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PANSTEATITIS IN AFRICAN SHARPTOOTH CATFISH, *CLARIAS GARIEPINUS* (BURCHELL), IN THE KRUGER NATIONAL PARK, SOUTH AFRICA

by

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Submitted in fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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University of Pretoria

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DEDICATION

This work is dedicated to my family.

My parents, Fritz and Hildegard, who provided the example and set the foundations for a lifelong scientific quest for knowledge.

My wife, Philippa, who gave her unwavering support, love and understanding.

My sons, Carl, Richard and Nicholaus, whose keen interest and help allowed me to set the example for their respective academic achievements as budding scientists.

I thank you for the love, support and encouragement you gave me.

The confluence of the Olifants and lower Letaba rivers at the entrance to the Olifants Gorge in the Kruger National Park.Both rivers in full flood in January 2011.

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DECLARATION

This thesis is my own original work and has not been submitted in candidature for any other degree.

Candidate: K.D.A. Huchzermeyer

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SUMMARY

PANSTEATITIS IN AFRICAN SHARPTOOTH CATFISH, *CLARIAS GARIEPINUS* (BURCHELL), IN THE KRUGER NATIONAL PARK, SOUTH AFRICA

by

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In the Kruger National Park (KNP), pansteatitis in sharptooth catfish, *Clarias gariepinus* (Burchell), was shown to be a serious problem in the inlets to large man-made lakes fed by rivers arising in the polluted catchments of the Olifants and Sabie rivers. An increasing prevalence of pansteatitis was recorded in catfish from the Olifants River gorge. A low prevalence was found in catfish upstream of the gorge at two further sites. No pansteatitis was detected in catfish from a rain-filled dam distant from the potential pollution sources affecting the Olifants River and in rivers arising outside of the park that were not dammed. Analysis of stomach content indicated a higher prevalence of fish in the diet of catfish affected by pansteatitis than in those not affected. Significant pathology in catfish was limited to changes associated with a generalised necrosis and inflammation of adipose tissues (pansteatitis), and there was evidence that lesions accumulated over time. Similar pathology was found in a captive population of catfish with known nutritional pansteatitis. Pathology in other organs that might have been attributed to pollution could not be demonstrated. Examination of blood smears and measurement of haematocrit, blood haemoglobin, serum vitamin E and erythrocyte glutathione peroxidase values did not prove useful as monitoring tools, probably because of the episodic exposure to oxidative stress and the chronic nature of the condition. Pansteatitis-affected catfish, kept in an experimental pond for 11 months after

the inciting nutritional cause had been removed, retained steatitis lesions almost unaltered. Whereas lipolysis appeared to be reduced by pansteatitis, adipogenesis appeared to be unaffected. Juvenile catfish confined in experimental tanks with sediments from sites where pansteatitis occurred remained healthy, and no pathology developed after 14 months, suggesting that sediments were not directly toxic.

The results of the study present the first record of pansteatitis in both wild and farmed African sharptooth catfish and emphasize the ecological importance and complexity of nutritional oxidative stress in a disturbed aquatic environment. Nutrient entrapment and the consumption of phytoplankton-feeding fish rich in polyunsaturated fats, particularly silver carp, *Hypophthalmichthys molitrix* (Valenciennes), a species alien to Africa but present in the Olifants River, is proposed as the dietary cause of the pansteatitis.

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LIST OF ABBREVIATIONS

CR	Crocodile River
CROC	Consortium for the Restoration of the Olifants Catchment
DHA	docosahexaenoic acid C22:6n-3
ECC	extracellular ceroid
EH	Engelhard Dam sampling site, July 2010
EPA	eicosapentaenoic acid C20:5n-3
FK	van Ryssen Dam
GAF	Gomoris aldehyde fuchsin
GL	Olifants Gorge*
GPx	glutathione peroxidase
GSH	glutathione
H&E	haematoxylin and eosin
LK	Lunsklip Fisheries
KNP	Kruger National Park
LUV	Levuvhu River
M	Mamba Weir
MDA	malondialdehyde
MFO	mixed function oxygenases
OG	Olifants Gorge*
OGM	Olifants Gorge Mozambique border
OL	Olifants Gorge*
LOC	Olifants Gorge*
LOG	Olifants Gorge*
PAS	Periodic acid Schiff's
PCV	packed cell volume
SAP	South African Police

SOD	Superoxide dismutase
SP	Sabiepoort sampling site
ROS	reactive oxygen species
RV	Reënvoël Dam*
RVB	Reënvoël Dam*
SANParks	South African National Parks
TBARS	thiobarbituric acid reactive substances

(*denotes codes used relating to the same sampling sites at different times)

CHAPTER ONE: INTRODUCTION

1.1. History and Background of the Study

1.1.1 The crocodile mortality events

The Olifants River Gorge and Lower Letaba River, at the confluence with the Olifants River, in the Kruger National Park (KNP), are home to one of the densest populations of large Nile crocodile, *Crocodylus niloticus*Laurenti, in South Africa. During 2007, the rising level of Lake Massingir,in Mozambique, flooded many of the rapids and pools in the Olifants Gorge. Altered hydrodynamics have resulted in the deposition of clay-rich sediments within the aquatic habitat of the area. During the autumn and winter of 2008 and 2009, large numbers of adult crocodiles were found dead in this area. Some 180 specimens out of a known population of at least 600 were found dead in 2008 alone. Autopsies performed by KNP veterinarians revealed exceptionally fat carcasses with an abnormal hardening of the fat. Histological examination of tissue specimens by Drs E. Lane (National Zoological Gardens, Pretoria, South Africa), J. Steyl and F.W. Huchzermeyer (Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa) confirmed an inflammation of the fat typical of pansteatitis.

1.1.2 Organisational response to the crocodile mortality

In response to the crocodile deaths in 2008, the Consortium for the Restoration of the Olifants Catchment (CROC) was founded as a multidisciplinary initiative. CROC provides the following preamble: *"Crocodile catastrophe – implications for mankind*

- *It is increasingly clear that the crocodile deaths in the Olifants Basin are symptomatic of a serious and growing environmental problem in which a tipping point has been reached /crossed with dramatic unexpected effects.*

- *Such a top predator collapse indicates prolonged and cumulative ecosystem stress caused by human activities in which the implementation of our legislated environmental controls and monitoring response proved inadequate.*

- *There are serious implications for human health and well-being if the situation continues and river health is not restored."*

A collaborative team effort was initiated, including KNP researchers, various universities, government departments and private sector consultants, to investigate various

aspects,including fish pathology, that may have played a role leading to the development of pansteatitis in the crocodiles.

A fish survey in the Olifants Gorge conducted by the author during August 2008, at the request of South African National Parks (SANParks), indicated that some sharptooth catfish, *Clarias gariepinus* (Burchell),carried large stores of fat that appeared to be affected by steatitis. Other fish species appeared to be healthy. The author subsequently confirmed pansteatitis in these catfish by histological examination of affected adipose tissues. This finding by the author formed the basis for the research that led to this PhD study. As catfish are known to form part of the diet of large crocodiles, the investigation was focussed on a study of the pathology in catfish inhabiting the Olifants Gorge. On 8 July 2009, for the first time, a large fish mortality event was observed within the Olifants Gorge. Affected fish were almost exclusively large sharptooth catfish and were found in the area overlying the clay-rich deposits at the point where the gorge widens into the dam. Fish carcasses were observed to be very fat. The fish kill remained localized in space and time, and no mortalities were observed in either the Olifants or Letaba rivers up-stream of the gorge, and fish in Lake Massingir appeared unaffected. Pansteatitis was confirmed by the author in live catfish sampled from the Olifants Gorge around this time by SANParks scientists.

It was assumed that pansteatitis in catfish and crocodiles in the Olifants Gorge was linked and associated with pollution. This studywas based on the assumption that pollution-associated pathology in fish in the Olifants River in the KNP preceded the pansteatitis syndrome that caused the deaths of crocodiles during the winter of 2008 and that certain pathological indicators may be used to monitor the incidence of pansteatitisin catfish along the river. This information may indirectly reflect the risk that pansteatitis poses to crocodiles in the same section of the river.

1.2. The Olifants River Catchment and Crocodile Mortalities

The Olifants River originates on the Highveld plateau of Mpumalanga Province in South Africa, then flows eastwards down the escarpment and traverses the KNP where it is joined by the Letaba River at the entrance to the Olifants Gorge. The gorge extends through the Lebombo mountains for approximately 9 km, exiting into Lake Massingir in Mozambique. From here the Olifants River continues through Mozambique before discharging into the Indian Ocean. The Olifants River is regarded as one of the most threatened aquatic

ecosystems in Mpumalanga Province of South Africa (Ashton 2010; de Villiers & Mkwelo 2009; Heath, Coleman & Engelbrecht 2010). Since large numbers of Nile crocodiles died from pansteatitis in the Olifants Gorge during 2008 (Ferreira & Pienaar 2011), a link has been sought to the consequences of human activity in the catchment of the Olifants and Letaba Rivers. The Olifants catchment has been heavily impacted by human activity including mining, coal-fired electricity generation, industrial and urban wastewater discharges, agricultural practices and water impoundments (Heath *et al*. 2010), whereas the Letaba catchment has been impacted by agriculture and human settlements. Lake Massingir sustains a considerable freshwater fishery.

Within the upper Olifants catchment lies Lake Loskop. Nile crocodile mortalities in this lake have coincided with periodic mass die-offs of fish since 2003 (Botha, van Hoven & Guillette 2011;Driescher 2007). During 2007, acid mine seepage was held responsible for a large scale fish mortality, and consumption of dead and rotting fish was found to be the most likely cause of the deaths of significant numbers of crocodiles and terrapins as a result of pansteatitis (J. Myburgh and co-workers, Faculty of Veterinary Science, University of Pretoria, pers. comm. 2009).Yellow discolouration of fat in Mozambique tilapia *Oreochromis mossambicus* (Peters) has been observed in Lake Loskop, and this change has been attributed to bioaccumulation of aluminium and iron via various species of phytoplankton on which these fish feed (Oberholster, Myburgh, Ashton, Coetzee & Botha 2011).

Between Lake Loskop and the KNP, the Olifants River has been dammed to form Lake Flag Boshielo. This lake maintains a healthy population of crocodiles, and fish mortalities are uncommon (A. Hoffman, Mpumalanga Parks Board, Marble Hall, pers. comm. 2012), indicating that mine pollution is not affecting this section of river. It has been proposed that the phytoplankton composition of this lake has been depleted by presence of silver carp, *Hypophthalmichthys molitrix* (Valenciennes) (Lübcker 2011), and is likely to differ from that of Lake Loskop.

The Massingir dam in Mozambique was constructed between 1972 and 1977 but the lake was never allowed to fill to capacity due to seepage from the dam wall. Rehabilitation of the dam and installation of the sluices was completed in late 2006. As a result the head-waters of Lake Massingir extended into and flooded parts of the Olifants Gorge in the KNP during the subsequent summer. This has brought about a drastic alteration of the aquatic habitat in the

Gorge. The sediment-rich burden of the Olifants River is now deposited annually in the Olifants Gorge where the flow of the river has been slowed by Lake Massingir. In the KNP, the deaths of crocodiles during the winter of 2008 (Ferreira & Pienaar 2011; Huchzermeyer, Govender, Pienaar & Deacon 2011), was thought to be linked to the altered habitat of the gorge in some way. However, no coincidental fish die-off was observed. The number of crocodile deaths has declined during the subsequent winters. Crocodile deaths in the Olifants Gorge continue to be restricted to the winter months, and, as with the 2008 crocodile mortalities,SANParks veterinarians established the cause of death as pansteatitis. Autopsies performed on some of the crocodiles revealed exceptionally fat carcasses with an abnormal hardening and yellow discolouration of the fat. Histological examination confirmed an inflammation of the fat typical of pansteatitis (E. Lane and co-workers, National Zoological Gardens,Pretoria & Faculty of Veterinary Science, University of Pretoria, pers. comm. 2008). Impaired movement, an inability to swim and eventual emaciation have been observed in surviving crocodiles in the Olifants Gorge. Apparently healthy crocodiles as well as those obviously affected were observed in the same area.Bioaccumulation of a pollutant was not confirmed in the crocodiles with pansteatitis, and the cause, though probably linked to pollution and hydrodynamic change, is unknown.

During periods of flooding the Olifants River carries large loads of silt. From time to time this is exacerbated when the Phalaborwa Barrage, on the Olifants River just west of the KNP, releases water to prevent debris build-up from damaging the sluice gates and to create space to accommodate the increased flow. Downstream, occasional fish kills have resulted from the oxygen depletion in the Olifants River caused by the high silt burden in the released water. During such episodes, in February 1999 and January 2004, a large number of silver carp were identified amongst the dead fish in the Olifants River within KNP, confirming the presence of this species during these months (J. Venter, SANParks, Skukuza, pers. comm. 2012).

Mamba Weir, on the Olifants River, lies just inside the western boundary of the KNP and this section of river differs from the Olifants Gorge in that the regular scouring, when the Phalaborwa barrage is opened, removes sediment build-up from the weir. Riparian vegetation along this section of the Olifants River includes sycomore fig trees, *Ficus sycomorus* L., which are absent on the steep banks of the Olifants Gorge. sycomore figs are a favoured food source for catfish.

A large phosphate mine is situated near the town of Phalaborwa (Ba-Phalaborwa Municipality) near the western entry point of the Olifants River into the KNP. For a number of years prior to 2004, and once in 2008, abnormally high phosphate levels were recorded in the Olifants River within the KNP (J. Venter, SANParks, Skukuza, pers. comm. 2012). These were ascribed to the discharge of tailings from the phosphate mine in Phalaborwa into the Selati River, a tributary of the Olifants River, and to municipal sewerage discharges from the town of Phalaborwa. This discharge was apparently discontinued after 2004 and, except during the winter of 2008, the measurement of phosphate levels in the Olifants River downstream in the KNP has shown acceptable limits (J. Venter, SANParks, Skukuza, pers. comm. 2012). Dissolved phosphate is often the limiting nutrient governing phytoplankton growth in fresh water. The high levels of phosphate reaching Lake Massingir may have been a significant stimulus for phytoplankton growth resulting in the blooms observed in 2008 (J. Myburgh, Faculty of Veterinary Science, University of Pretoria, pers. comm. 2008).

Implication of pollutants in the development of pansteatitis has potentially serious consequences. In the KNP the disease directly threatens biodiversity and the conservation of the Nile crocodile. The Olifants River serves as a source of drinking water and edible fish for communities living along its length, and both commercial and subsistence farmers rely on the river to irrigate crops and to supply water to livestock. Communities around Lake Massingir are dependent on the substantial inland fishery that has developed in this water body, and the impact of pollution from the industrial heartland of South Africa extending into Mozambique has international implications.

1.3. Justification for the study

A study of pansteatitis in sharptooth catfish will provide information on this nutritional disease in the KNP which is important for the conservation of keystone aquatic species such as the Nile crocodile. Pansteatitis is seldom encountered in free ranging wild animals, and as fish form part of the natural crocodilian diet an association between pathology in fish, changes in the aquatic environment and development of pansteatitis in crocodiles may be shown to exist. Pansteatitis has not been described previously in African sharptooth catfish. The species is ubiquitous and widespread, occurring throughout the African continent. Significant inland fisheries depend on this species in Mozambique and in countries to the north of South Africa. In South Africa the aquaculture potential of this species has not been fully exploited, but in many African countries as well as in Europe it is an important

aquaculture species that is farmed both intensively and extensively. This study will add valuable information on the nutrition of farmed catfish.

Discovery of the relationship of mortality in catfish and crocodiles and the impact of pollution will enable authorities to take steps to better conserve aquatic ecosystems and to prevent pollution entering conservation areas. The demise of crocodiles in the KNP has raised serious concerns about biodiversity conservation downstream of heavily polluted areas in the catchments of rivers traversing the park. The close spatial proximity of the Olifants Gorge, where pansteatitis occurs in crocodiles and catfish, to the freshwater fishery in Lake Massingir raises potential health concerns for people consuming fish from this lake.

1.4. Objective

The objective of this project was to study pansteatitis in African sharptooth catfish in the Olifants Gorge, to compare this to pathology in catfish from other sites within and outside of the KNP, to try to establish the probable causes of the pansteatitis in the catfish, to establish the relationship to the crocodile mortality and to identify improved procedures for monitoring fish health in KNP.

CHAPTER TWO: LITERATURE REVIEW

2.1. Introduction

A review of pertinent literature providing insight on the current knowledge of pansteatitis in various species of animal is provided in the first section of the review. From this review it is evident that pansteatitis, a nutritional disease, is the consequence of an oxidative stress process. The underlying biochemical and molecular process leading to the clinical picture of pansteatitis is the peroxidation of lipids within the adipose tissues of the animal. In situations of oxidative stress the antioxidant defences of an animal are overwhelmed by the release of reactive oxygen species, and the subsequent chain reactions causing damage to lipids, proteins and nucleic acids can result in a variety of pathologies. Polyunsaturated fat, consumed in large amounts by animals poorly adapted to such a diet, causes considerable oxidative stress, and this can lead to the development of pansteatitis. Lipid peroxidation may also be initiated by pro-oxidants other than polyunsaturated fats and may be the common pathogenic mechanism of numerous diseases, including certain neoplasias.

The second section of the review deals with the non-enzymatic metabolic role of vitamin E as an antioxidant and its protective role in biological membranes. Vitamin E is the major chain reaction-breaking antioxidant that protects tissues from the harmful effects of lipid peroxidation and the actions of other free radicals. High polyunsaturated fat intake can lead to higher vitamin E consumption in the liver and adipose tissues, and the ensuing hypovitaminosis E leaves these and other tissues vulnerable to oxidative attack. In many species pansteatitis is inextricably linked to vitamin E deficiency, either as a manifestation of the symptomatology of primary hypovitaminosis E or secondary to oxidative stress associated with lipid peroxidation.

In the third section of the review, the biochemical processes underlying oxidative stress and lipid peroxidation are explored. The roles of xenobiotics and particularly the effect of redox-cycling as an initiator of oxidative stress are discussed in the second part of this section. In the final section of the review the use of biomarkers in monitoring exposure to oxidative stress is examined, and applications and limitations of their use in field studies is discussed.

2.2. Pansteatitis

2.2.1. Pathology

The terms steatitis, necrotising steatitis, fat cell necrosis, generalised fat necrosis, pansteatitis and yellow fat disease have been used in the literature to describe a disease in animals resulting from necrosis of fat cells in one or more tissues. The term pansteatitis defines a generalised disorder of adipose tissues characterised by degeneration and necrosis of fat cells, inflammation and fibrosis, and accumulation of ceroid and lipopigment (Danse & Verschuren 1978a; de Bruijn, Veldhuis Kroeze & Sloet van Oldenruiterborgh-Oosterbaan 2006). The term steatosis is used to describe the underlying degeneration and necrosis of fat cells, whereas the term steatitis refers to the secondary inflammation and fibrosis that follow on fat cell breakdown (Danse & Verschuren 1978a). The characteristic yellow colour of steatitis-affected adipose tissues has led to the name yellow fat disease being used to describe the condition. In cats the disease has also been described as a sterile pigmentary panniculitis (Fytianou, Koutinas, Saridomichelakis, & Koutinas 2006). For consistency, where reference is made to the work of other authors the term pansteatitis will used where the author describes the generalised disorder and the term steatitis will be used where non-generalised lesions of fat cell necrosis are described.

The necrosis and inflammation in the adipose tissues results in a hardening of the affected tissue that, depending on whether in a subcutaneous or visceral location, may be palpable in some species (de Bruijn *et al*. 2006;Niza, Vilela & Ferreira 2003). In young horses the lesions may be extensive and consist of multinodular, focally haemorrhagic hard swellings of the adipose tissues with yellow-brown discolouration and an opaque appearance (de Bruijn *et al.* 2006). The pathology of steatitis has been reported in the literature for various species of fish (Begg, Bruno & McVicar 2000; Goodwin 2006; Helder 1979; Herman & Kircheis 1985; Roberts & Agius 2008;Roberts, Richards & Bullock 1979) and in these species is similar to that of warm blooded animals. In rainbow trout, *Oncorhynchus mykiss* (Walbaum), myopathy and anaemia have been described with both pansteatitis (Roberts *et al.* 1979) and vitamin E deficiency (Cowey, Degener, Tacon, Youngson & Bell 1984). Histological lesions of steatitis include severe degeneration and necrosis of fat cells. Macrophages and syncytial giant cells surround the necrotic fat cells and are often laden with pale brown lipopigment globules (Begg*et al.* 2000; de Bruijn *et al*. 2006). Affected tissues may be variably infiltrated by other leucocytes, including neutrophils, and in advanced stages in the horse, dystrophic calcification may occur (de Bruijn *et al.* 2006). In fish, the lesions of steatitis are

characterised by an inflammatory reaction dominated by macrophages progressing to epithelioid granulation tissue with fibrous connective tissue infiltration resulting in granuloma formation (Begg *et al*. 2000; Herman & Kircheis 1985).

Lipid peroxidation induces inflammation in the following way. Oxidative deterioration of polyunsaturated lipids, through the release of free radicals, initiates a sequence of events that lead to molecular damage of subcellular membranes and eventually to cell membrane damage and necrosis (Tappel 1973). Fish fats are more susceptible to autoxidation than other polyunsaturated fats due to their high content of long-chain polyunsaturated fatty acids, particularly eicosapentaenoic acid [C20:5(n-3)] (EPA) and docosahexaenoic acid [C22:6(n-3)] (DHA) (Gonzalez, Schemmel, Dugan & Welsch 1992).The extracellular lipopigment (ceroid) released during fat cell necrosis accumulates and persists in areas of fat affected by steatitis and acts as an irritant evoking a foreign body-type inflammatory reaction. This is typified histologically by presence of Langhans giant cells, macrophages and other leukocytes (Ginn, Mansell & Rakich 2007). Danse and Verschuren (1978b) found that in steatitis-affected adipose tissues of rats, the associated damage to fat cell membranes resulted in reduced activation of lipase. The reduction in lipolysis in severely affected fat tissues renders these fat stores unavailable as a source of energy to the animal. This may explain the body weight loss and cachexia in the absence of fat atrophy associated with poor appetite seen in some species suffering from yellow fat disease.

In warm blooded animals, the disease is often painful and may lead to fever and malaise (de Bruijn *et al*. 2006; Ginn *et al.* 2007;Niza, *et al*. 2003). In cats, symptoms of fever and malaise arise after several weeks of feeding unsuitable fish-based diets (Ginn *et al.* 2007;Niza *et al*. 2003). Early cases can be treated by correcting the diet, giving oral vitamin E supplementation at 25 to 75 IU twice daily and reducing the inflammation in the adipose tissues by administering corticosteroids (White 2000). A delay in therapeutic intervention reduces the chances of success, and advanced cases may be refractory to treatment (Niza *et al*. 2003; White 2000). The prognosis in horses, where the disease mostly occurs in foals, is considered to be poor (de Bruijn *et al*. 2006). In fish, steatitis lesions have been reported as incidental finding from apparently healthy slaughter fish (Goodwin 2006). Similar findings of subclinical pansteatitis found at slaughter have been reported from American alligators,*Alligator mississippiensis*(Daudin) (Larsen, Buergelt, Cardeilhac&Jacobson 1983).

