} of 10^{-6}M). The primary catfish hepatocyte cell culture system, that was successfully established, confirms that it can be effectively used as an *in vitro* system for monitoring of aromatic hydrocarbon pollution in aquatic ecosystems.

While the sharptooth catfish is widely distributed in South Africa and easy to work with, the Nile crocodile is just the opposite. The distribution of wild Nile crocodiles in South Africa is mostly limited to conservation areas (e.g. Kruger National Park and ISimangaliso Wetland Park) (Botha et al., 2011; Combrink et al., 2013; Ferreira & Pienaar, 2011). However, large numbers of Nile crocodiles are kept on big commercial crocodile farms in southern Africa (personal communication, Stefan van As, Exotic Leather Cluster, 2013). Nile crocodiles are dangerous and anybody working with these animals, especially the mature crocodiles, must be extremely cautious. While the existence of Nile crocodiles on commercial farms is not threatened due to the large number of crocodiles in captivity, wild crocodiles (lower numbers) are definitely affected by obvious aquatic ecosystem changes and freshwater pollution in Africa (Botha et al., 2011; Combrink et al., 2011; Ferreira & Pienaar, 2011; Irwin & Irwin, 2006; Myburgh & Botha, 2009). The large number of crocodiles on commercial farms, close to the Faculty of Veterinary Science, supported our crocodile research by facilitating access to tissue samples, carcasses and live animals.

Over the last decade or two, farming with Nile crocodiles in South Africa has developed into a very important industry, generating foreign revenue through the export of raw skins and the selling of luxury leather goods. An Exotic Leather Cluster (ELC) was started in 2014 at the University of Pretoria to promote the complete value chain of the crocodile leather industry - from crocodile farmers to the manufacturing and trade in luxury leather goods (personal communication, Stefan van As, Exotic Leather Cluster, 2013). The establishment of ELC is indirectly linked to the NUFU programme. Due to the first crocodile research projects (e.g. blood and urine collection techniques, etc.) that were started, on-going relationships (e.g.

diagnostic services) developed with most of the commercial crocodile farmers in southern Africa. During the negotiations to establish ELC, it became clear that the Department of Paraclinical Sciences and the Faculty of Veterinary Science contributed significantly to promote the production, health and welfare of Nile crocodiles in the past and should, therefore, play an important technical role in this cluster that is financed by the Department of Trade and Industry (DTI).

Working on a charismatic species like the Nile crocodile has also other advantages. It stimulated international collaboration outside the NUFU group that is still going strongly. The author, Jan Myburgh visited the USA and Japan to participate in research projects, e.g. collecting blood and urine samples from America alligators in Florida, USA and was also invited to become a member of the IUCN Crocodile Specialist Group (CSG) and since has attended all the working meetings. Several papers about our work on Nile crocodiles were presented at the international CSG working meetings. Crocodile diagnostic services also expanded and crocodile farmers in close vicinity to the Faculty of Veterinary Science started to regularly submit carcasses for confirmation of the cause of death or runting (ill-thrift; poor doing). This not only contributed to the Ecotox research projects in the form of valuable material and carcasses (e.g. blood and urine collection papers), but also supplied enough material for three additional scientific papers (case reports) that are currently in preparation.

On-going monitoring of the health of farm and wild crocodiles is important. However, efficient and safe collection of diagnostic samples is essential, especially from a welfare point. With the growth of the commercial crocodile industry in southern Africa and anthropogenic changes to aquatic ecosystems, the collection of samples for diagnostic and research purposes from live farm and wild crocodiles will become more important in future. Although the procedure for the collection of blood from crocodilians (e.g. American alligator) is known (personal communication, Louis Guillette and Russell Lowers, 2010), this technique, as well as the clinical anatomy of the spinal vein in the Nile crocodile has not been investigated before. Blood is usually collected from the post-occipital neck area (personal communication, Louis Guillette and Russell Lowers, 2010). With our Nile crocodile blood collection project

(Chapter 8) we contributed significantly to promote safe and efficient blood sample collection from the Nile crocodile.

During our first Nile crocodile study we confirmed that the spinal vein runs within the vertebral canal, dorsal to, and closely associated with the spinal cord, and that it develops into a venous sinus, cranially, in the post-occipital region. As this blood vessel is a sinus in the area of blood collection (post occipital region), we proposed to call it the spinal venous sinus of the spinal vein. Our histological investigations confirmed that the spinal venous sinus is situated epidurally (extradural) in the Nile crocodile. The most interesting observation concerning the atlas (C_1) was that it consisted of four individual bones (Chapter 8).

It is important for the blood collector to be knowledgeable about the basic anatomy of the post-occipital region and the spinal venous sinus before attempting this procedure to increase the success rate and prevent complications and cruelty (Chapter 8). Although the spinal venous sinus is accessed blindly, the use of the external landmarks, identified during this investigation, makes it possible to be successful during most attempts. Our reported landmarks, therefore, simplifies venipuncture for any biologist or veterinarian to blindly access the spinal venous sinus, and also promote efficient and safe sample collection.

For venipuncture the spinal venous sinus is accessed through the interarcuate space between the atlas and axis (C_1 and C_2) by inserting a needle angled just off the perpendicular, in the midline through the cranio-dorsal cervical skin, just cranial to the cranial borders of the first cervical osteoderms. The same techniques can also be used for intravenous infusions when an epidural needle and catheter set is used (Chapter 8). Using the post-occipital spinal venous sinus for venipuncture has some advantages and disadvantages. The advantages are: the site of venipuncture is easy to determine using external landmarks; the animal is restrained in a natural position (lying on its ventral surface or belly) and the collection technique is relatively simple. In the case of large crocodiles the collector may sit on the reptile's thorax and safely collect the sample from the post-occipital area. Potential disadvantages are: the blood vessel wall could be lacerated; the spinal cord may be damaged; contamination of the blood sample by cerebrospinal fluid (CSF) is a possibility; and

in bigger crocodiles the blood vessel will be comparatively deeper and the opening between the atlas and axis, therefore, sometimes more difficult to find.