2.2.2. Aetiology

The pathogenesis of pansteatitis is closely linked to vitamin E intake and consumption of diets rich in polyunsaturated fats. The aetiological cause is a deficiency in the non-enzymatic role of vitamin E as an antioxidant (Danse & Verschuren 1978a; White 2000). This may be caused either by a diet containing insufficient vitamin E or by a relative deficiency of vitamin E brought about by consumption of polyunsaturated fats that destroy the vitamin E (White 2000). The high degree of polyunsaturation of lipids in fish make these particularly prone to oxidative deterioration (Baker & Davies 1996a), and feeding certain fish diets rich in polyunsaturated fatty acids, or rancid fish oils with low vitamin E content, has been implicated in many cases of pansteatitis in both warm and cold blooded animals (Davis & Gorham 1954; Roberts & Rodger 2001; Wallach & Hoessle 1968, White 2000). The concentration of endogenous vitamin E in stored fish oil, known to be rich in polyunsaturated fatty acids, was found to be inversely proportional to the degree of oxidation of the oil (Hung, Cho & Slinger 1980). Polyunsaturated fatty acids are particularly prone to oxidation and consume high levels of vitamin E to contain the harmful effects of lipid peroxidation, accounting for significant depletion of plasma vitamin E associated with consumption of rancid fish diets (Baker & Davies 1996b).

Pansteatitis has been associated with anaemia and myopathy, symptoms typical of vitamin E deficiency. In fish, the anaemia and myopathy appear to be linked to the concurrent hypovitaminosis E that often manifests with pansteatitis. As vitamin E protects against lipid peroxidation, tissue vitamin E levels tend to, within limits, increase with heightened unsaturated fat intake (Raynard, McVicar, Bell, Youngson, Knox& Fraser 1991). In fish feeding on high levels of polyunsaturated fat, however, the tissue vitamin E levels become exhausted within a relatively short period. Vitamin E levels in rainbow trout liver were found to be inversely proportional to dietary level of lipid unsaturation, showing a higher consumption of vitamin E associated with unsaturated lipid intake, and feeding of such diets may induce apparent vitamin E deficiency symptoms (Watanabe, Takeuchi, Wada&Uehara 1981). Under conditions of dietary oxidant overload, depletion of hepatic vitamin E has also been shown to occur in sharptooth catfish (Baker & Davies 1997a). Furthermore, consumption of rancid oils in the diet may hinder intestinal absorption of vitamin E, and oxidation of vitamin E in the diet prior to ingestion can contribute to suppressed vitamin E intake (Baker &Davies 1996b). Where vitamin E intake is insufficient to provide adequate

protection against the peroxidation of dietary unsaturated or rancid fats, necrosis and inflammation of the fatty tissues ensues giving rise to the clinical picture of pansteatitis.

The close relationship between the oxidation of polyunsaturated lipids and vitamin E deficiency explains the overlap in the symptomatology of pansteatitis and hypovitaminosis E seen in some species (Ginn *et al.* 2007; Post 1993; van Vleet&Valentine 2007). Reduced growth, anaemia, exudative diathesis (ascites, exophthalmos), myopathy, dermal depigmentation, fatty livers with yellow to orange colouration and pancreatic atrophy (Murai & Andrews 1974; Post 1993; Poston, Combs & Leibovitz 1976; Stewart 1993) are typical of vitamin E deficiency in fish, and myopathy and exudative diathesis have also been described with selenium deficiency (Gallagher 1993). Similar changes have been described in some cases of pansteatitis in fish (Roberts *et al*. 1979). However, these changes have not been described in all cases of pansteatitis reported in the literature, and difference in degree of hypovitaminosis E and selenium uptake may explain some of the variation in symptoms and pathology in affected tissues. Other poorly elucidated factors may play a role in the initiation of lesions, and species and tissue dependent sensitivity may be important for the pathogenesis (Danse & Verschuren1978a). Also, difference in fatty acid composition between various fat depots may account for differences in distribution of lesions in various species (Danse & Verschuren1978a, de Bruijn *et al*. 2006). Pansteatitis has been extensively studied in rats fed a vitamin E deficient diet supplemented with fish oil by Danse & Verschuren (1978a & b), and these authors suggest that the adipogenic and metabolic characteristics of fat depots may be important determinants for the progression of degenerative changes seen with the disease and may explain species differences in the locations of steatitis within the body. In horses and swine, linolenic acid [18:3(n-3)] is the most important polyunsaturated fatty acid in the fat depots, whereas in other species EPA [20:5(n-3)] and DHA [22:6(n-3)] may be important too, and differences in deposition and metabolic rates of various fat depots may influence the development of steatitis at different sites (Danse & Verschuren 1978a).

Goodwin (2006) described steatitis at the fin bases of channel catfish,*Ictalurus punctatus* (Rafinesque),leading to ulceration and fin loss with few changes elsewhere. In a similar case described in Atlantic halibut, *Hippoglossus hippoglossus* L., Bricknell, Bruno, Bowden and Smith (1996) found fat cell necrosis restricted to the sub-dermal fat deposits around the fin bases of the fish. As the incidence of lesions was restricted to the dorsal surface of the fish the authors postulated an imbalance of dietary oxidants resulting in membrane damage that

may have been aggravated by exposure to sunlight. The fin bases or pterygiophorial regions were similarly implicated in a case of steatitis described in wild common dab, *Limanda limanda* (L.), by Begg *et al.* (2000); however, in this case only the ventral regions of the fish were affected. The authors describe similar lesions in another free-living fish, the long rough dab, *Hippoglossoides platessoides* (Fabricius). The authors point out that there is little overlap in diet between these two species,and this suggests a non-dietary, possibly pollution related cause.

Fish diets have been widely implicated as a cause of pansteatitis in many species of animal. The disorder is known to occur in mink and swine when fed large amounts of fish meal or fish waste that result in a relative vitamin E deficiency, with symptoms appearing approximately one month after commencement of such a diet (Smith *et al*. 1972). In birds the disease has been described in captive fledgling boat billed herons, *Cochlearius cochlearius* (Linnaeus), as a consequence of hypovitaminosis E associated with a frozen fish diet(Pollock, Sleeman, Houle & Ramsay 1999). Vitamin E supplementation applied to the outside of the food fish was suspected to have been washed off the fish by the parent birds before they fed the fish to their young. The disease has frequently been described in the domestic cat, when fed unsuitable fish-based home-made diets (Fytianou *et al*. 2006; Niza*et al*. 2003). Marine fish are rich in polyunsaturated lipids. In herring the lipids are approximately 80% unsaturated compared with only 50% in beef fat (Geraci & St. Aubin 1980). The rate of lipid peroxidation in fish flesh is increased by light, heat, heavy metals and by the iron-rich haematin compounds that are abundant in dark-fleshed fishes (Geraci & St. Aubin 1980). In cats the disease is particularly associated with feeding of red fish, such as tuna, and diets containing cod liver oil (White 2000; van den Broek & Thoday 1994).

Pansteatitis has also been reported in various species of cultured fish fed diets containing relatively high levels of polyunsaturated fats. Fats of fish origin, particularly in the absence of sufficient vitamin E, have most commonly been implicated (Goodwin 2006; Herman & Kircheis 1985; Roberts & Agius 2008; Roberts*et al.* 1979). Under culture conditions, pansteatitis has been described in channel catfish (Goodwin 2006); guppy, *Poecilia reticulata* Peters (Helder 1979); rainbow trout (Roberts *et al*. 1979); Sunapee trout, *Salvelinus alpinusoquassa* Girard (Herman & Kircheis 1985); striped jack, *Caranx vinctus* Jordan & Gilbert (Wada, Hatai & Kubota 1991); Atlantic halibut (Bricknell *et al.*1996); white sturgeon,

Acipenser transmontanus Richardson (Guarda, Bertoja, Zoccarato, Tartari & Biolatti 1997) and northern bluefin tuna, *Thunnus thynnus* L. (Roberts & Agius 2008).

Captive reptiles fed fish diets are known to develop pansteatitis, and the disease has been described in snakes (Langham, Zydeck & Bennett 1971). In crocodilians, pansteatitis has been associated with consumption of large numbers of dead and rancid fish following large scale fish mortality (Huchzermeyer 2003; Ladds, Mangunwirjo, Sebayang & Daniels 1995) and with a change in type of fish fed (Wallach and Hoessle 1968). Pansteatitis was found as an incidental finding at slaughter in captive American alligators fed over a period of 14 years on predominantly freshwater fish in the form of whole fish, fish heads, skins and entrails (Larsen*et al.* 1983), whereas a captive South American caiman, *Caiman crocodilus* (L.), died acutely from pansteatitis following a 3 week period of anorexia after being fed a similar diet (Frye & Schelling 1973).

Reports of pansteatitis in free-ranging wild animals are rare. Pansteatitis has been reported in a wild juvenile red-tailed hawk, *Buteo jamaicensis* (Gmelin), normally a non-piscivorous bird (Wong, Mikaelian, Desnoyers & Fitzgerald 1999), in great blue herons, *Ardea herodias* (Mitchell) (Nichols, Campbell & Montali 1986), and in wild egrets and herons from a reservoir in Japan (Neagari, Arii, Udagawa, Onuma, Odaya, Kawasaki, Tenpaku, Hayama, Harada, Mizukami & Murata 2011), where the cause of the disease was unclear. In wild rabbits, pansteatitis has been described where these animals lived in close association with sea birds and are suspected to have eaten regurgitated fish at times when grazing was sparse (Jones, Howard & Gresham 1969). In wild fish, steatitis has been described from common dab and long rough dab (Begg *et al*. 2000). In Lake Loskop, acid mine seepage was found to be the most likely cause of a mass fish mortality that in 2007 lead to the deaths of significant numbers of crocodiles and terrapins as a result of pansteatitis (J. Myburgh and co-workers, Faculty of Veterinary Science, University of Pretoria, pers. comm. 2009). The 2008 episode of crocodile pansteatitis in the KNP differed in that no mass mortality of fish was observed in the affected region. Circumstantial evidence pointed to illegal fishing activity with gill nets as a possible source of dead, rancid fish.

An infectious cause of pansteatitis has seldom been implicated, although steatitis may develop secondary to other disease processes. An unidentified coccidian-like protozoon has been associated with steatitis in a red kangaroo, *Macropus rufus*Desmarest (Dubey &Hartley

1992). In captive marmosets (*Callithrix* spp.), pansteatitis, anaemia and myopathy were thought to be associated with vitamin E deficiency that may have been compounded by presence of chronic colitis (Juan-Salles, Prats, Resendes, Domingo, Hilton, Ruiz, Garner, Valls & Marco 2003). The lipotropic property of certain coxsackie viruses has been demonstrated in mice, and the resulting steatitis was comparable to certain forms of panniculitis in man (Goodman, Bunting & Melnick 1951). In man, the expression of coxsackie virus B-induced myocarditis has been associated with selenium deficiency and other nutritional factors, including polyunsaturated fat and vitamin E intake (Levander & Beck 1997). Steatitis in association with *Streptococcus iniae* infection has been reported in the Amazon River dolphin, *Inia geoffrensis*(Blainville), (Bonar & Wagner 2003) and under mariculture conditions in silver perch, *Bidyanus bidyanus*(Mitchell), (Deng, Peng, Chen, Chen & Chang 2012). In the latter case steatitis was accompanied by severe lipidosis in the affected fish, and a dietary cause was suggested. A congenital cause has been proposed to explain the occurrence of some cases of pansteatitis in foals. Seasonal influences may account for low vitamin E transfer from dam to foal, and oxidation of adipose tissues in the foal might be initiated by infectious agents (de Bruijn *et al*. 2006).

2.3. Vitamin E

Under normal metabolic conditions oxyradical production is balanced by cellular antioxidant capacities (Burton 1994). The non-enzymatic role of the fat-soluble vitamin E, and its molecular function as an antioxidant, is well documented, and it is known that Vitamin E plays an important role in nature as an *in vivo* antioxidant preventing the oxidative conversion of polyunsaturated fats into lipid hydroperoxides (Burton 1994; Bus, Aust & Gibson 1976; Hove 1955; Stewart 1993). Lipid peroxides are held responsible for the multiple changes observed in animals deprived of vitamin E (Hove 1955). Several exogenous substances have been reported in the literature to promote the peroxidation of fats, including consumption of unsaturated fat (Burton 1994; Demopoulos 1973). These reactions can be mitigated by the presence of adequate dietary vitamin E (Baker *et al*. 1997; Bell, Cowey, Adron & Shanks 1985; Cowey *et al*. 1984; Fytianou *et al*. 2006; Moccia, Hung, Slinger & Ferguson 1984; Murai & Andrews 1974; Post 1993; Smith 1979; Watanabe *et al*. 1981).

Vitamin E is the generic name used to describe the four tocopherols and four tocotrienols that make up this group of lipid soluble substances (Burton 1994; Kumar, Cotran & Robbins 1997). Of these, α-tocopherol is the most biologically active (Burton 1994; Puertollano,

Puertollano, Álvarez de Cienfuegos & de Pablo 2011; Stewart 1993). For reasons of consistency the term vitamin E will be used to refer to α-tocopherol in this thesis. Vitamin E is absorbed from the diet via the small intestine and is taken up by the lymph in chylomicrons derived from the intestine (Burton 1994). As with other fat soluble vitamins this requires normal biliary and pancreatic function (Kumar *et al.* 1997). In the circulation, lipoprotein lipase bound to the vascular endothelium rapidly catabolises chylomicrons to free fatty acids and monoacylglycerols allowing the transfer of fatty acids and vitamin E to tissues (Burton 1994). At the same time chylomicron components and vitamin E are transferred to plasma, where vitamin E rapidly equilibrates with low density lipoproteinin the plasma (Burton 1994; Kumar *et al.* 1997). From the liver vitamin E is secreted into plasma in triacylglycerol-rich, very low density lipoproteins. The hydrolysis of these lipoproteins by lipoprotein lipase delivers fatty acids and vitamin E to tissues (Burton 1994). In contrast to vitamin A, which is primarily stored in the liver, vitamin E is stored throughout the body, mainly in adipose tissue and to a lesser extent in liver and muscle (Baker & Davies 1997a; Kumar *et al*. 1997).

Vitamin E is the main chain-breaking lipid soluble antioxidant that reduces damage to lipid membranes caused by reactive oxygen species (ROS) (Puertollano *et al.* 2011). In rats fed a vitamin E deficient diet, lipid peroxidation in tissues was enhanced by concurrent selenium deficiency (Awad, Morrow, Hill, Roberts & Burk 1994). In fish, as in mammals, glutathione peroxidase, an enzyme that recycles glutathione and removes further ROS to balance the redox state of the cell, is selenium dependant (Bell *et al.* 1985). Apart from its antioxidant role, vitamin E is involved in a number of other metabolic functions, including cell membrane integrity, enzyme and heme synthesis, and steroidogenesis (Geraci & St. Aubin 1980).

Dependency on lipoprotein polyunsaturated fats for normal metabolic function is more pronounced in poikilothermic (cold blooded) animals than in homothermic (warm blooded) animals (Agius & Agbede 1984; Hazel 1979; Hulbert 2003). The elongated and desaturated derivatives of linoleic acid (n-6) and α-linolenic acid (n-3) are essential fatty acids that affect the fluidity, flexibility and permeability of membranes (Steffens 1997). Poikilothermic animals such as fish and crocodiles depend on dietary polyunsaturated fats to maintain this membrane fluidity and normal metabolic function, especially at colder ambient temperatures, and are thus particularly sensitive to the effects of lipid autoxidation (Hazel 1979; Hulbert 2003). In trout this is expressed by a greater susceptibility to vitamin E deficiency at low

water temperatures (Cowey *et al*. 1984). Stabilising lipids is an important *in vivo* function of vitamin E thatextends beyond the life of the animal and is important in the storage stability of meat products. The risk of post-mortal oxidative lipid breakdown (rancidity) is magnified in fish as a result of the high polyunsaturated fatty acid content of fish flesh. In many parts of Africa, lack of cooling facilities and poor sanitary conditions lead to rapid deterioration of harvested fish. In freshwater fisheries, such as Lake Massingir, smoked fish are a popular product and are often kept for several weeks and shipped long distances before being consumed. Where vitamin E levels in the tissues are already low as a result of *in vivo* radical-mediated damage, there is a potentially increased risk of radical attack on human tissues after consumption of such fish products (Baker & Davies 1996a).

2.4. Oxidative Stress and *in vivo* Lipid Peroxidation

2.4.1. Free radical attack

Free radical reactions are common in normal cellular functions, but uncontrolled free radical reactions may be the initial molecular events resulting in various pathological processes (Demopoulos 1973). Lipid peroxidation is common to oxidative stress reactions, whether dietary or xenobiotic in nature. Although polyunsaturated fats are the most abundant and vulnerable target of free radical attack, structural and functional damage also occurs to carbohydrate, protein and DNA containing structures leading to various pathologies including neoplasia (Burton 1994; Porter 1989).Autoxidation, the spontaneous reaction of molecular oxygen with radicals, is held responsible for numerous deteriorative processes, including rancidity, that develop in foodstuffs (Porter 1989). The propensity of autoxidation to initiate chain reactionscan greatly amplify damage to lipids. This can lead to the sometimes far reaching consequences of free radical exposure (Burton 1994; Porter 1989).

Free radicals are chemical species that have a lone, unpaired electron in an outer orbit, a state that can exist in diverse compounds and that initiates relatively nonspecific hydrogen abstraction and chemical addition reactions (Demopoulos 1973; Tappel 1973). In unsaturated fatty acids, the presence of a double bond weakens the carbon-hydrogen bond of the carbon atom adjacent to a carbon with an unsaturated bond, making these particularly prone to peroxidation (Demopoulos 1973).With increasing number of double bonds in a fatty acid, the susceptibility to radical reactions is increased. Polyunsaturated fats are the most vulnerable and most abundant target of free radical reactions, the damage being caused either by free radicals themselves or by lipid peroxidation products such as aldehydes (Burton, 1994).

Peroxidation of polyunsaturated lipids results directly from the oxidative effects of oxygen, but the reaction needs to be initiated in some way to overcome the dissociation energy of an allylic bond in the polyunsaturated fatty acid (Burton 1994; Minotti & Aust 1992).

Lipid peroxidation can be initiated by redox cycling of various transition metals. Iron, an abundant metal in the body and needed for oxygen transportation, has been shown to be a potent initiator of lipid peroxidation (Demopoulos 1973; Kibanova, Nieto-Camacho & Cervini-Silva 2009; Minotti & Aust 1992). Unsaturated fatty acids make up an integral part of the plasma membranes of cells and of the membranes of organelles. The unsaturated bonds of fatty acids are situated in the nonpolar hydrophobic mid-zone of cell membranes where they are protected from oxidative damage (Bus *et al.* 1976; Demopoulos 1973; Minotti & Aust 1992). The rancidity of fats exposed to air follows a radical reaction initiated and catalysed by metals such as iron and copper, particularly if in complexes, such as heme, as these are more soluble in the nonpolar environment of the mid-zone of membranes (Demopoulos 1973). As the allylic carbon bonds of unsaturated fatty acids are partially activated, small amounts of pro-oxidants will initiate complete activation, resulting in abstraction of an allylic hydrogen. This sets in motion the series of structurally significant chain reactionstermed lipid peroxidation and exerts a profound effect on membrane structure and function (Demopoulos 1973;Kelly, Havrilla, Brady, Abramo & Levin 1998;Minotti & Aust 1992). Lipid free radicals released from decomposition of lipid hydroperoxides initiate the subsequent lipid peroxidation of cell membranes with far reaching consequences (Bus *et al.* 1976).

Pro-oxidant or oxyradical production in aquatic organisms has been linked to anthropogenic activity resulting in pollution of the aquatic environment (Bainy, Saito, Carvalho & Janqueira 1996; Winston 1991; Winston & Di Giulio 1991). A link between pollution-induced *in vivo* lipid autoxidation in fish and the subsequent development of pansteatitis in crocodiles and fish has not previously been elucidated.

2.4.2. Xenobiotics as pro-oxidants

Oxidative stress is an important component of toxicology, and increasing evidence suggests that it is also of ecological significance, particularly where pollutants are carried in the aquatic environment (Kelly *et al.* 1998). The *in vivo* reduction in tissue vitamin E brought about by the inherent instability of polyunsaturated fatty acids may increase expression of

other xenobiotic oxidants where dietary polyunsaturated fat intake is high. Oberholster *et al.* (2011) working in Lake Loskop, a polluted water body in the upper Olifants River catchment, have documented the bioaccumulation of iron and aluminium by phytoplankton species in this lake. Raised levels of both aluminium and iron in the fat of Mozambique tilapia from some sites in Lake Loskop were thought to derive from ingestion of phytoplankton species, and may have been responsible for the yellow discolouration of the fat of these fish. Redox cycling of iron is known to be an initiator of lipid peroxidation (Baker*et al.*1997; Demopoulos 1973; Di Giulio, Washburn, Wenning, Winston & Jewell 1989; Kibanova *et al.* 2009; Minotti & Aust 1992; Tappel 1973) and depletion of tissue vitamin E levels by high dietary iron intake may render polyunsaturated fats in the tissues of the fish vulnerable to peroxidation (Baker *et al.* 1997).

The acquisition of iron by teleost fishes has been reviewed by Bury and Grosell (2003). Although iron is one of the most abundant metals on earth, biologically unavailable colloidal hydrous iron oxides predominate in naturally oxygenated freshwater environments with near neutral pH. When these complex with organic matter in freshwater environments they settle out, and in the anoxic zone of sediments the ferric iron (Fe^{3+}) reduces to ferrous iron (Fe^{2+}), which may leach back into the water. In freshwater fish, iron is readily taken up by the gill and intestinal epithelia in the ferrous state, and, although iron may be assimilated from both the water and the diet, uptake from the aquatic environment forms a considerable part of the iron homeostasis of freshwater fish. To regulate cellular iron concentrations the intracellular protein ferritin takes up ferrous iron and oxidises this to ferric iron which is incorporated into the ferritin molecule (Bury & Grosell 2003). The lipid peroxidation potential of iron is countered by the strong iron-binding properties of lactoferrin and transferrin at physiological degrees of iron saturation (Gutteridge, Paterson, Segal & Halliwell 1981). There is no known regulatory excretory pathway for excretion of iron. Homeostasis is regulated by absorption from the diet, although some iron may be lost through breakdown of haemoglobin and excretion through bile and by sloughing of intestinal epithelium (Bury & Grosell 2003). Baker and Davies (1997b) have proposed that transient lipid damage instigated by elevated dietary intake of iron in sharptooth catfish may be responsible for lowering hepatic vitamin E levels.

A number of other vitamin E antagonists involved in catalytic peroxidation of unsaturated fats are known for their pro-oxidant effect. Hove (1955) described the vitamin E antagonistic

effects of cod liver oil; tri-o-cresyl phosphate (TOCP), a plasticizer, solvent and gasoline additive; carbon tetrachloride (CCL_4); pyridine; some sulphonamides; and sodium sulphite, although the biochemical action of these poisons may be more complex. Tri-o-cresyl phosphate in the diet of lambs was shown by Draper, James and Johnson (1952) to induce a disease identical to "stiff lamb disease"; the symptoms could be prevented by administration of vitamin E. In silver poisoning, Diplock, Green, Bunyan, Mchale and Muthy (1967) showed that, in the rat, the dietary stress of silver on vitamin E function was independent of peroxidation of unsaturated fat and that the anti-vitamin E effect of silver was not a pro-oxidant effect. On the other hand iron, was demonstrated by Baker *et al.* (1997) to increase fatty acid peroxidation in hepatic and cardiac tissue in sharptooth catfish consuming sub-lethal levels of dietary iron sulphate (ferrous iron).

Xenobiotics capable of redox cycling are particularly relevant to toxicology (Kelly *et al*., 1998). Those capable of redox cycling include: quinones (menadione, pyrene quinone, adriamycin and mitomycin C), bipyridyl herbicides (paraquat, diquat and 1,10-phenanthroline), aromatic nitro compounds (nitrofurantoin, nitropyrene and misonidazole), aromatic hydroxylamines, some dyes, some transition metals, and metal chelates (Fe-EDTA and Cu-bleomycin) (Di Giulio *et al.* 1989; Kelly*et al*. 1998). Other chemicals that are either free radicals or hydrophobic may induce free radical reactions in the presence of unsaturated fatty acids and include the alkyl polyhalides (CCL_4, DDT, halothane), alcohols, and the chemical carcinogens 4-amino biphenyl and dimethyl 4-amino biphenyl (Demopoulos 1973). It has been proposed that the oxidative tissue damage, reflected in hepatic lipid peroxidation and DNA damage resulting from exposure to the polyhalogenated hydrocarbons, lindane, DDT, chlordane and endrin may contribute to the toxic manifestations of these xenobiotics (Hassoun, Bagchi, Bagchi & Stohs 1993). In mice and rats exposed to dietary dieldrin, a reduction in hepatic and serum vitamin E level was observed, suggesting oxidative challenge (Bachowski, Xu, Stevenson, Walborg & Klaunig 1998).Pansteatitis has, however, not been reported with exposure to these xenobiotics.

The herbicide, paraquat, has been used as an oxidant model causing lipid peroxidation of cell membranes in both humans and animals (Åkerman, Amcoff, Tjärnlund, Fogelberg, Torrissen & Balk 2002; Bus *et al.* 1976; Parvez and Raisuddin 2005). The cyclical reduction-oxidation of paraquat results in generation of superoxide radicals which dismutate to singlet oxygen, a free radical species that has a lone, unpaired electron. Singlet oxygen reacts with unsaturated

lipids in cell membranes to form lipid hydroperoxides. The chain reaction leading to the membrane destructive process of lipid peroxidation results from the spontaneous decomposition of lipid hydroperoxides to lipid free radicals (Bus*et al.*1976) (Figure 2.1). Imbalances in generation and removal of radical species result in oxidative stress that has the potential to cause biological injury (Kelly *et al*. 1998). Basic biological processes such as cellular respiration and the action of certain enzymes lead to production of reactive oxygen species within the body. Certain xenobiotics have the ability to enhance the cellular production of such oxyradicals within cells (Kelly *et al*. 1998).

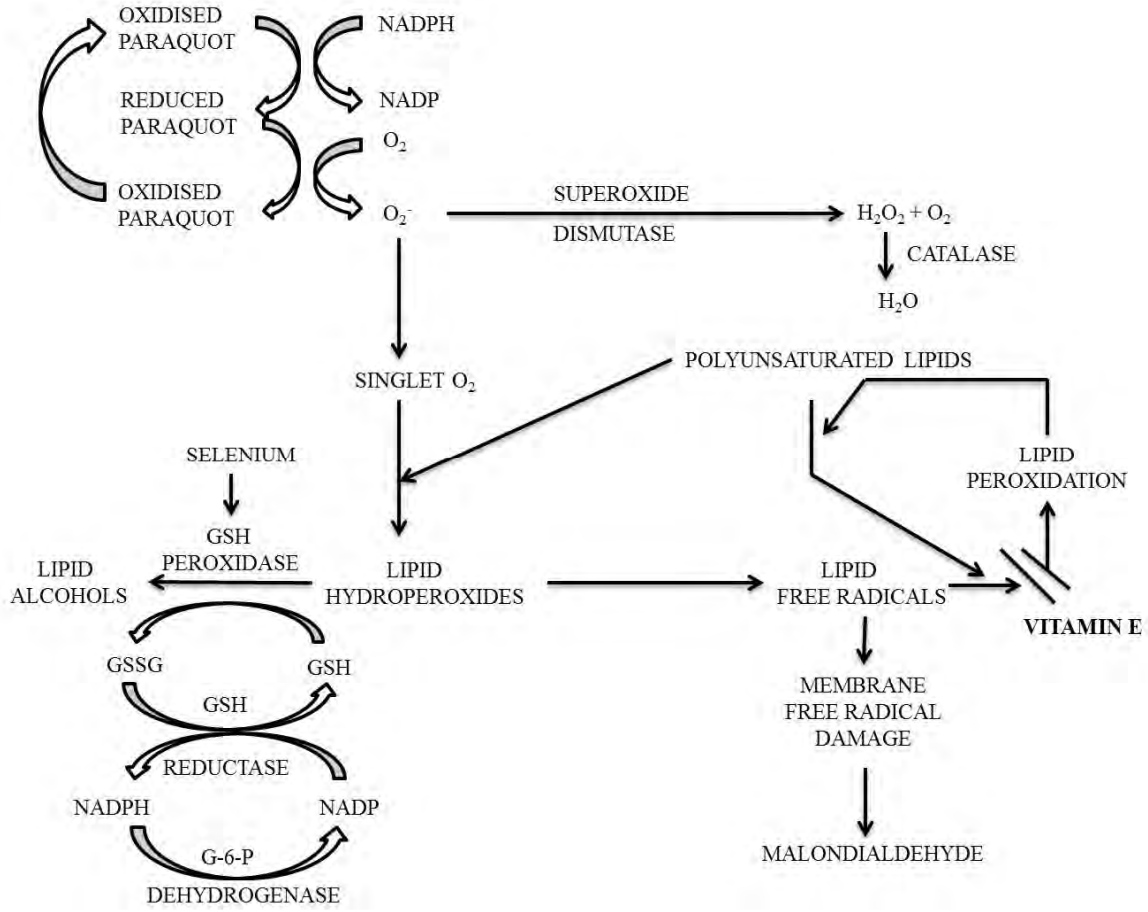

Figure 2.1: Schematic presentation of the effect of redox cycling of a pro-oxidant on polyunsaturated lipids. Proposed mechanism of paraquat toxicity (from Bus *et al*. 1976) [GSH=glutathione].

Mammalian organisms use three defence mechanisms to counter the destructive process caused by oxidative stress. Superoxide dismutase scavenges toxic superoxide radicals, endogenous antioxidants such as vitamin E terminate the free radical chain reaction of lipid

peroxidation and glutathione peroxidase enzymatically reduces the unstable lipid hydroperoxides to stable lipid alcohols, thus preventing further formation of free radicals (Bus *et al*. 1976). Vitamin E acts as hydrogen atom donor thereby preventing the reactive lipid peroxyl radicals from abstracting hydrogen atoms from *in vivo* sources such as DNA and proteins (Kelly*et al.* 1998). With deficiency of the antioxidants selenium, vitamin E and glutathione the toxicity of xenobiotics such as paraquat is significantly increased (Bus *et al.* 1976).

2.5. Bio-monitoring

Histopathological tissue changes in fish have been proposed as a sensitive tool for assessing exposure to pollution (Adams, Brown & Goede 1993;Bernet, Schmidt, Meier, Burkhardt-Holm & Wahli 1999; Heath, du Preez, Genthe & Avenant-Oldewage 2004; Roberts & Agius 2003), and the role of fish in sediment toxicity assessments has been reviewed by Halare, Seiler and Hollert (2011). These authors stress the importance of benthic rather than pelagic fish species in such studies. Histology has been used to monitor the health status of tigerfish, *Hydrocynus vittatus* Castelnau, lowveld large scale yellowfish, *Labeobarbus marequensis* (Smith), rednose labeo, *Labeo rosae* Steindachner and redeye labeo, *L. cylindricus* Peters, fish representing different trophic levels in the Olifants River in the KNP, and showed that these species were in a healthy state (Wagenaar, Smith & Smit 2012a; Wagenaar, Smith & Smit 2012b).

The cellular responses to oxidative stress in fish are similar to those of mammals. These include activation of low molecular weight radical scavengers such as glutathione (GSH), vitamin E and ascorbic acid as well as the antioxidant enzymes, superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) (Kelly *et al*. 1998, Winston 1991). Increased activity of these enzymes has been associated with exposure to redox-cycling compounds (Di Giulio *et al.* 1989). As in mammals, glutathione peroxidase activity in salmonids has been shown to be dependent on selenium (Bell *et al.*1985; Poston *et al.* 1976). These endogenous antioxidant defence mechanisms and related biomarkers have been used in mammalian and fish tissues to measure the effects of pro-oxidant exposure (Awad *et al.* 1994; Parvez & Raisuddin 2006). Amongst mammals, cats are known to be particularly sensitive to the pro-oxidant effect of ingested polyunsaturated fats. Measurable changes in the levels of vitamin E and glutathione peroxidase have been demonstrated in the blood of kittens exposed to experimental diets rich in polyunsaturated fats(Fytianou*et al.*2006).

A prerequisite for use of biomarkers is that the response should be a selective, primary response detectable in the living organism before the onset of more serious pathological consequences, but not all biochemical responses meet these criteria (Di Giulio *et al.* 1989). Interpretation of such changes in *in vivo* field studies may not be straightforward. The influence of variables that are difficult to control such as age, physiological and reproductive state, season and water quality, affecting both the organism and toxicant bio-availability, can influence the quantifiable endpoint of bio-monitoring (Di Giulio *et al.* 1989). Depending on the type of oxidative stressor, the responses of antioxidant enzyme activity may be variable; they may be elevated as a result of induction or suppressed as a toxic reaction to a stressor, or with exposure to multiple stressors both responses may occur simultaneously resulting in no net difference (Di Giulio *et al.* 1989). Oxidative stresshas been shown, particularly in cases of toxicant-induced inflammation, to lead to up-regulation of antioxidant enzymes (Kelly *et al.* 1998). In experimental situations the harmful influences of pro-oxidants have been demonstrated by negating these effects by antioxidant supplementation, particularly with vitamin E (Awad *et al*. 1994; Baker & Davies 1996a; Baker & Davies1996b;Bell *et al*. 1985; Cowey *et al*. 1984).

The thiobarbituric acid reactive substances (TBARS) test is a commonly used assay to measure malondialdehyde (MDA), one of the end products of lipid peroxidation, in plasma (Kelly *et al*. 1998, Hinchcliff & Piercy 2000). However, as thiobarbituric acid reacts with a number of other oxidation products, the test is non-specific (Kelly *et al*. 1998). Malondialdehyde is also rapidly metabolised and may not always be a reliable indicator of lipid peroxidation *in vivo* (Bus *et al.* 1976), and significant levels above the basal level must be present for accurate assessment of *in vivo* lipid peroxidation (Di Giulio *et al*. 1989). It has been suggested that ethane and pentane, measurable β-scission products of n-3 and n-6 fatty acids in the breath of small laboratory animals, might also be adapted to aquatic animals as a sensitive test of *in vivo* lipid peroxidation (Di Giulio *et al*. 1989).

Free radicals may also play a role in chemical carcinogenesis and induction of the mixed-function oxygenases (MFO), and MFO responses have been used as biomarkers for chemical exposure, particularly to hydrocarbon pollutants (Di Giulio *et al*. 1989). The effect of oxyradical damage on DNA from environmental genotoxicants such as polycyclic aromatic hydrocarbons, pesticides and metals has led to the development of several methods for

detecting DNA damage. Lee and Steinert (2003) have reviewed the use of single cell electrophoresis or the comet analysis for detecting DNA damage in marine and freshwater aquatic animals and suggest that this test has several advantages over other methods of determining DNA damage.

CHAPTER THREE: FIELD STUDY

3.1. Materials and Method

3.1.1. Introduction to the fieldwork

The focus of the fieldwork was on the sharptooth catfish, as this species was thought to be a major food source for crocodiles along the Olifants River gorge and lower Letaba River. Being an omnivorous benthic scavenger it is likely that this species would be preying on other weakened fish species in the system. Samples were collected and processed over a 2 year period between November 2009 and November 2011. Fish surveys were done by the author as early as August 2008 and, where relevant, data from fish sampled between August 2008 and November 2009, although not part of the structured study, have been included in the results. Collection of fish samples from the Olifants Gorge took place every six months.Winter samplings were done between June and September when river flow had subsided, most of the rainfall-related sediment load had been dropped and water temperatures had reached their winter minimum. Summer sampling was done between November and March when river flow and sediment load were high. Up to 25 fish were collected during each sampling episode in the Olifants Gorge. It was not always possible to catch as many fish as this from the reference sites. Fish were caught by baited hook and line and by netting. A range of fish species, including Mozambique tilapia, were included in samplings prior to November 2009. In September 2008 and June 2011, tigerfish were also sampled in the Olifants Gorge from the confluence of the Olifants and Letaba rivers.

3.1.2. Description of the study area

Samplings took place from the confluence of the Olifants and Letaba rivers (23°59′21.8″S 31°49′35.6″E), where the Olifants River enters a 9 km long gorge through the Lebombo Mountains, to where it enters Lake Massingir on the Mozambique border (23°57′48″S 31°52′97″E) (Figure 3.2). Catfish specimens were also collected from various other localities within and outside the KNP. These included a negative reference population in Reënvoël Dam (23°58′37.2″S 31°19′38.4″E) that has its entire catchment within the KNP, and a wild population in van Ryssen Dam (24°00′13.6″S 31°05′36.9″E) at the FOSKOR phosphate mine near the town of Phalaborwa (Ba-Phalaborwa Municipality) just west of the KNP. Upstream of the gorge fish were sampled at Mamba Weir (24°03′32″S 31°14′14″E), where the Olifants River enters the western boundary of the KNP. On the Letaba River fish were sampled from Engelhard Dam (23°50′19″S 31°28’28″E). Further south in the KNP samplings took place from the Sabiepoort (25°10'25.41"S 32°02'23.42"E), where the Sabie River enters Lake Corumana on the Mozambique border and from the Crocodile River (25°23′57.1″S 31°57′29.9″E) on the southern boundary of the Park. In the north of the KNP, fish were sampled from the Levuvhu River (22°25′51.0″S 31°18′04.4″E). Catfish were also sampled

from a farmed population at Lunsklip Fisheries (25°23′08.9″S 30°15′35″E) near Lydenburg (Thaba Chweu Municipality), Mpumalanga. These fish were fed almost exclusively an excess of trout slaughterhouse waste, rich in polyunsaturated fat. Slaughterhouse waste, consisting largely of fat rich innards, was dumped into the catfish pond where it was left to be consumed by the fish. Trout farmed at Lunsklip fisheries were fed a commercial trout ration which was top-dressed with additional marine fish oil.

3.1.3. Specimen collection

To minimise the influence of autolytic change on the gross and microscopic appearance of the organs and tissues of the sampled fish, all sampled fish were transported live to the relevant examination facilities and were kept alive until they could be examined. Setting up a field laboratory near the site of sampling was not always possible due to the logistical and safety considerations of working in the field in the KNP. Sharptooth catfish are air-breathing fish and can be transported live over relatively long distances without adverse effects. A portable holding tank (Minurphy Tarpaulins, Pietermaritzburg) was filled with river water at each field laboratory site. To minimise stress, the fish were released into the holding tank as soon as was practical after being caught and were kept there until they could be examined. A maximum of 20 fish could be examined in one day, but the number was dependent on the catch success of the fishermen. Up to 28 fish were collected on each sampling occasion. Two 250 L fish transport tanks belonging to SANParks were used to transport the fish. The catfish were either processed at the sampling site or transported live in fish transport tanks to various laboratory facilities.

All fish sampled prior to November 2009 were examined and dissected in field laboratories set up near the sampling sites along the river.Catfish caught in the Olifants Gorge and in Reënvoël Dam in November 2009 were transported live to Lydenburg and examined and dissected in the author's facility. In July 2010 the post-mortem facility, belonging to SANParks Scientific Services, in Skukuza was used and fish from the Olifants Gorge, Mamba Weir, Engelhard Dam and the Sabiepoort were transported live to Skukuza. In January 2011, a field laboratory was set up first near the confluence of the Olifants and Letaba Rivers and then at Reënvoël Dam, and fish were examined and dissected on site. Fish from van Ryssen Dam were brought live to the field laboratory at Reënvoël Dam. For the June 2011 samplings, a field laboratory was again set up near the confluence of the Olifants and Letaba rivers and fish from the Levuvhu River were transported live to this site. Tigerfish

sampled from the Olifants Gorge at the confluence of the Olifants and Letaba rivers were caught within a short walking distance from a field laboratory set up at the confluence. Tigerfish are sensitive to water quality and handling stress, and these fish were examined and dissected without delay as they were caught. For examination of catfish from the Crocodile River in June 2011 the post-mortem facility in Skukuza was used. The fish were transported live from the Crocodile River to Skukuza. Catfish from Lunsklip Fisheries were examined and processed on site at Lunsklip Fisheries.

3.1.4. Sampling and fish dissections

For examination fish were placed into a water bath containing benzocaine hydrochloride (Kyron Laboratories, Johannesburg) as anaesthetic at approximately 30 ppm. Once anaesthetised, the fish were removed from the anaesthetic bath, weighed and subjected to length measurements, body condition scoring and blood collection. For ease of handling the fish were placed into a PVC cradle made from a one meter section of PVC pipe, cut lengthwise through the middle, with rectangular plastic ends attached to prevent the cradle from rolling. Detailed data sheets were completed for all gross observations and measurements. Blood was collected through a 20 gauge hypodermic needle into a 5 ml syringe from the large vessels just ventral to the vertebral canal in the tail area caudal to the abdominal cavity or from the large blood vessels running through the kidney. Collected blood was directly transferred to both EDTA and serum tubes. EDTA tubes were gently shaken to avoid clotting and were wrapped immediately in aluminium foil to prevent exposure to sunlight. Fresh blood smears were made from each fish and air dried before being fixed and stained. Samples for serum were centrifuged to separate the blood from the serum after clotting had occurred.

The catfish were then euthanized by being kept in the benzocaine hydrochloride anaesthetic bath until all life signs had ceased. Gross examination and post-mortem dissection were performed. The following adipose tissues were carefully examined for gross signs of pansteatitis: mesenteric fat, pectoral fat, coronary fat, intermuscular and hypodermal fat, and fat surrounding the brain. The mesenteric fat was removed from each fish and weighed. Tissue specimens from all organs were collected and immediately fixed in 10% buffered formalin.

The major part of the visceral fat of sharptooth catfish is stored within the mesenteries, forming a discrete body towards the caudal portion of the abdominal cavity. A further discrete body of fat originating from the hypodermal fat layer is situated behind the pectoral fin. This fat cushion overlies an extension of the anterior kidney and liver into the hypodermal space, a feature unique to this species. For the purposes of this manuscript these two discrete fat depots will be referred to as mesenteric and pectoral fat respectively. Samples of liver, mesenteric fat, pectoral fat and eyes were collected on ice for toxicological examination by co-workers. Pectoral and mesenteric fat were collected for determination of fatty acid composition. The bony structures surrounding the swim bladders of the catfish were opened to allow inspection of the swim bladders. Otoliths were collected from all specimens for age determinations.

As each organ system was examined the relevant observations were recorded. The following small tissue samples were collected in 10% buffered formalin from each fish. Tissue blocks, approximately 15 mm long, 10 mm wide and at least 5 mm thick, were cut from the mesenteric fat, pectoral fat, brain fat, liver, pancreas, spleen, cranial and caudal kidney, heart and gonad. A cross-sectional wedge of muscle and skin, including intermuscular fat, approximately 5 mm thick, was cut from the body of each fish. The wedge was cut from the tail region, just caudal to the cloaca, and extended from the pterygiophores on the ventral midline into the lateral musculature. One gill arch was removed from one side of the fish, and the soft tissue of the arch with a number of attached primary gill filaments was dissected free of the bony arch. The entire brain was removed from the cranium and also fixed in 10% buffered formalin. Intestine was included only on some occasions. Sagittal otoliths were removed from each sampled fish and placed into small paper envelopes and left to dry. The number codeallocated to each fish was recorded on the respective specimen bottle and otolith envelope.

Sampling from the Sabiepoort and from van Ryssen Dam had not been included in the original protocol. As a result of time constraints and limited facilities at these two sampling sites, no blood samples or weights of fat tissues were collected from these fish.

3.1.5. Gross and histological examinations

All information gleaned from the gross and histological examinations was recorded in table form and later transcribed to Excel spread sheets. Each fish was given a sampling number and

code relevant to the sampling site and date. The fish identification number was written onto a square of paper and placed onto the relevant fish for photographic documentation. Each fish was photographed before the start of the dissection. A second photograph was taken of each fish after the abdominal wall had been removed exposing the abdominal organs. All interesting pathology was photographed. The originally assigned code and number of each fish was used to identify all samples collected from the fish. Laboratory results, including the histological examination of the tissues, were linked to each individual fish via this number. During the initial data collection from the gross examination, written descriptions were recorded and numerical scores were ascribed to each observation. Apart from sample collection for further laboratory evaluation, the length and weight of each fish was measured, and the condition score was recorded. A scoring system on a scale of one to five was systematically applied to all organs and tissues, with one showing the greatest degree of abnormality and five showing no abnormality.

3.1.6. Laboratory Work

Preparation of histological sections was done by the histopathology laboratory of the Pathology Section, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria. Tissue specimens, fixed in 10% formalin, were processed using standard histological technique, and paraffin wax sections were cut at 5µm. All specimens were mounted on glass slides and stained with haematoxylin and eosin (H&E). A selected number of sections from fish with severe pansteatitis, sampled from Lunsklip Fisheries and from the Olifants Gorge in November 2009, were in addition stained with Periodic acid Schiff's (PAS), Gomoris aldehyde fuchsin (GAF) and Perl's Prussian blue stain. Sections were prepared from the following organs and tissues of all sampled fish: mesenteric fat, pectoral fat, hypodermal and intermuscular fat, brain fat, liver, spleen, kidney, pancreas, heart, gonad, muscle, skin, gills and brain. Sections of the intestines were prepared from a selected number of fish. Blood smears were fixed and stained with a CAM's quick stain (Kyro-Quick stain, Kyron Laboratories) in the author's veterinary clinic. Otoliths were embedded in resin and sectioned transversely at 0.4 mm thickness by microtome according to the method of Weyl and Booth (2008)at the Department of Ichthyology and Fisheries Science, Rhodes University, Grahamstown. Mounted sections were examined under the light microscope by the author. Growth zones were counted to determine the ages of the fish (Figure 3.1a & 3.1b).