During our investigations it transpired that the length of the bevel of the hypodermic needle is one of the most important predisposing factors for the development of complications. A longer bevel would increase the risk of damage to the blood vessel wall being damaged during the blood collection procedure. This is particularly relevant considering that the length of the bevel, when inserted perpendicularly into the blood vessel of a smaller crocodile, is nearly as long as the total diameter of the venous sinus. This creates a situation where the sharp tip of the needle is already touching or penetrating the ventral wall of the blood vessel before the needle opening comes in contact with the blood (Chapter 8). The design of short bevel needles, specifically for crocodilian work, is something to consider in the future.

The successful mastering of this venipuncture technique affords any crocodile researcher or veterinarian the opportunity to safely collect blood samples which can be used for an array of investigations or specific tests, for example diagnostic clinical pathology, hormone concentrations, pharmacokinetic studies, etc.

Despite the fact that urine is commonly screened as a diagnostic sample in humans and animals (Latimer, 2011), it has not been routinely used in crocodilians. The most obvious reason is probably that a practical method of urine collection was not known or reported (Chapter 9). Another contributing factor was that the anatomy of the cloaca of the Nile crocodile and other crocodilians has never been thoroughly investigated.

In our second Nile crocodile study we confirmed that it is possible to collect a relatively clean urine sample from the cloaca with a dog urinary catheter. For urine collection the “urinary chamber” is accessed via the uroproctodeal sphincter after inserting the catheter through the cloacal opening and gently pushing it in a cranial direction (Chapter 9). The technique that we developed is atraumatic and simple, making this a practical method that can be used by anybody interested in obtaining a urine sample from a Nile crocodile.

Anatomically, the cloaca is divided into three compartments, the proctodeum, urodeum and coprodeum. We discovered that in the Nile crocodile urine accumulates in a common chamber formed by the urodeum and coprodeum (Chapter 9). Our observations differed significantly from the findings reported by Kuchel and Franklin (2000) who worked on the estuarine crocodile (*Crocodylus porosus*). They reported that the three chambers of the cloaca are separated by "tight muscular sphincters" and that the urodeum forms the largest chamber. In marked contrast, it was clear from our study that in the Nile crocodile, the urodeum and coprodeum form a single urinary chamber, separated from each other by a rudimentary coprodeal fold (Chapter 9). In the estuarine crocodile, the coprodeum is illustrated as being small and that faecal material accumulates in this compartment. Contrary to their findings, we surmised that in the Nile crocodile faecal material is temporarily stored in a very short rectum, which is separated from the urinary chamber by the rectocoprodeal sphincter. Whether these significant differences in cloacal morphology represent species-specific peculiarities or reflect differences in interpretation need further investigation.

The reported method for the atraumatic collection of urine samples from the Nile crocodile (Chapter 9) opens new fields for future research. Chemical pollutants, pharmaceutical drugs and steroid hormones or their breakdown products are excreted in urine (McClellan-Green et al., 2006). A urine sample could therefore be considered to be a more appropriate sample, if compared to blood, for the detection of metabolised and water soluble pollutants, hormones and drugs.

Future projects directly linked to this project that should be completed or implemented:

1. Blood biochemistry of the Nile crocodile. A well planned project to determine normal reference ranges for the Nile crocodile is required, as well as to evaluate the diagnostic value (interpretation) of blood results.
2. A urine sample can be used for diagnostic purposes. However, an in-depth study to determine the diagnostic value of Nile crocodile urine samples should be planned. Going hand-in-hand with the urine chemistry research is kidney physiology.

3. A Nile crocodile primary hepatocyte system was initiated during this PhD project and it was found to be working well. Final validation must be done before it can be used as a primary cell culture monitoring assay.
4. Use of sharptooth catfish primary hepatocytes and gill filaments to monitor PAH pollution in the upper Olifants catchment.
5. Thyroid anatomy and function in the Nile crocodile and sharptooth catfish. The thyroid is known to be affected by aquatic pollutants. Monitoring of thyroid function could be an important parameter to determine aquatic ecosystem health.
6. Although a primary hepatocyte system was successfully established for the sharptooth catfish we discovered interesting gaps in the current understanding of the liver anatomy. For example, all sharptooth catfish livers have thin bilateral stalks (like an umbilicus) of liver tissue transversing the body wall and ending under the skin as slightly enlarged bodies. What is the reason for this evolutionary development?
7. Sharptooth catfish blood chemistry and endocrinology also need more attention.

In conclusion, the focus of the Nile crocodile projects was on the establishment of sample collection techniques. However, the development of the blood and urine sample collection techniques, not only confirmed the normal anatomy of the sample collection sites, but also created the potential for screening of blood and urine samples for an array of chemicals and clinical parameters. Screening of the blood samples, collected from apparently healthy African sharptooth catfish, contributed to our database of “normal” laboratory values, as well as evaluating the potential diagnostic value of standard veterinary clinical pathology tests. The establishment of specific cell culture assays for the African sharptooth catfish contributed to the implementation of innovative *in vitro* techniques that can be used to effectively monitor aquatic animal health. The momentum that was generated with the five research projects (Chapters 5 to 9) should kick-start new innovative ideas and investigations to study the health monitoring of aquatic ecosystems in southern Africa. In addition, a large number of publications and new research questions emanated from the NUFU-funded collaborative research programme and this PhD project.

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