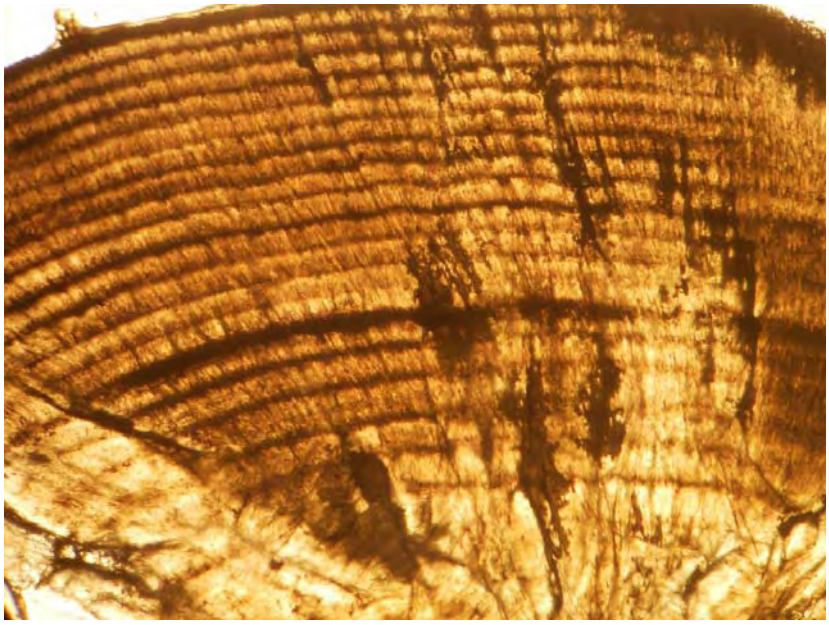

Figure 3.1a: Example of growth rings in the otolith of a 19 year old catfish from the Olifants Gorge.

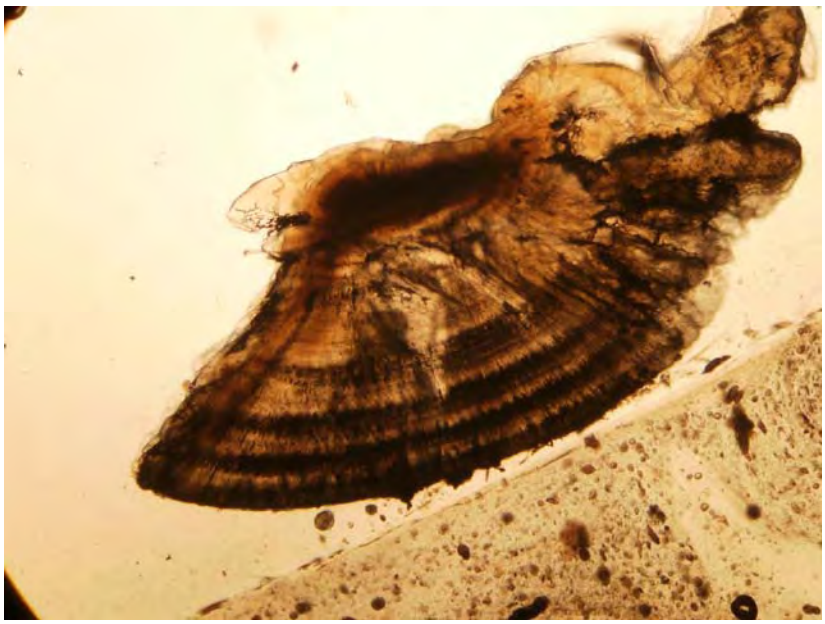

Figure 3.1b: Example of growth rings in the otolith of a 4 year old catfish from the Olifants Gorge.

Histological sections were examined by the author by standard light microscopy for presence of pathology. Relevant pathology was photographed by the author at thePathology Section, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria. Stained blood smears made from all fish collected from the Olifants Gorge, Lunsklip Fisheries and Reënvoël Dam during November 2009 were examined under a standard light

microscope. One hundred erythrocytes were counted, and the proportion of polychromatocytes determined and recorded. This was repeated over two further areas of the same blood smear. The average of the three counts was taken to be representative of a specific blood smear. The amount of crenation of the erythrocytes was noted.

To determine the haematocrit of the fish, microhaematocrit tubes were filled and sealed before centrifugation on a field micro-centrifuge. Packed cell volume (PCV) was expressed as percentage of the height of the red cell column compared to the total column height. All changes were recorded in detail and the relevant information added to the fish data sheets.

Blood glutathione peroxidase and vitamin E measurements have been used in studies of the acute effects of steatitis in kittens (Fytianou *et al*. 2006) and dietary vitamin E and selenium deficiency in rainbow trout (Bell *et al.* 1985).The measurement of these blood parameters was included in this study in an attempt to identify tests suitable for non-lethal monitoring of pansteatitis in catfish of the KNP. Serum samples and blood samples collected in EDTA were immediately put on ice or refrigerated and submitted to IDEXX Laboratories (Johannesburg) for determination of haemoglobin and erythrocyte glutathione peroxidase values. Serum vitamin E determinations were done by the laboratory of Ampath Pathologists on behalf of IDEXX laboratories.

3.1.7. Statistical analysis

Presence of grossly visible signs of pansteatitis was used to determine pansteatitis prevalence. For further statistical evaluation, data were grouped into two categories: those collected from fish with pansteatitis (category 1) and those collected from fish where pansteatitis could not be demonstrated either macroscopically or histologically (category 2). Where an observation could be categorised into presence or absence of the observation, the frequency distribution was analysed using the chi-squared test or contingency analysis (Agresti & Franklin 2007). To test the effect of age on pansteatitis in catfish from the Olifants Gorge, the frequency of pansteatitis in catfish below and above 3 years of age was examined. A further test was done to compare the mean ages of catfish with and without pansteatitis. To test the effect of mass and length on pansteatitis incidence, the means for body mass and length of catfish with and without pansteatitis were compared. To allow the effect of condition factor on pansteatitis incidence to be tested, mass and length data were converted to express condition factor [$C=(\text{mass}/\text{length}^3)10^5$].

Apart from the descriptive recordings of gross pathology and histopathology observations, all observations were assigned a numerical score on a scale of one to five, where five denoted no observable abnormality and one denoted severe abnormality. As these scores represented frequencies of less than n=5, the non-parametric Kruskall-Wallis and Mann-Whitney U tests(Agresti & Franklin 2007) were used to test for differences inthe frequency of histopathology observations of various parameters between fish with and without pansteatitis from the Olifants Gorge sites. The same tests were used to analyse whether significant variances also existed for the frequency of a specific observation between sampling sites. The tests were performed on the following data sets: data from all Olifants Gorge samplings (Test data=sites where pansteatitis was found), data from Reënvoël Dam (RV and RVB) samplings (Ref data=sites where no pansteatitis was found), data from the Lunsklip Fisheries sampling (LK=positive reference site with high prevalence of pansteatitis).

T-tests were used to compare mean blood chemistry and haematological values between steatitis positive and steatitis negative fish sampled from the Olifants Gorge and from Lunsklip Fisheries. Type 1 error levels below 0.05 (5%) were accepted as significant. A second set of data was arranged to compare means of all data for a particular variable between sampling sites without differentiating whether the samples were obtained from fish with or without steatitis. The data were statistically analysed using analysis of variance followed by the post-hoc Tukey HSD Test (Agresti & Franklin 2007). Where necessary, the non-parametric Kruskall-Wallis test was used (Agresti & Franklin2007). For comparison of the percentage of fish with suppressed serum vitamin E values between sites, the chi-squared test was used.

All statistical analyses were done using Statistica 10 (Statsoft).

3.2. Results

3.2.1. Prevalence of pansteatitis

3.2.1.1. Prevalence in free living catfish

The observations on prevalence of pansteatitis in catfish in rivers and dams in the KNP have been published and the manuscript is attached to the thesis (Huchzermeyer 2012) [see Appendix A.2].

The most distinctive pathology observed in catfish from the Olifants Gorge was centred in the adipose tissues of the fish. At localities where pansteatitis was observed in the sampled catfish, the mesenteric fat was most frequently and most severely affected by pansteatitis. Pansteatitis was only occasionally observed in the pectoral, intermuscular and hypodermal fat and in the fat surrounding the brain simultaneously with pansteatitis in the mesenteric fat. Pansteatitis was never observed in the coronary fat. Presence of macroscopic lesions of fat necrosis and associated inflammation of the adipose tissues was used to determine pansteatitis prevalence in the KNP rivers (Figure 3.2).

A high prevalence of pansteatitis was repeatedly identified in catfish sampled from the Olifants Gorge between August 2009 and July 2011. Pansteatitis prevalence similar to that found in fish from the Olifants Gorge was detected in catfish sampled from the Sabiepoort during a single sampling in July 2010. Lesions in the adipose tissues were identical to those observed in fish from the Olifants Gorge, and splenomegaly and pancreatic atrophy was similarly observed. Lower pansteatitis prevalence was observed in fish sampled from Engelhard Dam. However, the severity of pansteatitis lesions in one fish from this site was comparable to that of severely affected fish from the Olifants Gorge and Lunsklip Fisheries (see section 3.2.1.3). In catfish sampled from Mamba Weir a low prevalence of pansteatitis was noted. Macroscopically no pansteatitis could be identified in fish sampled from Reënvoël Dam during November 2009 and again during a repeat sampling in January 2011. Similarly, pansteatitis could not be detected in fish sampled from van Ryssen Dam (Figure 3.2). Pansteatitis could also not be demonstrated in catfishsampled from the Levuvhu and Crocodile rivers (Table 3.1).

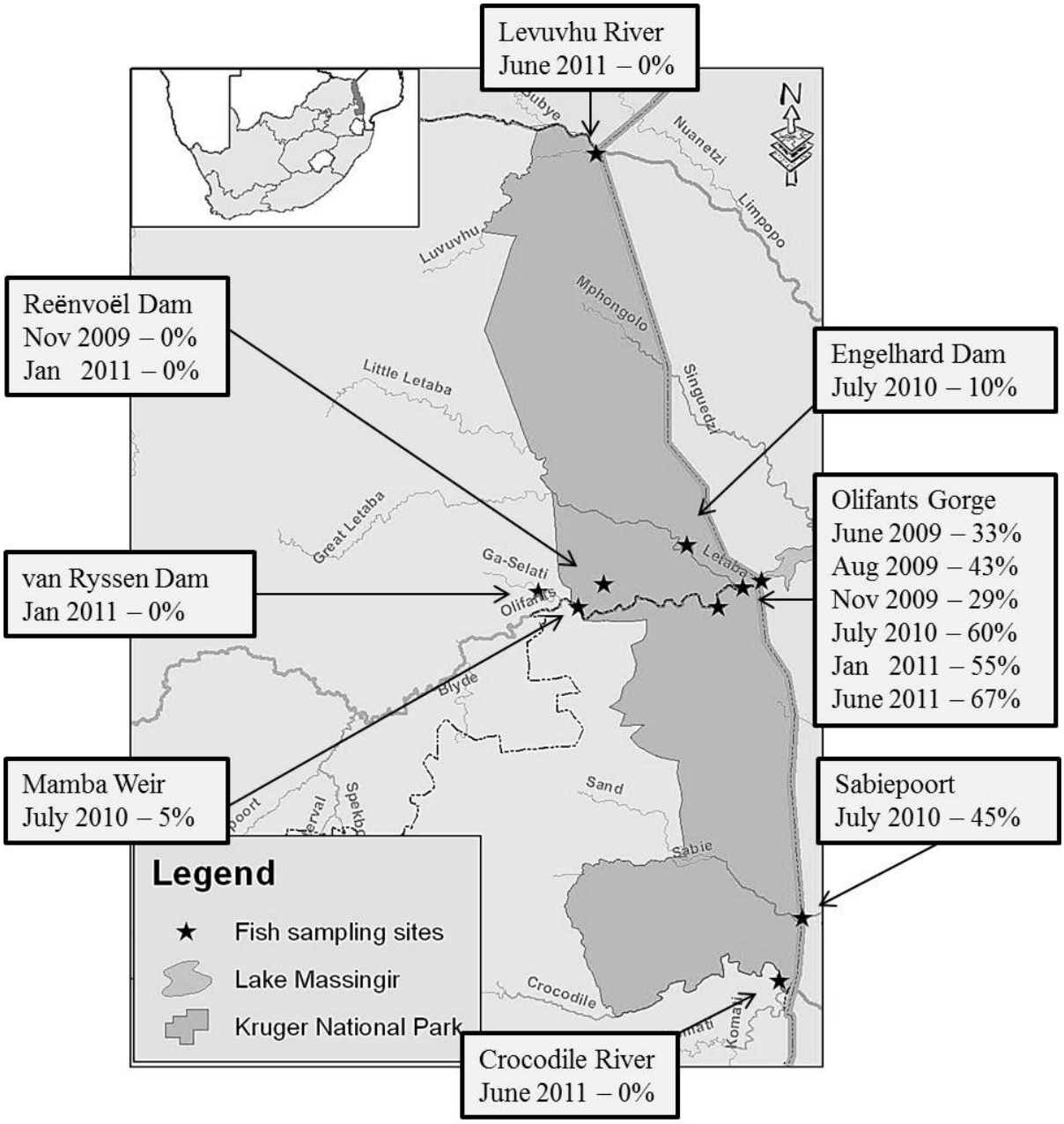

Figure 3.2: Macroscopic pansteatitis prevalence as percentage of sampled catfish from various sampling sites in the Kruger National Park during the period2009-2011.

Table 3.1: Prevalence of macroscopically detectable pansteatitis lesions in the Olifants Gorge and other reference populations of catfish in and around KNP

Date	Sampling site	% fish with pansteatitis	Total fish sampled
June 2009	Olifants Gorge	33	9
August 2009	Olifants Gorge	43	14
November 2009	Olifants Gorge	29	21
November 2009	Reënvoël Dam	0	28
July 2010	Olifants Gorge	60	25
July 2010	Mamba Weir	5	20
July 2010	Engelhard Dam	10	21
July 2010	Sabiepoort	45	11
January 2011	Olifants Gorge	55	22
January 2011	Reënvoël Dam	0	13
January 2011	van Ryssen Dam	0	10
June 2011	Olifants Gorge	67	21
June 2011	Levuvhu River	0	14
June 2011	Crocodile River	0	20

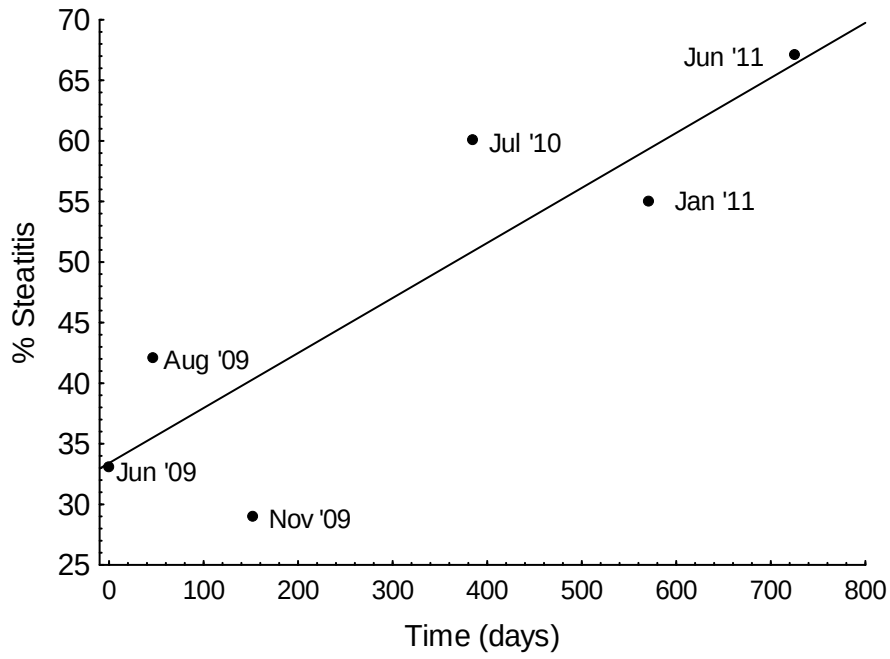

Figure 3.3: Prevalence of pansteatitis in catfishsampled from various localities along the Olifants and lower Letaba rivers in the Kruger National Park from 2009 to 2011 (Correlation coefficient: r = 0.87, significance: p = 0.02).

Most catfish from the Olifants Gorge, as well as from the Sabiepoort,stored relatively larger amounts of fat compared to catfish sampled from other localities in KNP. However the amount of fat carried was considerably less than that carried by the fish from Lunsklip Fisheries. A decline over time in amount of stored fat in catfish sampled from the Olifants Gorge was noted during repeat samplings between November 2009 and June 2011. Mesenteric fat made up 4.6% of body mass in the most obese specimen from the Olifants Gorge sampled during November 2009, whereas it only constituted 0.9% of body mass in the most obese specimen sampled during June 2011. By contrast, the most obese fish sampled from Lunsklip Fisheries carried more than 12% of body mass as mesenteric fat (see section 3.2.1.3). Fish sampled from the Olifants Gorge during June 2011 were distinctly leaner than fish sampled during July 2010 and several wasted fish were caught from the Olifants Gorge during the 2011 samplings. One of these fish was extremely emaciated but nevertheless had a reasonable amount of fat stored in the mesenteric adipose tissue. Pansteatitis was evident in the adipose tissue of this fish. Most catfish sampled from Engelhard Dam, Mamba Weir, Reënvoël Dam and van Ryssen Dam were lean (Table 3.2). Fish from the Levuvhu River carried more fat in their adipose tissues than fish sampled from the Olifants Gorge during the

same period, whilst fish from the Crocodile River were notably leaner than fish from the Olifants Gorge (Table 3.2).

Table 3.2: Mesenteric adipose tissue mass relative to body mass of catfish sampled from the Olifants Gorge and other sites on various dates.Olifants Gorge (OG), Engelhard Dam (EH), Mamba Weir (M), Reënvoël Dam (RV), van Ryssen Dam (FK), Levuvhu River (LUV) and Crocodile River (CR)

Sampling site	Date	Fat % of body mass				
		Mean	Standard Deviation	Sample variance	Range	n
OG	Nov-09	1.17	1.36	1.86	0.08-4.59	20
OG	Jul-10	1.12	1.02	1.05	0.00-3.68	25
OG	Jan-11	0.40	0.80	0.64	0.02-3.80	22
OG	Jun-11	0.18	0.20	0.04	0.02-0.88	21
EH	Jul-10	0.19	0.40	0.16	0.00-1.57	21
M	Jul-10	0.16	0.24	0.06	0.00-0.74	20
RV	Jul-10	0.32	0.29	0.08	0.00-0.88	13
FK	Jul-10	0.05	0.08	0.01	0.00-0.26	10
LUV	Jun-11	0.96	0.78	0.61	0.03-2.20	14
CR	Jun-11	0.14	0.16	0.03	0.00-0.53	20

Repeated samplings have taken place in the Olifants Gorge since 2009, and an increase in prevalence of pansteatitis was detected (Figure 3.3).The ages of catfish sampled from the Olifants Gorge ranged from 1 to 19 years, and pansteatitis was observed in catfish from 3 to 19 years old. Comparison of the number of fish with pansteatitis under 3 years of age with the number above 3 years indicated a significant effect of age on pansteatitis incidence in the Olifants Gorge (Chi-squared Test, $p<0.002$). A comparison between the mean ages of fish with and without pansteatitis in the Olifants Gorge indicated a higher frequency of pansteatitis in older fish ($p=0.021$) (Figure 3.4). Severity of pansteatitis, however, did not vary with age. There was also no variation in pansteatitis incidence between male and female catfish. Ages of catfish sampled from Reënvoël Dam, where no pansteatitis was found, similarly ranged from 1 to 19 years with both male and female fish represented.

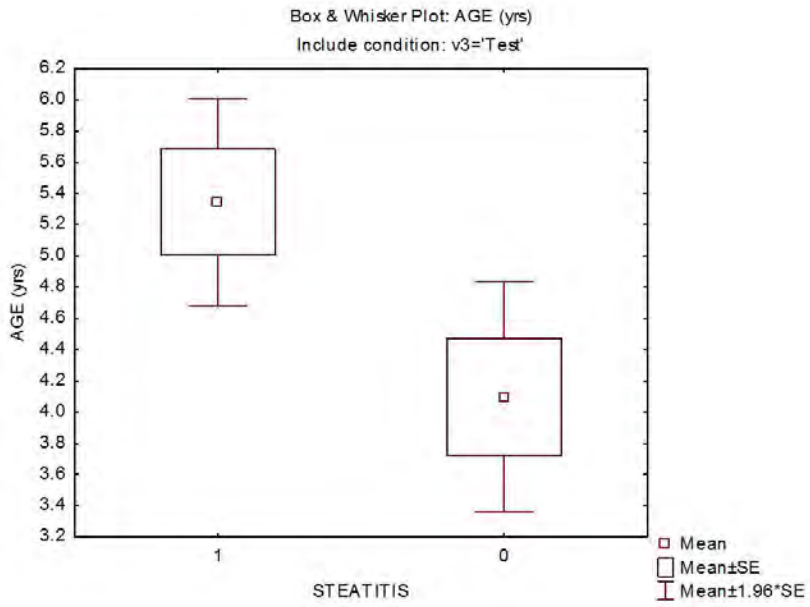

Figure 3.4: Box and whisker plot showing variation in age between catfish with and without pansteatitis sampled from all Olifants Gorge sites (1 denotes fish with pansteatitis incidence, 0 denotes fish without pansteatitis).

Pansteatitis also varied with mass and length in the Olifants Gorge catfish (Figures 3.5 & 3.6), and significant differences in prevalence of pansteatitis were noted between mass and length classes (Chi-squared Test, P<0.001). There was, however, no variation between condition factor and pansteatitis incidence (T Test, p=0.59).

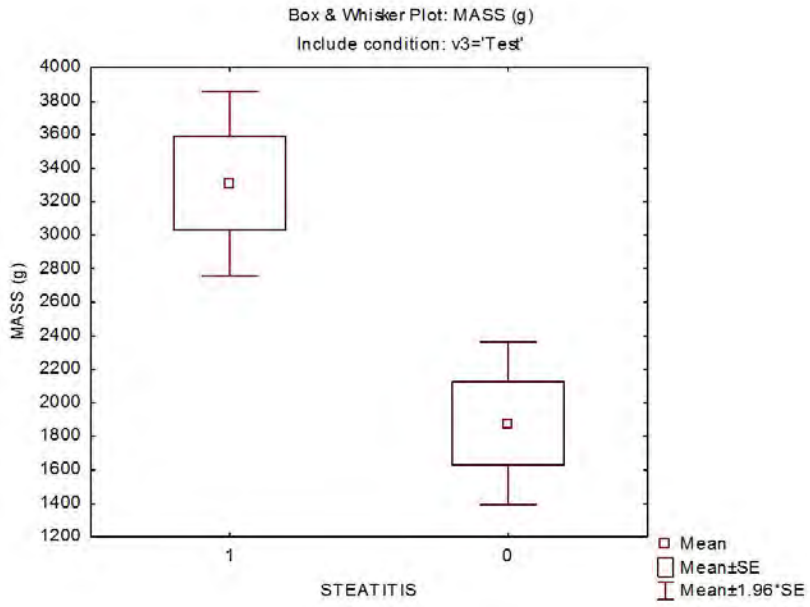

Figure 3.5: Box and whisker plot showing variation in body mass between catfish with and without pansteatitis sampled from all Olifants Gorge sites (1 denotes fish with pansteatitis incidence, 0 denotes fish without pansteatitis).

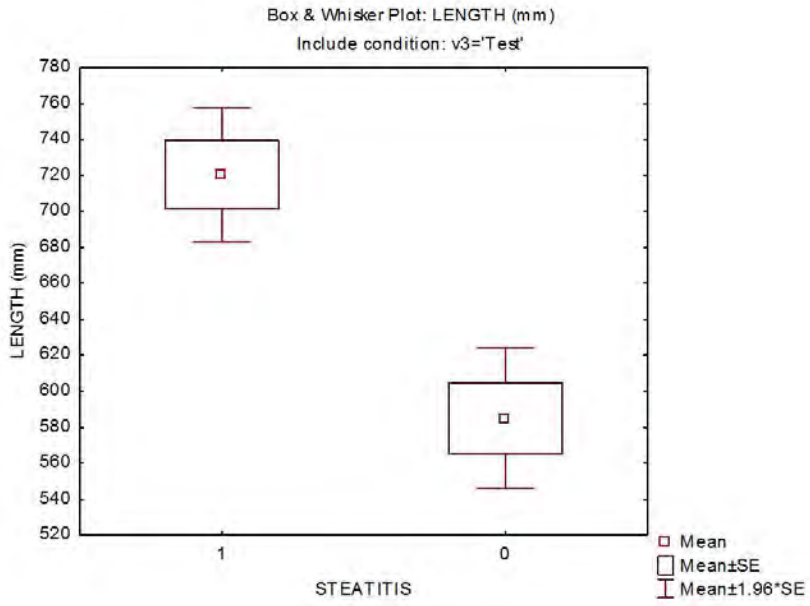

Figure 3.6: Box and whisker plot showing variation in body length between catfish with and without pansteatitis sampled from all Olifants Gorge sites (1 denotes fish with pansteatitis incidence, 0 denotes fish without pansteatitis).

3.2.1.2. Stomach content relative to prevalence

The observations on stomach content relative to pansteatitis prevalence in catfish in rivers and dams in the KNP have been accepted for publication in a manuscript that addresses the food-web of the respective aquatic ecosystems in the KNP using stable isotopes (Woodborne, Huchzermeyer, Govender, Pienaar, Hall, Myburgh, Deacon, Venter & Lücker 2012) [see Appendix A.3]. A further manuscript dealing with a comparison of lipid properties of healthy and pansteatitis-affected catfish relative to diet has been accepted for publication and is in press (Huchzermeyer, Osthoff, Hugo & Govender in press) [see Appendix A.4]. Both manuscripts have been attached to the thesis.

The sharptooth catfish is an omnivorous benthic scavenger and an active hunter. In the Olifants Gorge, catfish stomach contents consisted predominantly of fish. On the Mozambique border where the Olifants River flows into Lake Massingir and where the sand bottomed pools and rapids have been covered with clay deposits, stomach and intestinal content of sampled catfish consisted of algal detritus and clay. At those sampling sites where pansteatitis was prevalent in catfish, this was repeatedly linked to presence of fish remnants in the stomach contents. Although stomach content only revealed what had been ingested

prior to sampling, a relative relationship between fish in the diet and presence of pansteatitis existed as illustrated in the triplot in Figure 3.7.

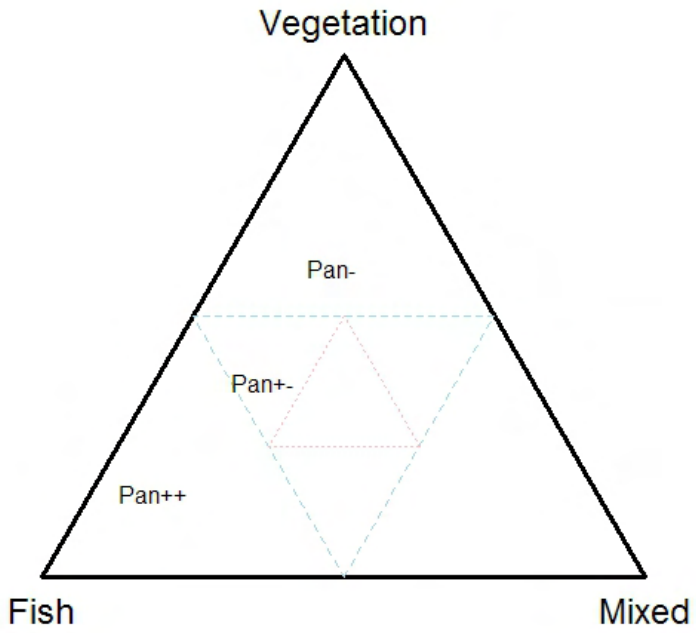

Figure 3.7: Stomach content analysis of sharptooth catfish showing that the population with pansteatitis (Pan++) at a site where pansteatitis was prevalent had stomach contents with a higher proportion of fish than vegetation, or invertebrates and detritus (mixed) when compared with the population of fish that did not have pansteatitis (Pan+-) from areas that had pansteatitis prevalence, and the population from areas without pansteatitis prevalence (Pan-). (Triplot preparation courtesy of S. Woodborne, Centre for Scientific and Industrial Research, Pretoria).

Fish remnants in stomach content were often in an advanced stage of digestion and consisted of bones and scales from noticeably large fish as well as occasional pectoral spines of *Synodontis* spp. fish. In a few cases these spines had migrated through the stomach wall and were found lying in the mesenteric cavity with only a mild associated inflammatory reaction. Intestinal content was often considerable and appeared whitish grey and pasty in catfish where bones and scales were present in the stomach. This was distinct from the black brown intestinal content associated with invertebrate and plant stomach content. Almost all catfish sampled in the Olifants Gorge during the peak flow of January 2011 had full stomachs, the ingesta consisting of fish as well as insects and small reptiles that had been washed into the

river during the flood conditions. Despite the murky turbulent water, catfish appeared to feed with ease under these conditions. During the winter samplings when the water in the Olifants River is relatively clear far fewer sampled catfish had significant amounts of ingesta in the stomach.

Stomach content of catfish sampled from the Sabiepoort consisted mainly of fish remnants, although stomachs of several of these fish contained recently ingested crocodile fat with visible signs of steatitis still present. The stomachs of catfish sampled from Mamba Weir contained predominantly the fruit of sycomore fig trees, which overhang the embankment of this stretch of the river. Although more than half of sampled catfish from Reënvoël Dam had empty stomachs, invertebrate and mixed detritus, vegetation and fish were represented in the ingesta of the remaining fish. Recognizable remnants of Mozambique tilapia were found in the stomach content of most catfish sampled from van Ryssen Dam. The majority of catfish sampled from the Crocodile River during June 2011 had stomachs distended with filamentous algae. Microscopic examination of fluid expressed from the stomach contents revealed that large numbers of diatoms had been ingested with the filamentous algae. Stomach content of catfish sampled from the Levuvhu River, during June 2011, consisted of algae and sycomore figs.

3.2.1.3. Prevalence in a captive farmed population of catfish

Lesions identical to those found in the adipose tissues of catfish from the Olifants Gorge, the Sabiepoort and Engelhard Dam were discovered by the author in a captive population of catfish at Lunsklip Fisheries. The majority of these fish had severe visible changes in the fat associated with pansteatitis (Table 3.3). This provided the study with an identified positive control for evaluation of gross pathology and histology observed in catfish in the KNP. Although pansteatitis was observed macroscopically in 66% of fish sampled from Lunsklip Fisheries, 95% of these fish showed steatitis on histological examination. Many of these fish had very large mesenteric fat reserves (Table 3.4).

Table 3.3: Prevalence of macroscopically detectable pansteatitis lesions in catfish sampled from a captive population at Lunsklip Fisheries (LK)

Date	Sampling site	% fish with pansteatitis	Total fish sampled
Nov-09	LK	66	21

Table 3.4: Mesenteric adipose tissue mass relative to body mass of catfish sampled from Lunsklip Fisheries (LK)

Date	Sampling site	Fat % of body mass				
		Mean	Standard deviation	Sample variance	Range	n
Nov-09	LK	4.61	3.45	11.89	1.01-12.07	21

These fish were from the remnants of a previously farmed population of catfish that had been retained in an earth pond. They were fed the untreated fish-waste from a trout slaughterhouse on the farm. The fat-rich innards of the trout were fed to an excess that left an oily scum, visible on the water surface along the edges of the dam. The stomach contents of these fish consisted largely of oily fish remnants. The fish carried considerably more mesenteric fat than the fish from the Olifants Gorge and other sampling sites (Tables 3.2 & 3.4). In many cases the fat was severely affected by pansteatitis. The sampled catfish were large, weighing from 2.82 to 11.5 kg and ranged in age from 5 to 13 years. On observing the dam in broad daylight, numerous large catfish could be seen swimming sluggishly near the surface with either the dorsal and tail fin or one pectoral fin protruding above the surface of the water. These fish appeared to have lost the negative buoyancy that is usual for this species. As these catfish were caught by scoop net, the sampling bias was towards weaker fish.

3.2.1.4. Discussion of prevalence

Pansteatitis was identified in catfish sampled from the Olifants River at the confluence with the Letaba River where the Olifants River enters the 9 km long gorge that opens into Lake Massingir in Mozambique. This same area has been the epicentre of the recent crocodile mortalities. During repeat samplings, an increasing prevalence of pansteatitis affecting up to 67% of catfish in this section of river was identified. A lower prevalence of pansteatitis was found in catfish sampled from Engelhard Dam on the Letaba River a few kilometres upstream of the confluence with the Olifants River and in catfish sampled from Mamba Weir where the

Olifants River enters the western boundary of the KNP. In the Sabiepoort of the KNP, the Sabie River flows through a gorge before entering Lake Corumana in Mozambique. Here pansteatitis prevalence was similar to that in fish from the Olifants Gorge. Crocodile deaths from pansteatitis have also been observed in the Sabiepoort (D Govender, SANParks, Skukuza, pers. comm. 2010). It is well documented that the Olifants River, draining the eastern side of the Mpumalanga Highveld, has been extensively affected by anthropogenic activity (Ashton 2010; de Villiers & Mkwelo 2009; Heath *et al*. 2010).There is, however, little similarity in pollution impacts between the respective catchments of the Sabie, Letaba and Olifants rivers. Pansteatitis could not be detected in fish from the Levuvhu and Crocodile rivers, both of which drain catchment areas subject to divergent anthropogenic impact and neither of which are dammed in or near the KNP. Neither could pansteatitis be demonstrated in fish from Reënvoël Dam, which is an entirely rain-fed water body within KNP and distant from potential pollution sources affecting the Olifants River. It is possible that pansteatitis in catfish in Engelhard Dam was associated with upstream movement of fish from the Olifants Letaba confluence.

Catfish are known to prey on a wide variety of organisms including invertebrates and fish, and their feeding preferences change with size, fish being the preferred prey of large catfish (Bruton 1979). They are also known to switch their feeding activity to prey on the most abundant and vulnerable fish species available (Bruton 1979). In the Olifants Gorge, pansteatitis was positively correlated to age, weight and length of catfish and no pansteatitis was found in fish less than 3 years of age. As length and weight were correlated to age, it is likely that larger catfish were able to catch and kill larger prey. This may imply a change in preference of food source when catfish reach a certain size in the Olifants Gorge and in the Sabiepoort. This was reflected in the stomach content analysis, which indicated a greater prevalence of fish in the diet of catfish with pansteatitis when compared to catfish without pansteatitis. Stable isotope analysis of muscle tissue of catfish from sites with pansteatitis indicated a dietary shift by catfish to a higher trophic level, and a change in feeding behaviour to a limited range of species (Woodborne *et al*. 2012) [see Appendix A.3]. In most cases, the fish remnants in the stomachs were in an advanced stage of digestion and it was not possible to identify prey species.

The debilitating effects of advanced pansteatitis were evident in the captive catfish from Lunsklip Fisheries. The large amounts of fat trapped in the adipose tissues and impaired

muscular activity of these fish appeared to interfere with the sounding abilities of the fish. Healthy catfish prefer to remain close to the bottom of pools and rivers and are seldom seen near the surface of water bodies during the daytime, other than to gulp air into the suprabranchial air-breathing organ. Sluggish behaviour and positive buoyancy in pansteatitis-affected fish make them easy prey in a habitat where crocodiles occur.

3.2.2. Pathology of pansteatitis in catfish

3.2.2.1. Gross pathology of pansteatitis

The detailed pathology and histopathology of the organs and the specific lesions associated with fat necrosis in catfish from the Olifants Gorge have been published (Huchzermeyer *et al.* 2011) and the manuscript is attached to the thesis (see Appendix A.1). A report on the findings of a preliminary study to identify pathology present in fish in the lower Olifants River has been prepared for the Water Research Commission and is also attached to the thesis (see Appendix B.1).

Necrosis and associated steatitis was observed repeatedly in the adipose tissues and, other than severity, there was no distinction between the lesions observed in catfish sampled from the Olifants Gorge, Engelhard Dam, Sabiepoort and the positive reference population at Lunsklip Fisheries. Steatitis lesions presented as distinct white and brown spots (Figure 3.8a) consisting of small focally disseminated to coalescing granulomata up to 5 mm in diameter. In more advanced cases lesions were characterized by a brown colour, sometimes with an orange coloured centre. Affected mesenteric fat, in severe cases, had a rubbery consistency, and brown granulomata were confluent throughout the fat (Figures 3.8b & 3.8c).

In catfish with pansteatitis from both the Olifants Gorge and Lunsklip Fisheries, lesions were mostly restricted to the mesenteric fat tissue. In severely affected fish the caudal section of the mesenteric fat body was often adherent to the hind gut and the caudal section of the gonads (Figure 3.9). In milder cases granulomata were more densely concentrated on the parietal aspect of the fat body closest to the mesenteric insertion (Figure 3.8a). Layers of fat with differing severity of steatitis were observed in some fish. Presence of both coalescing granulomata and scarring of the adipose tissues together with more recent lesions characterized by focal brown spots in the fat appeared to indicate an on-going incitement of fat necrosis in catfish of the Olifants Gorge. Occasional fish showed steatitis in the pectoral fat cushion (Figure 3.10a) and the intermuscular fat (Figure 3.10b & 3.10c). Steatitis could not be demonstrated in the epicardial fat. In fish with generalised pansteatitis, lesions could however be demonstrated in the intracranial fat surrounding the brain (Figure 3.10d).

Figure 3.8a: Early steatitis lesions in catfish sampled from the Olifants Gorge during July 2010. Note the small sharply circumscribed foci of fat cell necrosis and associated ceroid deposition imparting the characteristic brown colour (arrow).

Figure 3.8b: Cross-section of mesenteric fat with advanced pansteatitis from a catfish sampled from the Engelhard Dam during July 2010. Note the diffuse brown granular appearance of the fat, the rough surface and virtually total absence of normal appearing fat.

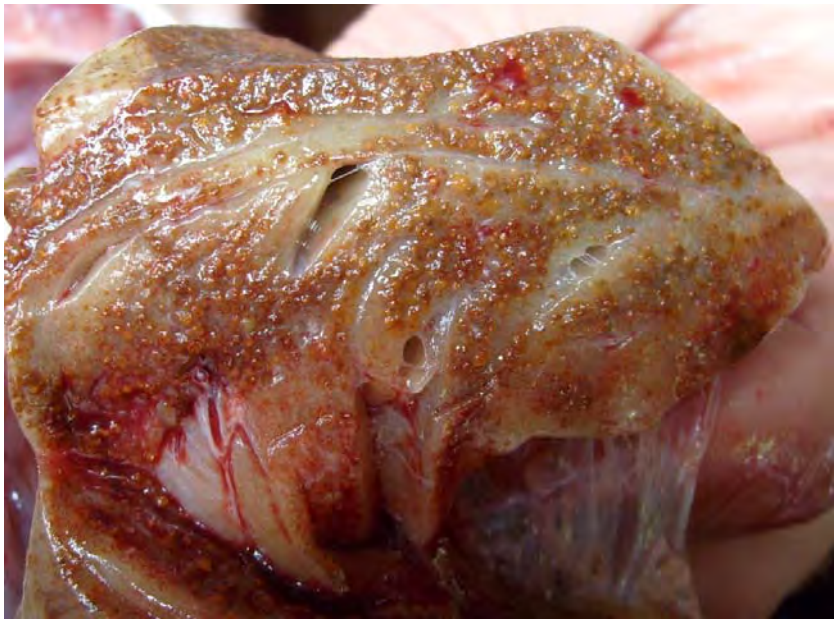

Figure 3.8c: Cross section of mesenteric adipose tissue from a catfish collected from the Olifants Gorge in November 2009 showing typical severe pansteatitis. Note brown granuloma formation within the adipose tissue.

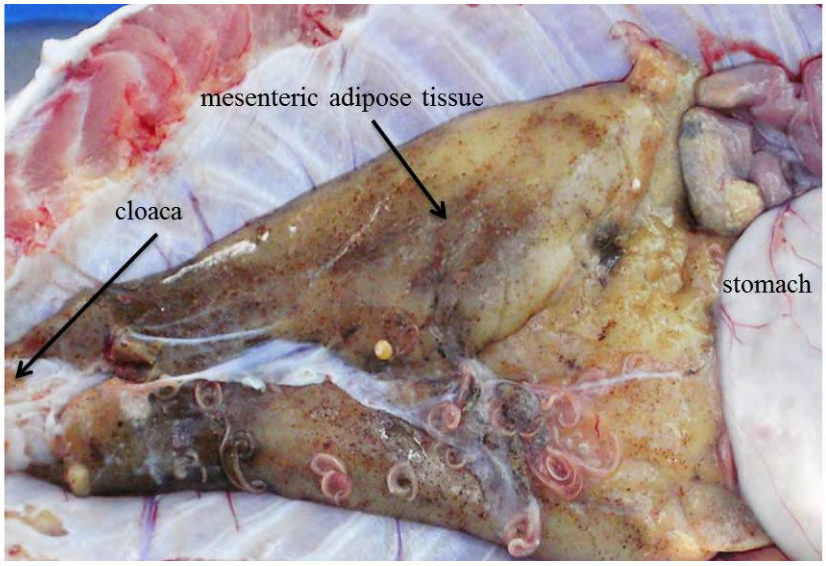

Figure 3.9: Pansteatitis of the mesenteric adipose tissues in a catfish sampled from the Olifants Gorge during June 2011. Note that the caudal portion of the mesenteric fat body appears more severely affected.

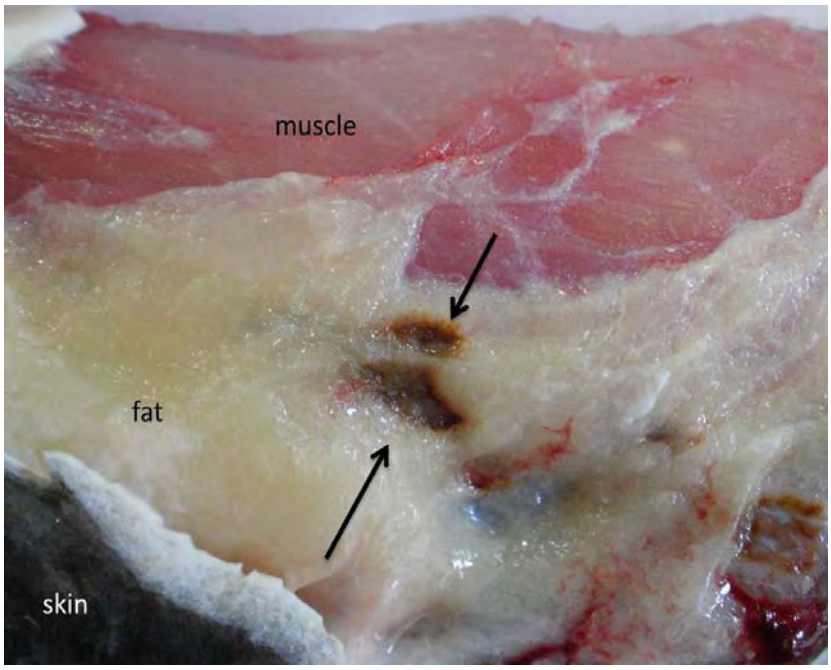

Figure 3.10a: Focal steatitis (arrows) in the pectoral fat of a catfish sampled from the Olifants Gorge during July 2010.

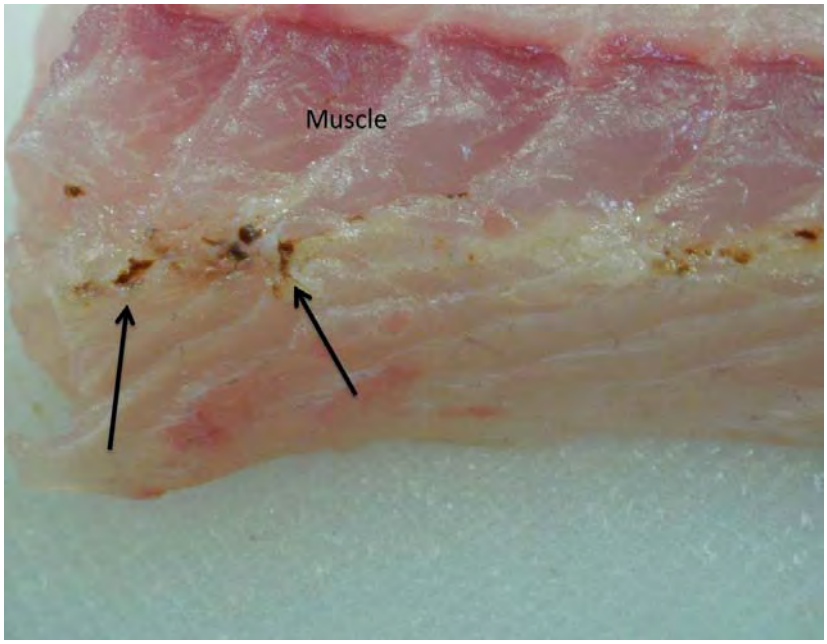

Figure 3.10b: Focal steatitis (arrows) in the intermuscular fat of a catfish sampled from the Olifants Gorge during July 2010.

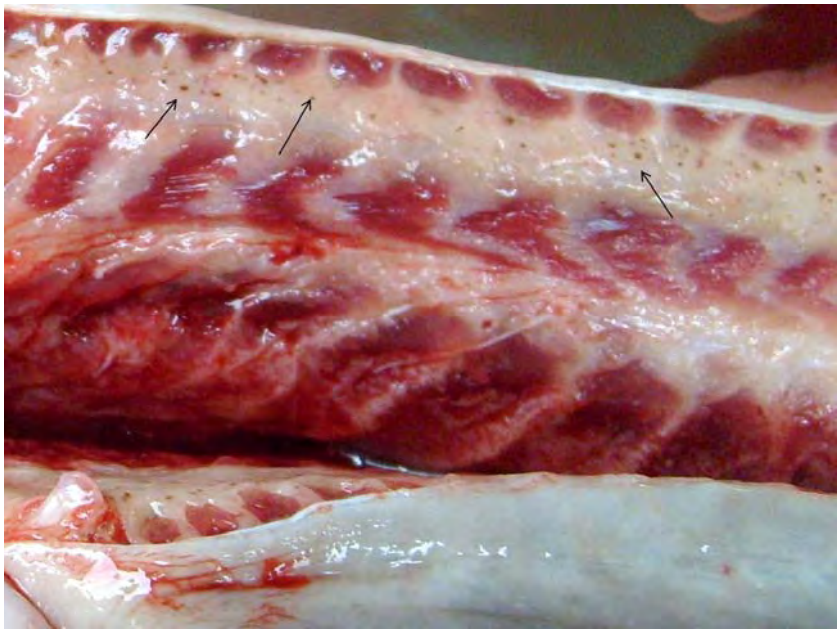

Figure 3.10c: Focal areas of steatitis (arrows) in the intermuscular fat of a catfish sampled from Lunsklip Fisheries during November 2009.

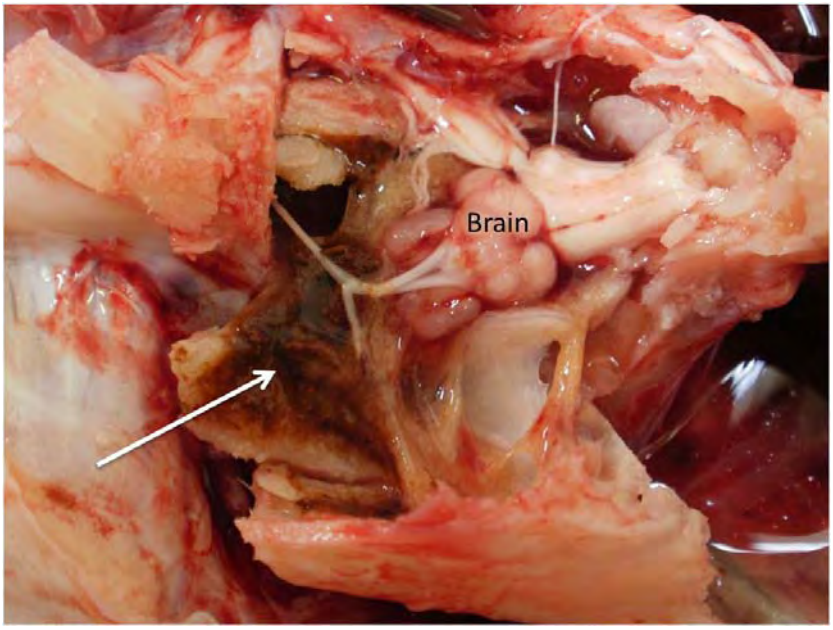

Figure 3.10d: Generalised steatitis in the fat surrounding the brain of a catfish sampled from the Olifants Gorge during July 2010. Note brown discolouration of fat (arrow) adhering to the opened cranium.

Catfish collected from the Olifants Gorge and lower Letaba River during the samplings prior to November 2009 presented with large amounts of variably coloured fat in the body cavity and between the muscles of the tail. The variation in colour, from a cream white to dark yellow, continued to characterize the mesenteric fat of catfish from all samplings in the Olifants Gorge, despite the reduction in quantity of mesenteric fat noted during subsequent samplings (Table 3.2). There was no correlation between fat colour and pansteatitis. Microscopic examination of histological sections of fat from sampled fish confirmed the macroscopic diagnosis of pansteatitis (Figures 3.16a and 3.16b).

Incidental pathology in adipose tissue associated with presence of parasites was noted in many fish and could be differentiated from changes associated with pansteatitis. Cysts of digenean parasites, varying in size from 2 to 15 mm in diameter, were occasionally noted in the mesenteric adipose tissues of fish sampled from the Olifants Gorge. Cysts mostly appeared as well circumscribed, hard, white nodules, sometimes focally disseminated throughout the mesenteric fat (Figure 3.11). On incision they consisted of a dense connective tissue capsule surrounding a central parasitic larva. In some cases an irregular brown discolouration as a result of melanin deposition was noted in the adjoining tissue. Such granulomata were distinguishable from those caused by fat necrosis. *Contracaecum* spp. larval nematodes were present in variable numbers within the peritoneal cavity of most fish sampled from the Olifants Gorge. Brown discolouration from melanin deposition in focal areas of the mesenteries overlying the mesenteric fat was occasionally noted in the presence of severe infestation with *Contracaecum* spp. larvae. This was particularly evident in catfish sampled from van Ryssen Dam (Figure 3.12). The dark colour associated with melanin deposition around larval nematodes and digenean trematodes differed distinctly from the discolouration associated with pansteatitis.Both lipopigment and ceroid were absent from such lesions.

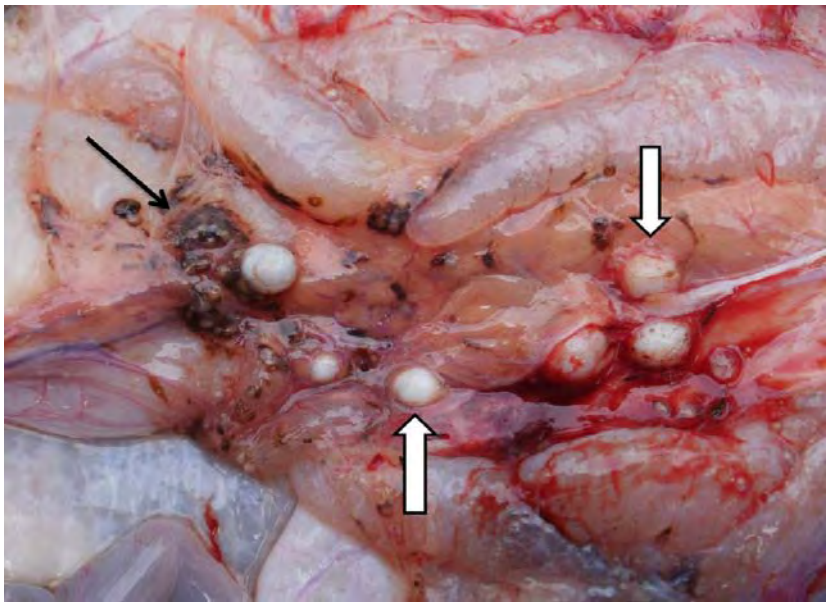

Figure 3.11: Melanin deposition (black arrow) associated with digenean trematode cysts (block arrows) in the caudal mesenteric fat between the male accessory sexual glands of a catfish sampled from Reënvoël Dam in KNP.

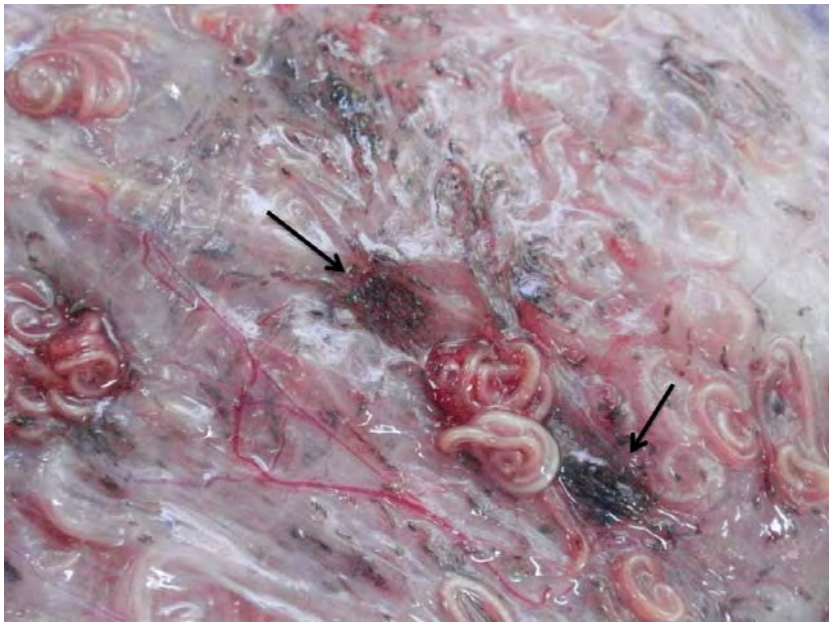

Figure 3.12: Melanin deposition (arrows) in the vicinity of larval nematodes in the mesentery overlying the mesenteric fat in a catfish sampled from van Ryssen Dam.

Gills of catfish sampled from the Olifants Gorge generally appeared to be in a good condition. During the early samplings in the Olifants Gorge during 2008 and 2009, gills of

many catfish appeared paler than normal and mildly hyperplastic. During later samplings this was no longer evident. Furthermore gill pallor appeared to be affected by temperature of the holding water and length of time that fish were kept in the holding tanks. With warmer water and longer holding periods gills appeared paler. Deformities of the gill cartilage associated with intense infestation by digenean trematode metacercariae were evident in some fish sampled from the Olifants Gorge, Mamba Weir, Engelhard Dam and Reënvoël Dam (Figure 3.13).

Figure 3.13: Gill arch of catfish sampled from the Olifants Gorge during November 2009 showing severe infestation of the cartilage of the primary gill lamellae by encysted digenean trematode metacercariae.

Livers of fish from the Olifants Gorge varied in colour but appeared more orange, fatty and swollen than in fish sampled elsewhere (Figure 3.14a). Livers of fish with good fat reserves often showed small focal deposits of fat on the surface (Figure 3.14b). Pale zones, sometimes observed in parts of the liver, occasionally extended into the hypodermal lobe (Figure 3.14c).Most of the pansteatitis-affected fish from the Olifants Gorge and Lunsklip Fisheries

had enlarged and rounded spleens with a rough surface (Figure 3.15a & 3.15b); the healthy spleen of catfish is an oval flat structure with sharp edges and a smooth surface. Atrophy of the pancreas was evident macroscopically in fish suffering from pansteatitis.

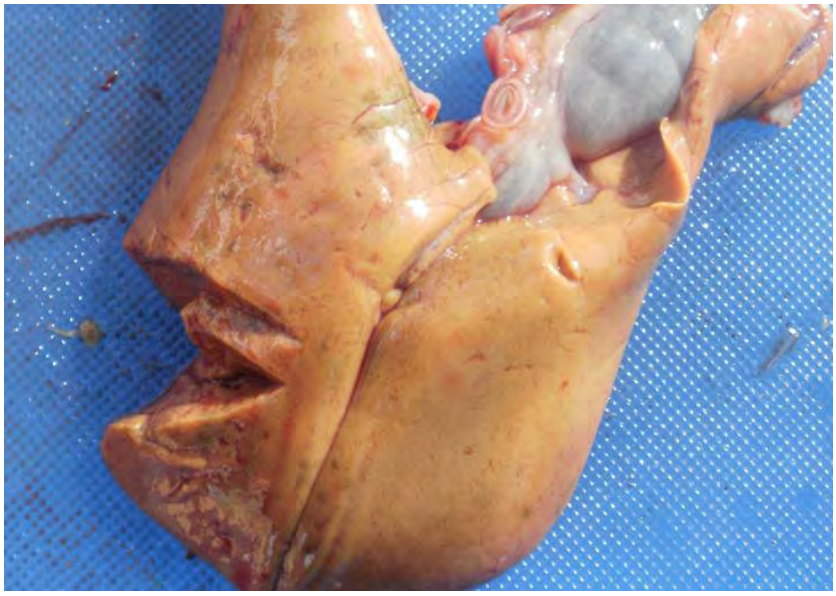

Figure 3.14a: Swollen fatty liver of a catfish suffering from pansteatitis, sampled from the Olifants Gorge during June 2011.

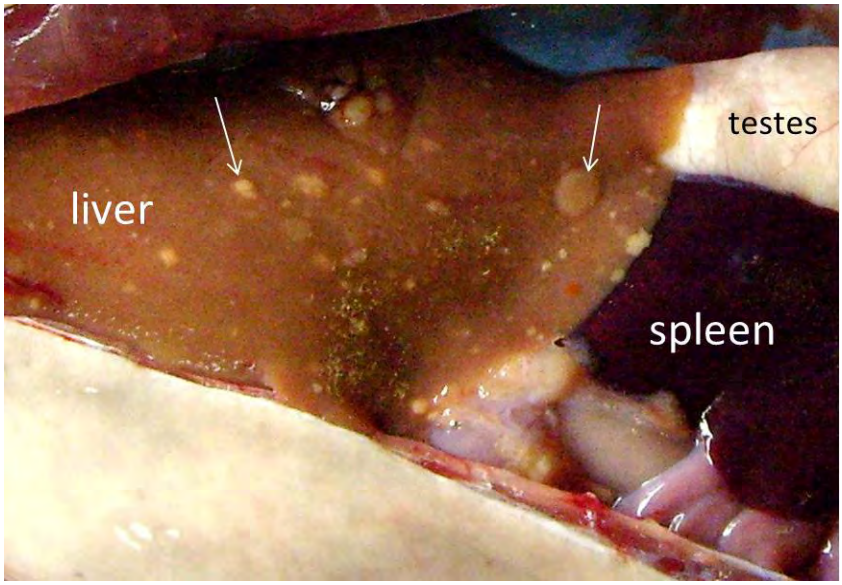

Figure 3.14b: Focal fat deposits beneath the liver capsule from a catfish with pansteatitis sampled from Lunsklip Fisheries during November 2009.

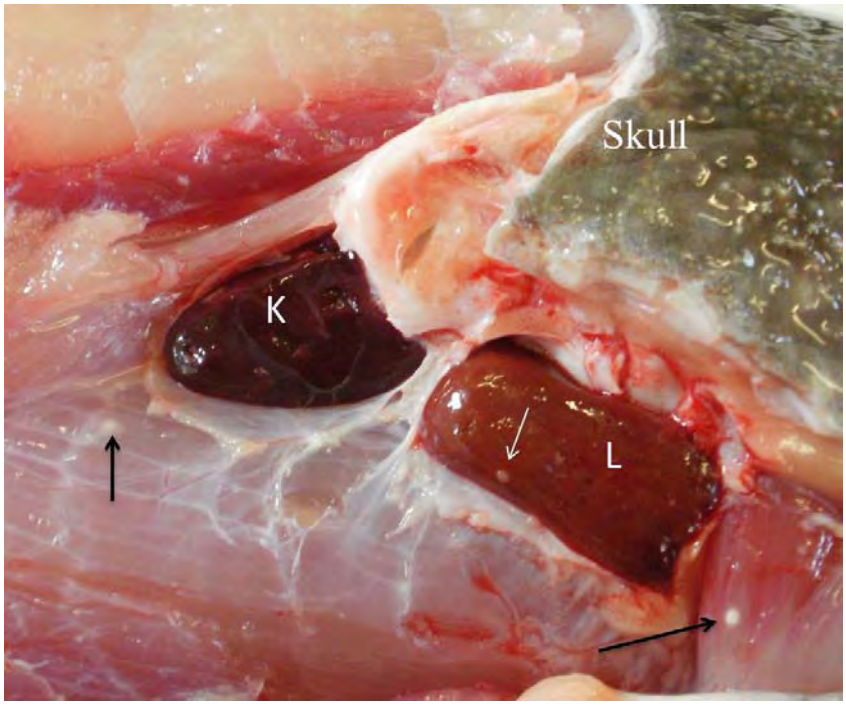

Figure 3.14c: Position of the external lobes of the liver (L) and cranial kidney (K) beneath the pectoral fat cushion caudoventral to the caudal margin of the skull of sharptooth catfish. Note the focal fat deposits visible on the surface of the liver in this catfish sampled from the Olifants Gorge. Arrows point to encysted digenean trematode larvae in the musculature.

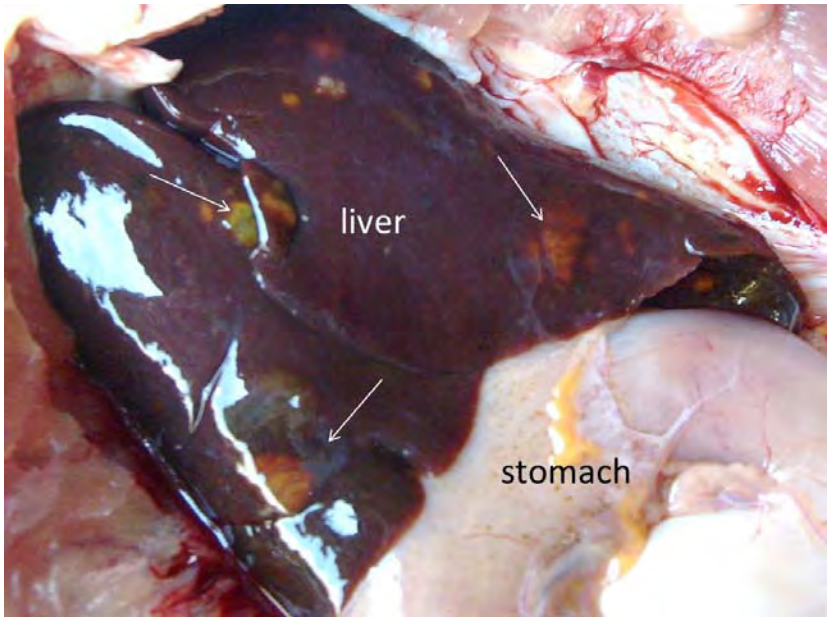

Figure 3.14d: Liver from a catfish sampled from Reënvoël Dam during November 2009. Arrows show damage associated with parasitic cysts in the liver parenchyma.

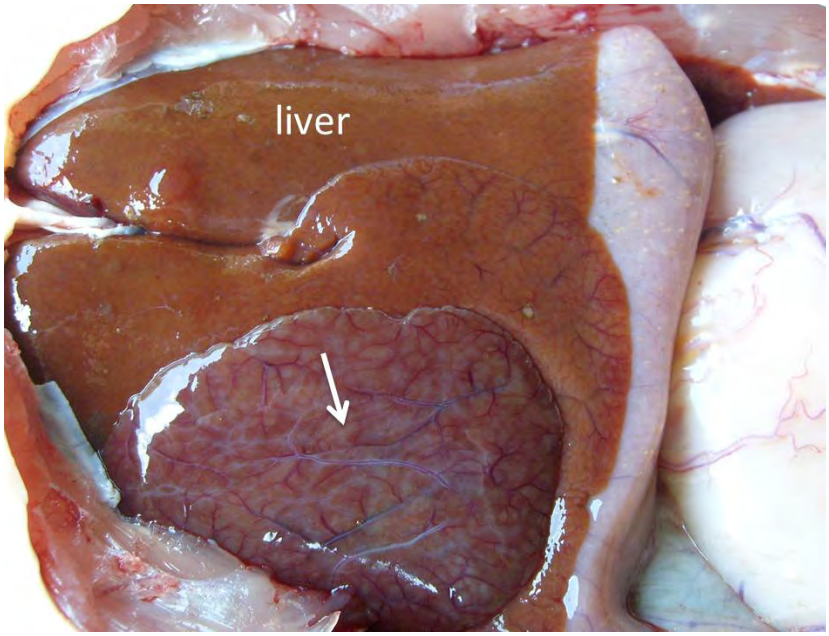

Figure 3.14e: Outgrowth of regenerating hepatic tissue (arrow), probably associated with parasitism, in a liver from a catfish sampled from Reënvoël Dam during November 2009. Note the normal brown colour of the liver.

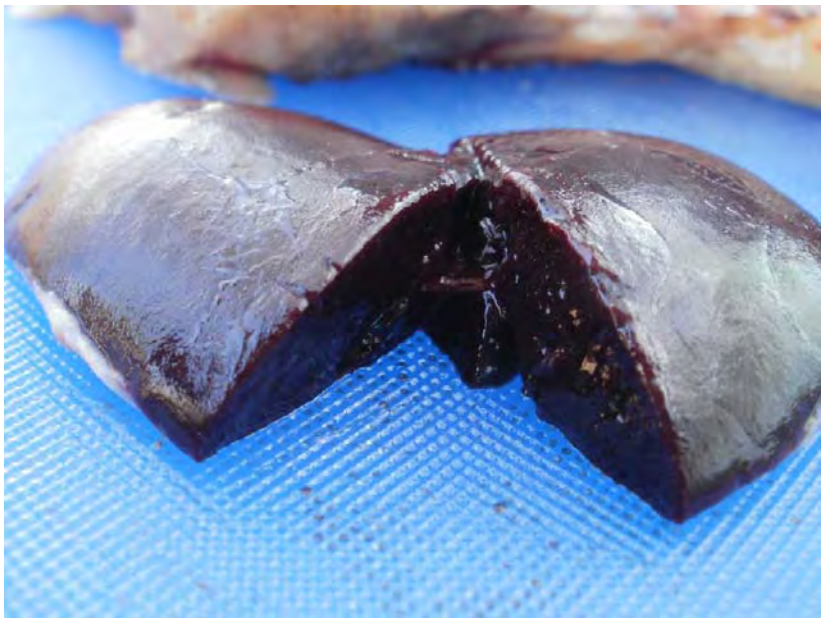

Figure 3.15a: Enlarged spleen of a catfish from the Olifants Gorge suffering from pansteatitis.

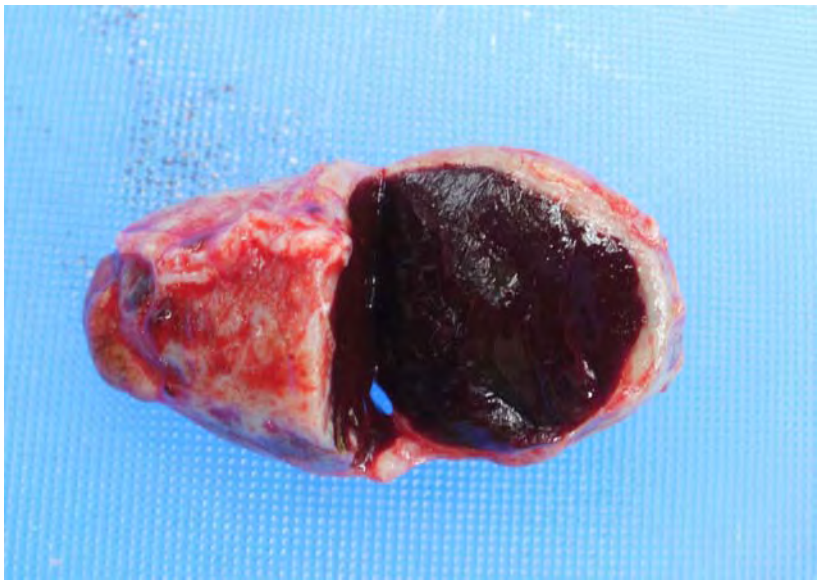

Figure 3.15b: Spleen of a catfish from Lunsklip Fisheries suffering from severe chronic pansteatitis showing prominent capsular thickening and splenomegaly. Note the rounded appearance of the normally flat spleen.

Catfish sampled from Reënvoël Dam showed no pansteatitis and were used as a negative control population (Table 3.1). The gills of catfish sampled from Reënvoël Dam during November 2009 appeared normal and in good condition despite heavy parasite burdens. Livers showed no fatty change, but varying pathology was observed associated with high levels of parasitosis, including outgrowths of regenerating liver tissue on the dorsal and ventral surface of some livers (Figures 3.14d & 3.14e). *Contracaecum* spp. larval nematodes were present in variable numbers within the peritoneal cavity of most catfish sampled from Reënvoël Dam, and parasitic granulomata were frequently present in large numbers in the mesenteric adipose tissue. Brown melanisation of focal areas of the mesenteries overlying the mesenteric fat in the presence of *Contracaecum* spp. larvae and in the vicinity of larval digenean trematode cysts was occasionally noted (Figure 3.11). Compared to other sampling sites, fish from Reënvoël Dam carried the heaviest burdens of digenean trematode cysts in the organs and musculature. Similar deposits of melanin were observed in association with heavy *Contracaecum* spp. larval burdens in catfish from van Ryssen Dam (Figure 3.12).

3.2.2.2. Histopathology of pansteatitis

3.2.2.2.1. Histopathology of the adipose tissues

Various granulomatous reactions were observed in the fat tissues of catfish. Parasitic granulomata were distinguishable from foci of inflammation and granuloma formation associated with non-parasitic causes. The histological appearance of non-parasitic granulomata in the adipose tissues was typical of lesions expected with steatitis (Figures 3.16a & 3.16b). These lesions were similar in appearance in all fish sampled with macroscopic pansteatitis, including the fish suffering from nutritional pansteatitis at Lunsklip Fisheries.

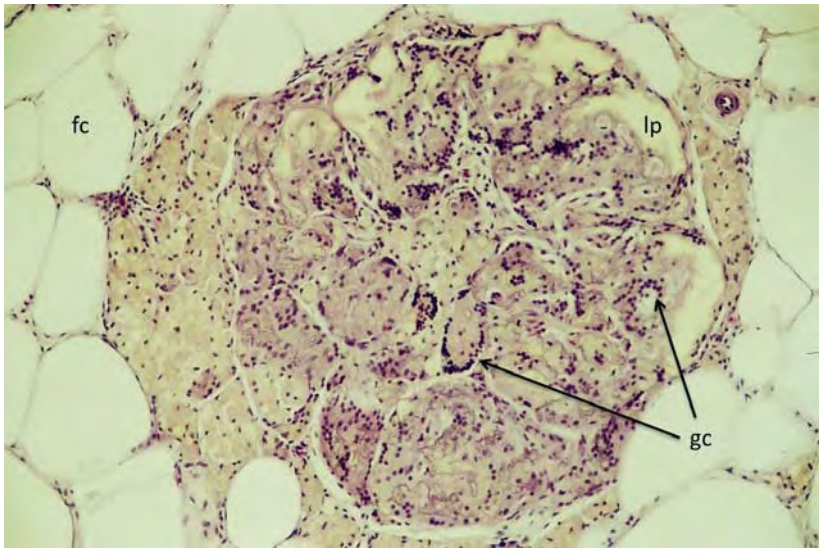

Figure 3.16a: Giant cell formation (arrows) in a steatitis lesion in adipose tissue from a catfishsampled from the Olifants Gorge in July 2009. Giant cells (gc), lipopigment (lp), adipocytes (fc) (H&E X100).

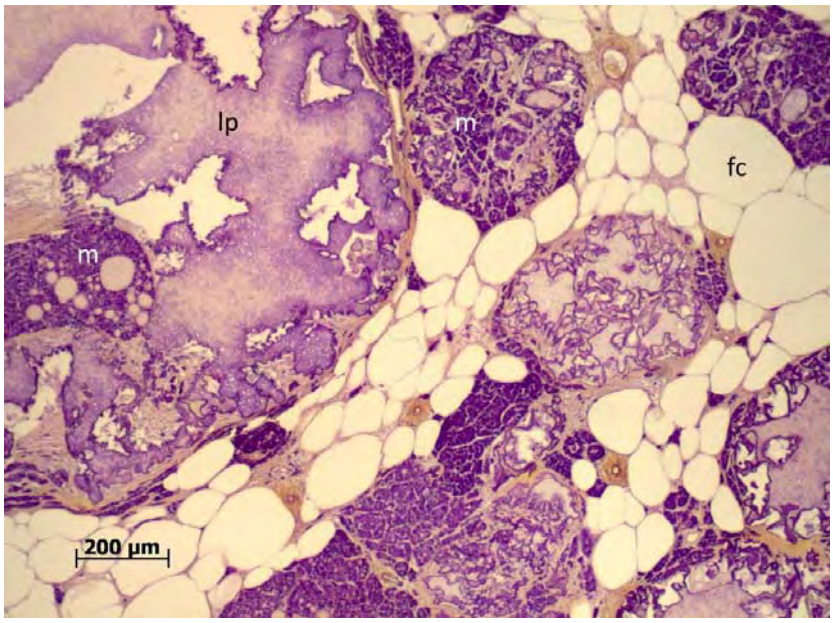

Figure 3.16b: Positive staining ceroid pigment (purple) within the lipopigment remnants (lp) of ruptured adipocytes and macrophages (m) in mesenteric fat of a catfish sampled from the Olifants Gorge during November 2009. Adipocytes (fc) (GAF).

In haematoxylin and eosin stained sections of affected fat, varyingly sized foci consisting of ruptured adipocytes contained a characteristic pigment identified as extracellular ceroid-type (ECC) lipopigment (Elleder 1991), also called preceroid (Jolly & Dalefield 1990). This pigment, typical of oxidative damage to fat cells, appeared as yellow, granular and refractive inclusions of varying size. ECC lipopigment was also observed to be phagocytised by the macrophages surrounding steatitis lesions. Necrotic adipocytes and associated cell breakdown debris were surrounded by a dense mass of macrophages containing intracellular ceroid (Figure 3.16b). The lesions were associated with the presence of variable numbers of fibroblasts and connective tissue deposition. However, macrophage aggregations associated with fat cell necrosis in the adipose tissues did not contain haemosiderin (Table 3.5).Lesions were focally disseminated throughout the affected mesenteric adipose tissue and represented the brown granulomata noted macroscopically. Lipopigment- and ceroid-containing macrophage aggregations within the interstitium of the fat tissues in the absence of adipocyte necrosis were noted in a few fish, and were considered to be indicative of mild or early oxidative damage to the fat. Presence of ceroid in the macrophages was confirmed by staining with GAF (Figure 3.16b) and PAS stains. Multinucleate Langhans giant cells were

invariably associated with the inflammatory response surrounding necrotic areas of fat (Figure 3.16a).

In some lesions, smaller macrophages were arranged in the form of a sheath surrounding the ruptured fat cells. Advanced cases presented with a clear or lipopigment containing central lacuna surrounded by organised layers of epithelioid cells that in places coalesced and became embedded in fibrous connective tissue (Figure 3.16c). Clear lacunae were an artefact of sectioning where the central pigmented area of fat breakdown products had been lost during sectioning.

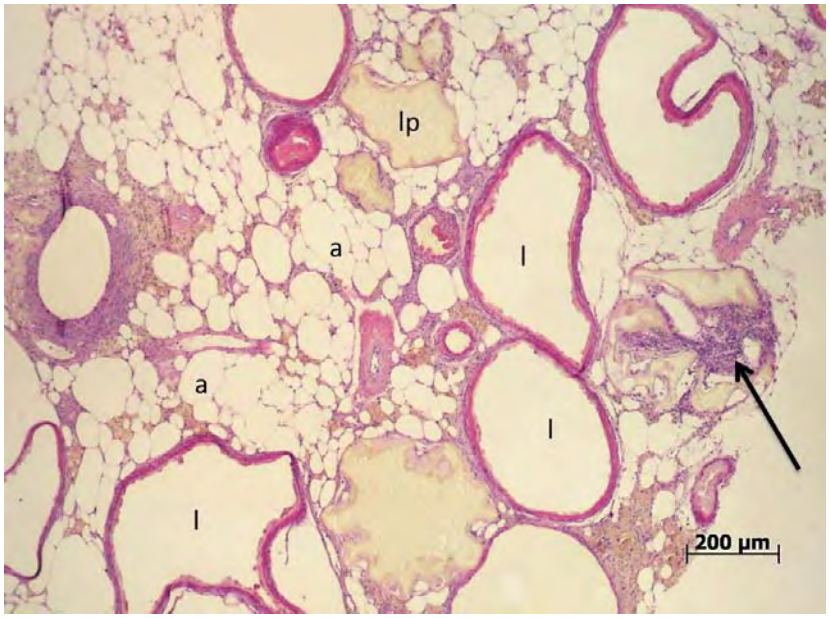

Figure 3.16c: Advanced stage of fat necrosis and steatitis in mesenteric fat of a catfish sampled from the Olifants Gorge during June 2011. Note the apparently empty lacunae (l) where necrotic remnants of oxidised fat (lp) have been lost during processing, surrounded by a macrophage sheath. Adipocytes (a), foreign body giant cells (arrow) (H&E).

Lesions in the adipose tissues were focal and often roughly circular in shape in mild cases, and disseminated and coalescing throughout the adipose tissue in severe cases. Surrounding adipocytes often appeared normal, although in severe cases they were reduced in number and displaced by the associated inflammatory reaction.

Steatitis was observed in both atrophied adipose tissue (Figure 3.16d) and in adipose tissue where adipocytes were replete with fat. The focal distribution of granulomata in mildly affected fish resulted in lesions sometimes being missed during the sectioning process. Such cases, although positive for pansteatitis on macroscopic evaluation, could not be identified on histological evaluation alone.

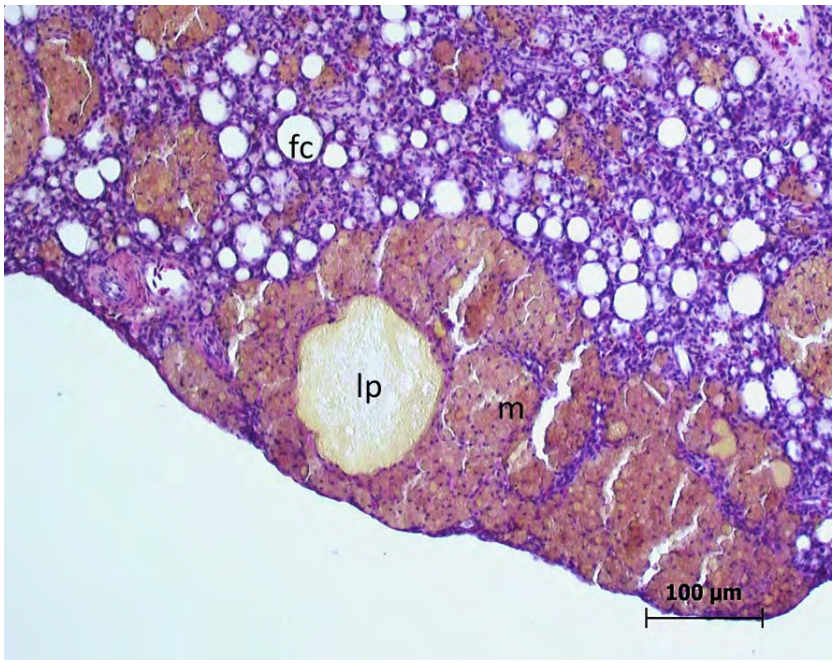

Figure 3.16d: Atrophied mesenteric adipose tissue showing inflammation typical of steatitis in a catfish sampled from the Olifants Gorge during June 2011. Note small size of adipocytes (fc) and aggregates of macrophages (m) containing ceroid, surrounding areas of fat necrosis containing lipopigment (lp) (H&E).

In many catfish with pansteatitis, only the mesenteric adipose tissues were affected. Where other fat tissues were affected, lesions in these were less severe than in the mesenteric fat. In a small number of catfish with pansteatitis the fat surrounding the brain and the pectoral adipose tissue was affected. Lesions in the pectoral fat appeared similar to those in the mesenteric fat (Figure 3.17a). In the intracranial fat the large areas of extracellular ceroid-type lipopigment, noted in association with steatitis in other adipose tissues, were absent, and the lesions consisted of dense aggregations of ceroid- and lipopigment-containing macrophages and a mild, sometimes focal, mixed inflammatory cell infiltration (Figure 3.17b). A fibroblast and connective tissue reaction was absent from lesions in this fat.

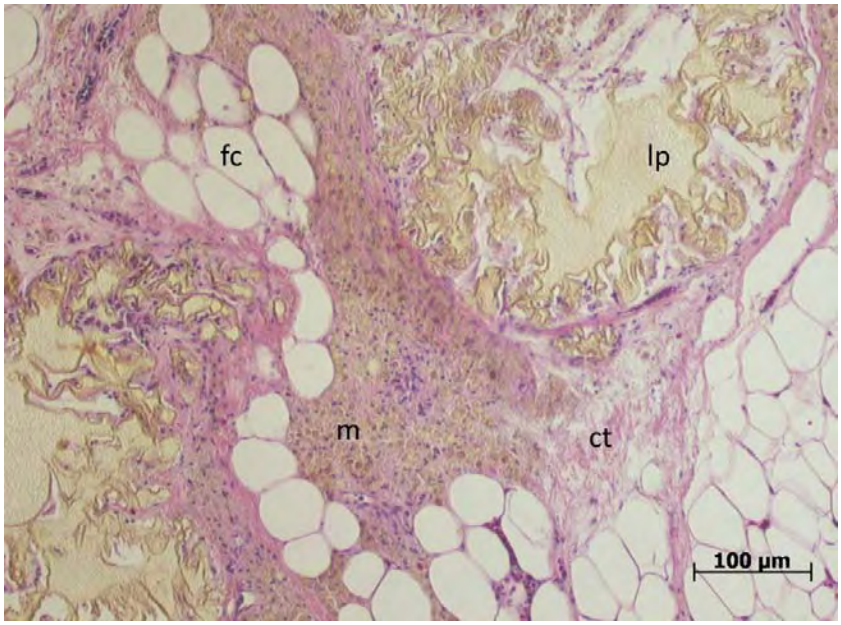

Figure 3.17a: Section of pectoral fat from a catfish sampled from the Olifants Gorge in June 2011 showing extensive extracellular ceroid-type lipopigment (lp) surrounded by connective tissue (ct) and macrophages (m). Adipocytes (fc) (H&E).

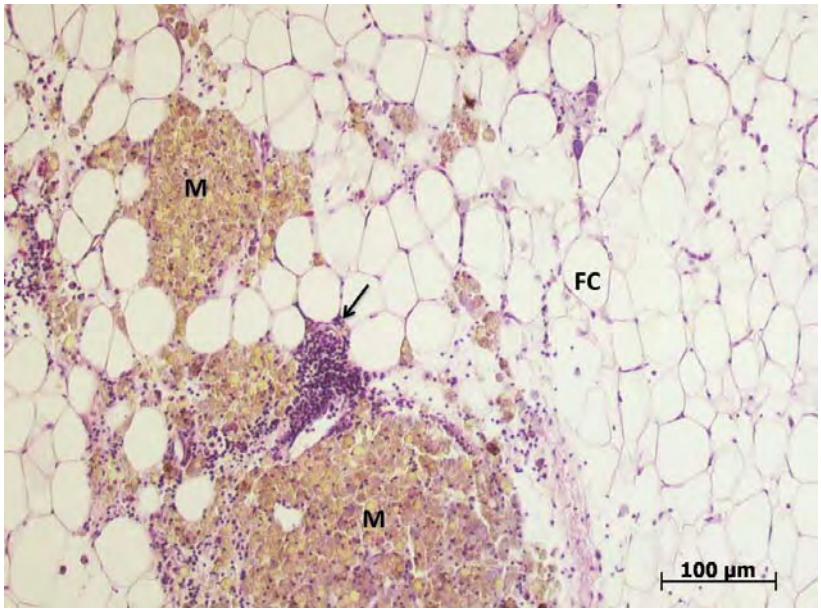

Figure 3.17b: Lipopigment-containing macrophages associated with steatitis in the fat surrounding the brain of a catfish sampled from the Olifants Gorge during June 2011. Focus of mixed inflammatory cells (arrow), macrophages (M), adipocytes (FC) (H&E).

Parasitic granulomata of varying sizes were common in the mesenteric, hypodermal and intermuscular fat but were not observed in the pectoral fat (Figures 3.18a, 3.18b & 3.18c). These granulomata were distinct from granulomata caused by steatitis. Parasitic granulomata showed a greater infiltration of fibroblasts and greater collagen deposition in the capsule than observed with granulomata associated with steatitis. Macrophage clusters on the periphery of parasitic granulomata were less intense and usually in the form of melanomacrophage centres. In haematoxylin and eosin stained sections, these appeared mildly basophilic in colour with variable amounts of brown melanin pigment. Ceroid- and lipopigment-containing macrophages were not generally associated with parasitic granulomata, and were infrequent in the vicinity of parasites in the mesenteric adipose tissues.

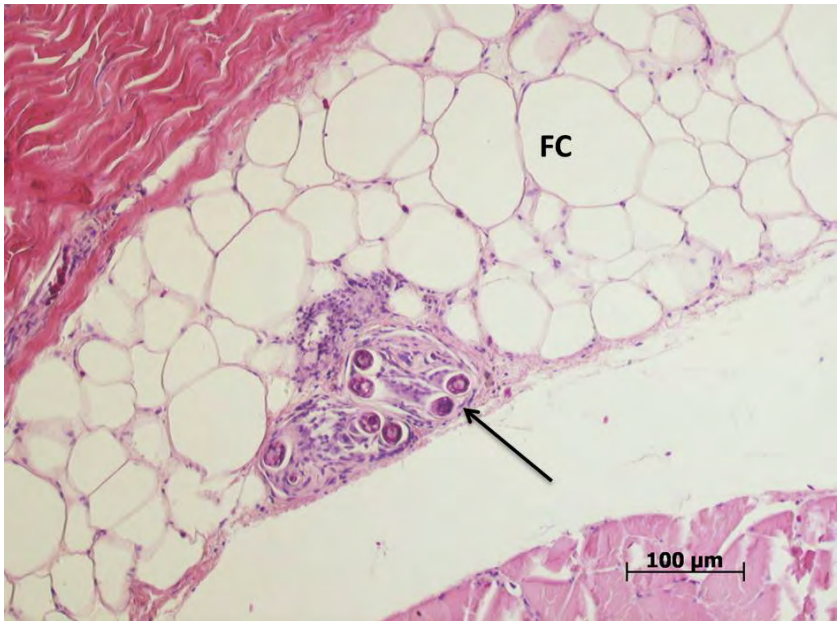

Figure 3.18a: Granuloma caused by migrating larval nematodes (arrow) in the hypodermal fat of a catfish sampled from the Olifants Gorge. Adipocytes (FC) (H&E).

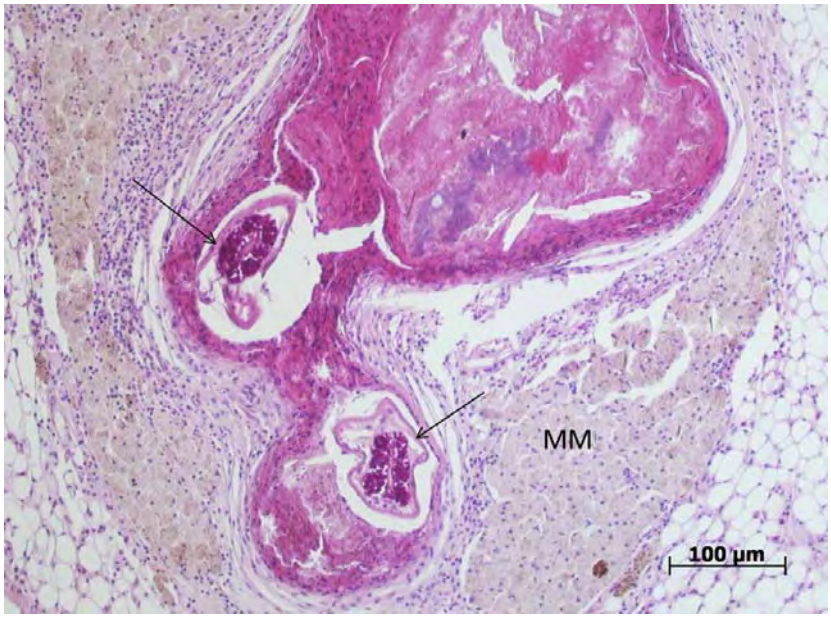

Figure 3.18b: Granuloma formation in the mesenteric fat of a sharptooth catfish associated with larval nematodes (arrows) sampled from Reënvoël Dam. Note, in this case, the extensive melanomacrophage reaction (MM), but absence of lipopigment (H&E).

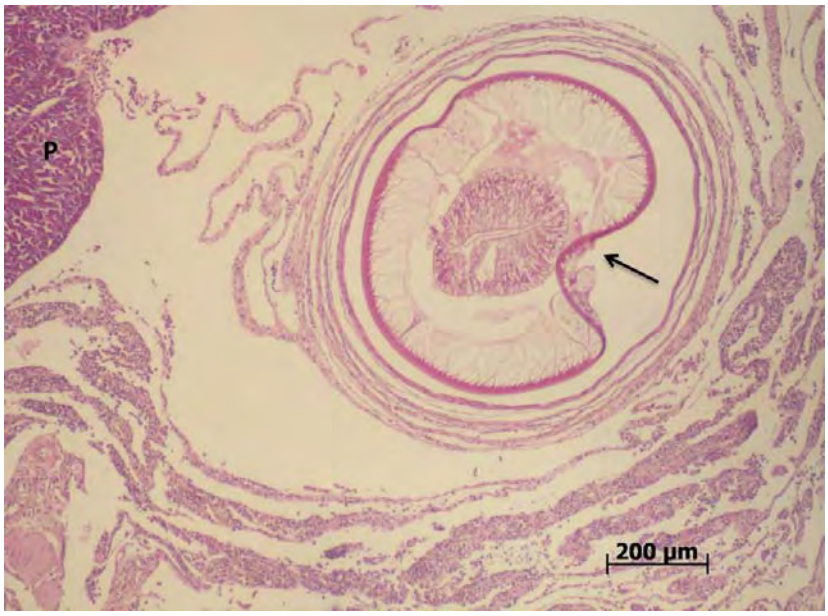

Figure 3.18c: Large encysted nematode larva (arrow) in the mesenteric fat attached to the pancreas (P) of a catfish sampled from van Ryssen Dam (H&E).

3.2.2.2.2. Histopathology of other organs

Varying degrees of hepatic lipidosis, often within distinct foci, were observed in the livers of fish suffering from pansteatitis. Special stains established the presence of ceroid, a golden brown breakdown product of oxidised unsaturated fat, in the hepatocytes of these fish, as well as large amounts of haemosiderin (Figures 3.19a and 3.19b). Presence of haemosiderin was confirmed by use of Perl's Prussian blue stain (Table 3.6). In places, well-demarcated foci of fat vacuolation contained distinctly less iron than in surrounding hepatocytes. In other areas the haemosiderin appeared clumped within the zone of fat vacuolation (Figure 3.19b). Such areas of fat vacuolation were also observed in livers of some catfish from Reënvoël Dam in the absence of pansteatitis.

A large focus of hepatocellular disorganization was observed in the liver of one fish from Lunsklip Fisheries suffering from severe pansteatitis. The affected area showed eosinophilia of hepatocytes and tracts of fibroblasts and associated inflammatory round cells infiltrating the liver parenchyma. The periphery of the focus was demarcated by a zone of melanomacrophage aggregations. The central area of the lesion appeared partitioned by fibrous tracts with islands of hepatocytes undergoing degeneration and necrosis. A clearly demarcated zone of disorganization with enlarged hepatocytes, devoid of pigment, arranged in loose whorls with mild fibroplasia, surrounding dilated vascular spaces was observed occasionally in catfish from Reënvoël Dam and from the Olifants Gorge. Adventitious macrophages were numerous in the livers of fish with pansteatitis, containing large deposits of both ceroid and haemosiderin, imparting a pronounced golden brown colour in the haematoxylin and eosin stained sections, with the macrophages staining strongly for iron with Perl's Prussian blue stain (Table 3.5). Melanomacrophages in all organs of older fish were replete with melanin. Variable numbers of inflammatory cells associated with ducts and blood vessels were observed in the livers of older fish particularly.

Granulomata associated with well-encapsulated larval nematodes of varying sizes were a common histological finding in many liver sections of fish from both the Olifants Gorge and Reënvoël Dam and other sampling sites in KNP, but were not observed in livers of catfish from Lunsklip Fisheries. These granulomata were characterised by a central cavity, filled with amorphous tissue debris, surrounded by a fibrous capsule of varying thickness. The nematode larvae could either be observed on the perimeter of the cavity or within the connective tissue capsule. Although some cysts were surrounded by a variable mixed

inflammatory cell reaction, melanomacrophages were usually absent from these reactions (Figure 3.20).

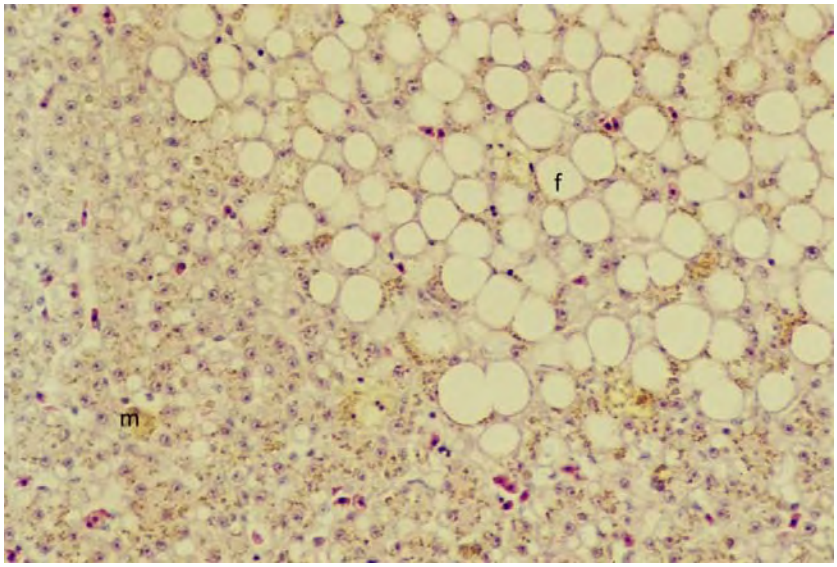

Figure 3.19a: Liver section of a catfish sampled from the Olifants Gorge during November 2009. Note distinct focus of fat vacuoles (f) (H&E, X200).

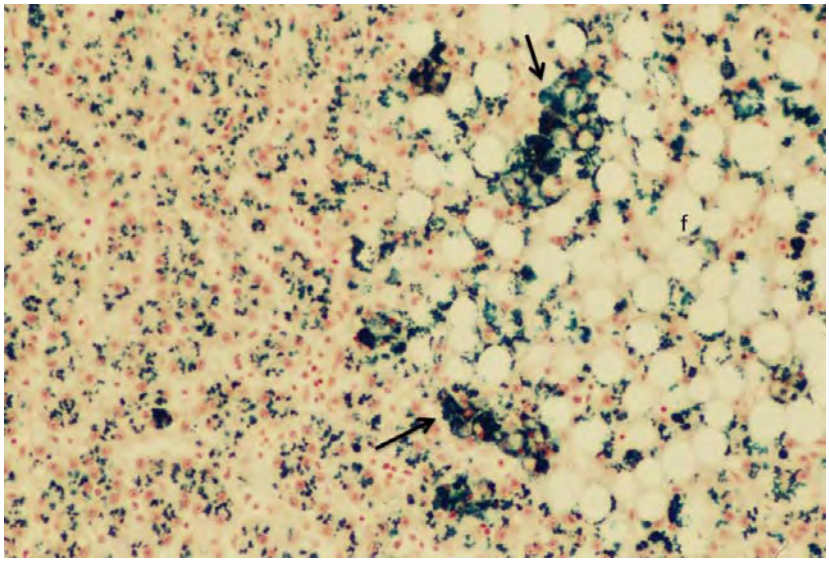

Figure 3.19b: Liver section of a catfish sampled from the Olifants Gorge during November 2009. Note distinct focus of fat vacuoles (f) and clustering of haemosiderin (arrows) on the perimeter of this focus (Perl's Prussian blue, X200).

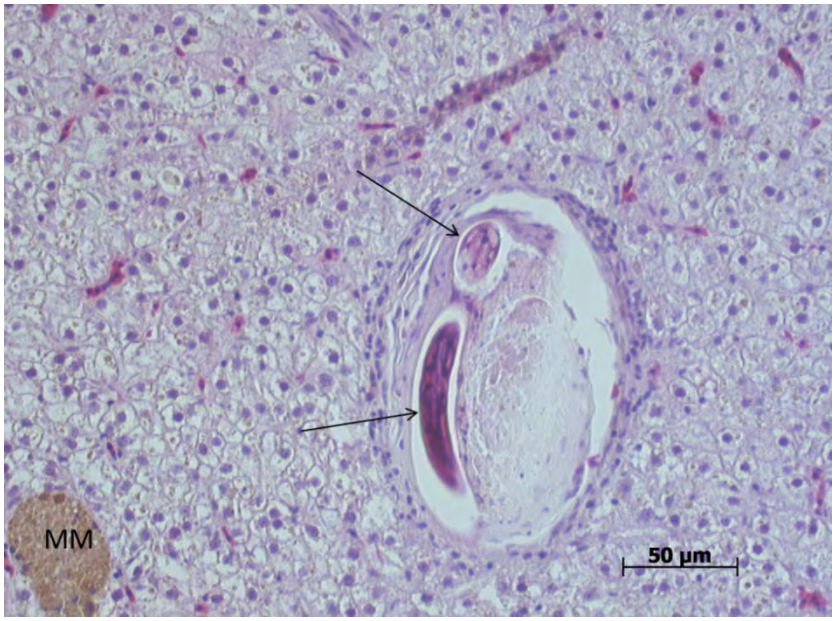

Figure 3.20: Granuloma in the liver of a catfish sampled from the Levuvhu River containing larval nematodes (arrows). Note amorphous tissue debris within the cyst adjacent to the parasites. Melanomacrophage centre (MM) (H&E).

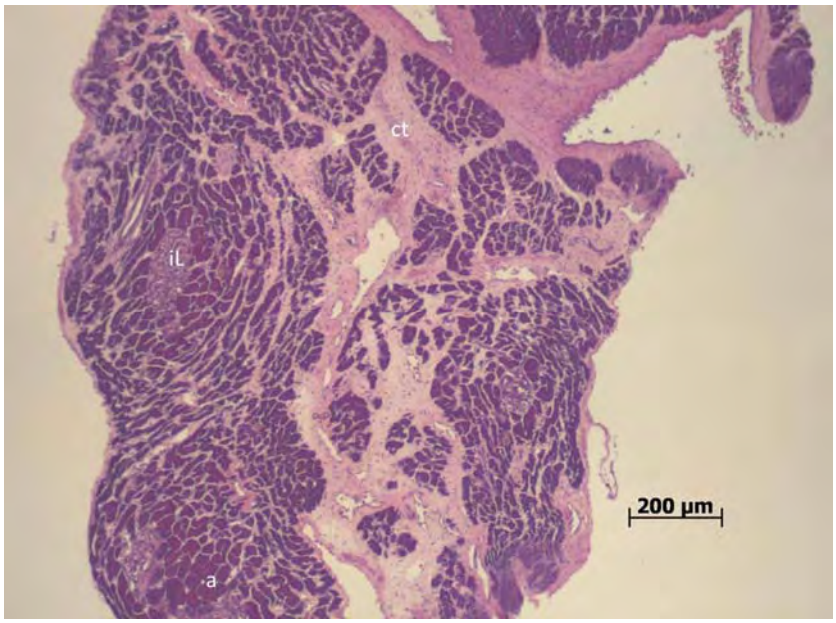

Figure 3.21: Pancreatic atrophy in a catfish suffering from pansteatitis, sampled from the Olifants Gorge during June 2011. Note prominence of connective tissue (ct) extending between groups of acinar cells (a). Islet of Langerhans (iL) (H&E).

Pancreatic acinar and islet of Langerhans cells appeared normal in all of the fish. The variable prominence of pancreatic tissues noted macroscopically was explained by atrophy of the organ, which was most prominent in fish affected by pansteatitis (Figure 3.21). Macrophages associated with the pancreas of fish suffering from pansteatitis did not contain haemosiderin. No specific pathology was observed in the intestines of the sampled fish.

In fish from all sites, variable numbers of focally disseminated clusters of dense basophilic lymphocytes were noted in the cranial and caudal kidney representing variation in the normal lymphocytic tissue within this organ. Melanomacrophages in the kidneys of fish with pansteatitis were replete with ceroid. Haemosiderin was also observed in the macrophages of the kidney in fish with pansteatitis but to a lesser extent than in the spleen and liver (Table 3.5). Positive Perl's staining material was observed in some of the renal tubular epithelial cells of such fish (Table 3.6).

The spleens of fish from various sites were variable in appearance depending on the numbers of erythrocytes held in the splenic sinusoids. Melanomacrophages in the spleen of fish with pansteatitis were replete with ceroid, and the splenic macrophages also carried large amounts of haemosiderin (Table 3.5).Encapsulated necrotic foci from degenerating parasites within the spleen were observed in two fish from the Olifants Gorge. Multiple cyst-like mineralized foci were observed in the spleen of one fish that was not suffering from pansteatitis. Small unidentified coccidian-type intracellular parasites were observed in macrophages within melanomacrophage centres of both the spleen and kidney of fish from the Olifants River and from Reënvoël Dam. These parasites were not observed in fish from Lunsklip Fisheries. No pathology was associated with the presence of this parasite.

Multiple focal cyst-like structures that appeared to be of thyroid origin were observed in the hearts of two fish with pansteatitis from Lunsklip Fisheries. The structures appeared to be lined by an epithelium and were filled with homogenous eosinophilic material. Myocardial lesions were not observed in fish from other sampling sites. No signs of fat necrosis could be detected in epicardial fat cells, where these were present on the hearts of sampled fish.

Gills in the catfish specimens collected in the Olifants Gorge during January 2009 presented with a two to three-fold increase in the thickness of the epithelium of the secondary lamellae. In many of these specimens the epithelial hyperplasia increased towards the base of the

secondary lamellae, imparting a wedge-shaped appearance. Such changes were less evident in fish sampled from the Olifants Gorge in November 2009, and during later samplings gills showed minimal signs of hyperplasia. Monogenean trematodes were occasionally visible between the secondary lamellae of the gills. Some fish showed deformity and hyperplasia of the cartilage of the primary gill lamellae as a result of infection with a digenean trematode, possibly *Centrocestus formosanus* (Nishigori) (Figures 3.22a & 3.22b). These parasites could be observed lying within cysts in the gill cartilage, where they appeared to feed off chondrocytes, causing considerable damage to the gill cartilage. Infection with this parasite and resultant gill cartilage deformity was also common in fish sampled from Reënvoël Dam. Only mild hyperplasia of the gill epithelium was evident in some fish from Lunsklip Fisheries; however the absence of digenean gill parasites from these fish was notable.

No lesions were observed in the brains of fish from any of the sampling sites. At some sampling sites large numbers of digenean trematodes (Fam. Diplostomidae) were observed surrounding the brain and within the brain fat (Figure 3.23). An inflammatory reaction with presence of macrophages was observed in association with these parasites, but they were never observed penetrating the brain tissues. There was no correlation between parasite presence and pansteatitis in the brain fat.

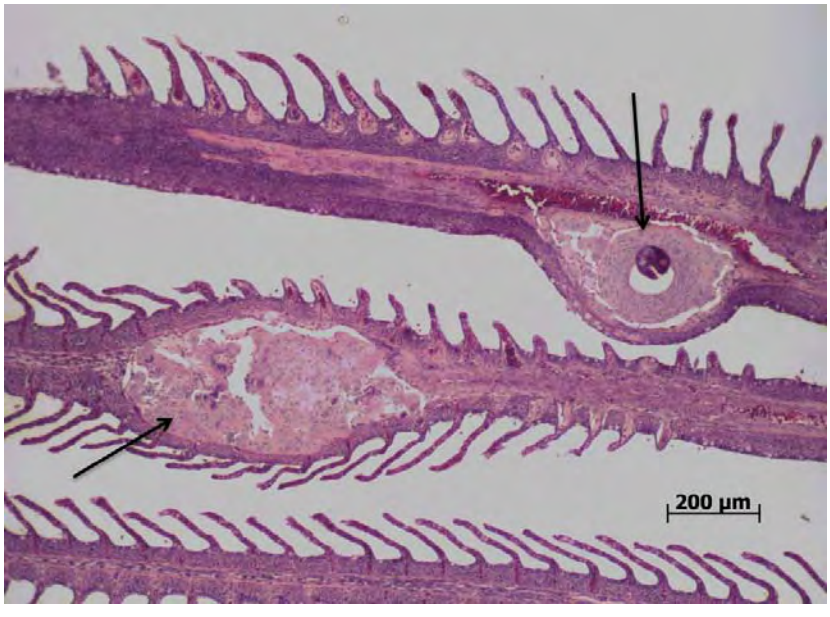

Figure 3.22a: Digenean trematode larva encysted within the cartilage of the primary lamellae of a catfish sampled from the Olifants Gorge during June 2011. Note hyperplastic changes in the cartilage (arrows) (H&E).

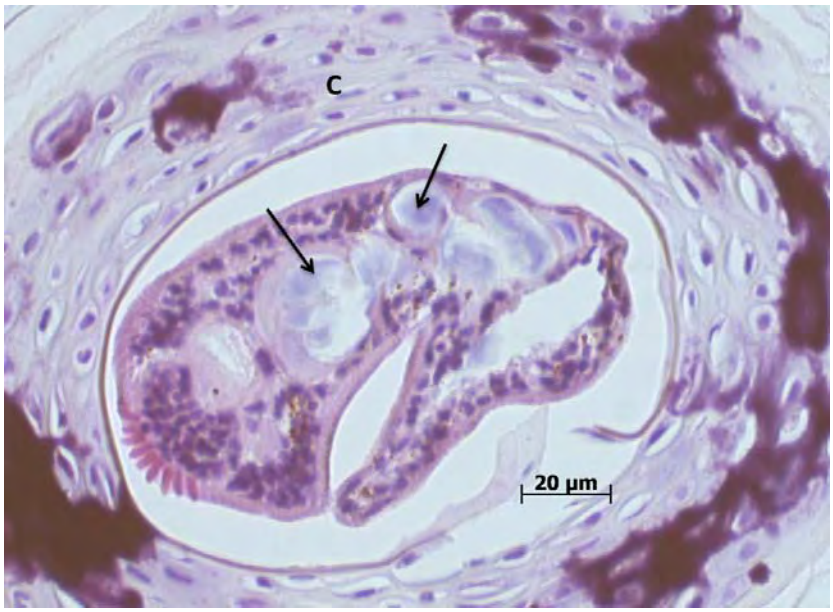

Figure 3.22b: Enlarged view of a digenean trematode, probably a metacercaria of *Centrocestus formosanus*, encysted in the gill cartilage (C) of a catfish from the Olifants Gorge. Note the ingested chondrocytes (arrows) (H&E).

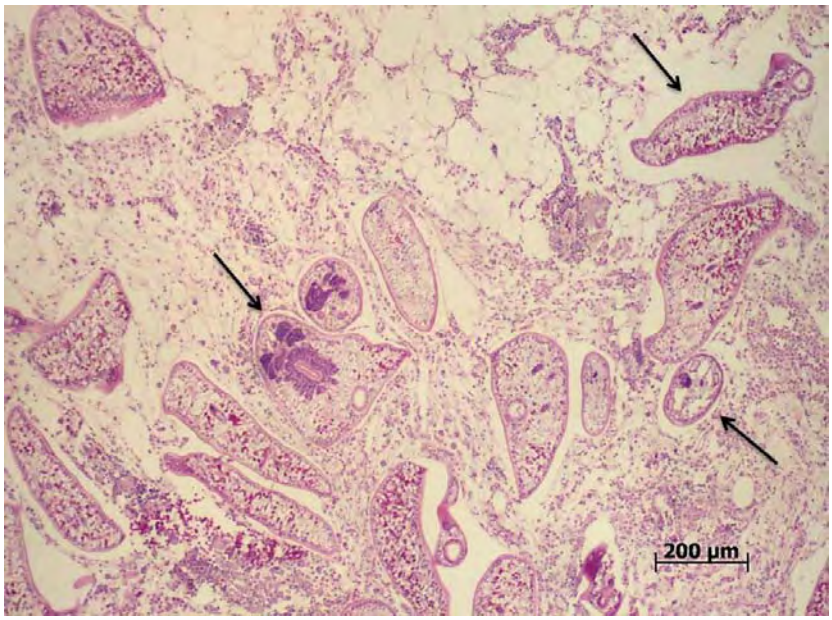

Figure 3.23: Digenean trematodes of the family Diplostomidae (arrows) within the cerebrospinal space adjacent to fat lining the cranium of a catfish sampled from Engelhard Dam during July 2010. Note the cellular reaction associated with presence of these parasites (H&E).

Maturity of gonads observed in sampled fish depended on age and sampling season. Older female fish showed large numbers of melanomacrophages within the ovaries. Sections of the ovaries of fish with pansteatitis showed numerous adventitious macrophage aggregates containing large amounts of haemosiderin (Table 3.5). In testicular tissue, melanomacrophages were seldom noted, and if present were devoid of haemosiderin. Gonadal development in all cases appeared to be normal, and no pathology was noted within the gonads. Development of intersex was not observed in sampled fish.

Muscle atrophy was observed in some fish suffering from pansteatitis. Other than the presence of parasitic cysts, no other pathology was observed in muscle tissue. The fibrous and round cell inflammatory reaction associated with parasites depended on type and stage of parasite but was not correlated with presence of steatitis in the intermuscular fat. Macrophages in the hypodermis did not contain haemosiderin in pansteatitis-affected fish. No specific pathology was observed in the skin of sampled fish.

Table 3.5: The staining properties of macrophages in various tissues of catfish with pansteatitis sampled from the Olifants Gorge in November 2009 (OG). Perl's Prussian blue. Staining intensity on a scale of 1 to 5 expressed as mean of the sample size n

Sampling site	Liver	Spleen	Cranial kidney	Caudal kidney	Ovary	Testes	Pancreas	Hypo-dermis	Fat	Brain
	(n11)	(n9)	(n3)	(n9)	(n3)	(n4)	(n3)	(n6)	(n11)	(n2)
OG	3.90	3.86	1.50	1.79	4.00	0.00	0.00	0.00	0.00	0.00

Table 3.6: Perl's Prussian blue staining properties of hepatocytes and renal tubular epithelial cells in catfish with pansteatitis sampled from the Olifants Gorge in November 2009 (OG). Perl's Prussian blue. Staining intensity on a scale of 1 to 5 expressed as mean of the sample size n

Sampling site	Hepatocytes (n11)	Tubular epithelial cells (n9)
OG	3.73	1.17

3.2.2.3. Statistical analysis

The numerical scores assigned to descriptive observations were used to detect differences between various pathology parameters and presence or absence of pansteatitis. Splenomegaly was observed frequently in catfish suffering from pansteatitis, and a significant effect of pansteatitis on spleen size was noted (Chi-squared Test, p=0.015). Pancreatic atrophy was often noted in fish suffering from pansteatitis but there was no significant difference in catfish with and without pansteatitis. There was no effect of parasitosis on incidence or severity of pansteatitis. A comparison of the severity of steatitis lesions in the mesenteric fat of catfish showed significant differences between the Olifants Gorge test sites and the reference sites (Kruskal-Wallis ANOVA by ranks, p<0.001), with the degree of severity greatest in catfish from Lunsklip Fisheries (Figure 3.24).

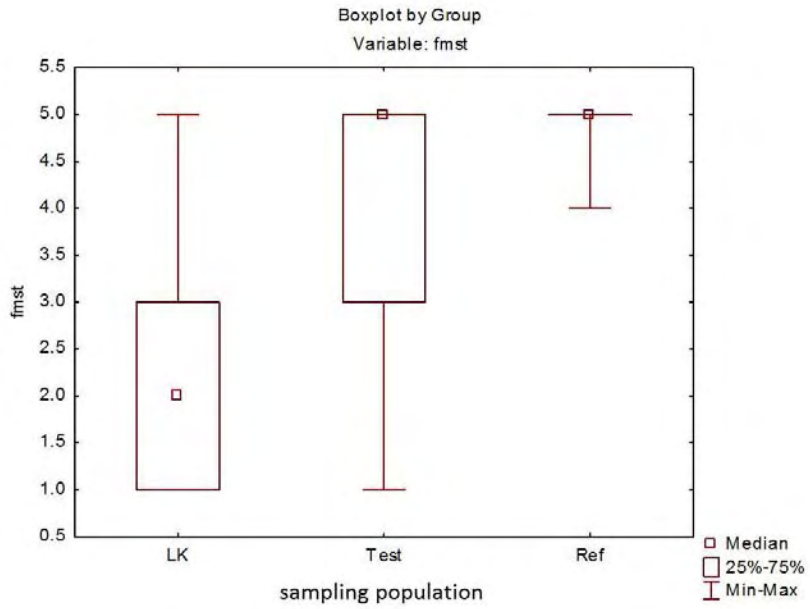

Figure 3.24: Comparison of steatitis severity in mesenteric fat of catfish sampled from LK (positive reference site), Test (all OG sites) and Ref (RV and RVB negative reference sites). Fmst=steatitis severity in mesenteric fat on a scale of 1 to 5 with 1 representing severe steatitis and 5 representing absence of steatitis.

There was no significant difference in degree of hepatic lipidosis between catfish with and without pansteatitis within the Olifants Gorge population of catfish. But when comparing all catfish sampled from Olifants Gorge to the catfish from Reënvoël Dam, a significantly higher proportion of the Olifants Gorge fish showed hepatic lipidosis (Kruskal Wallis Test, p<0.001), whereas there was no significant difference in hepatic lipidosis between fish from Lunsklip Fisheries and Reënvoël Dam. (Figure 3.25).

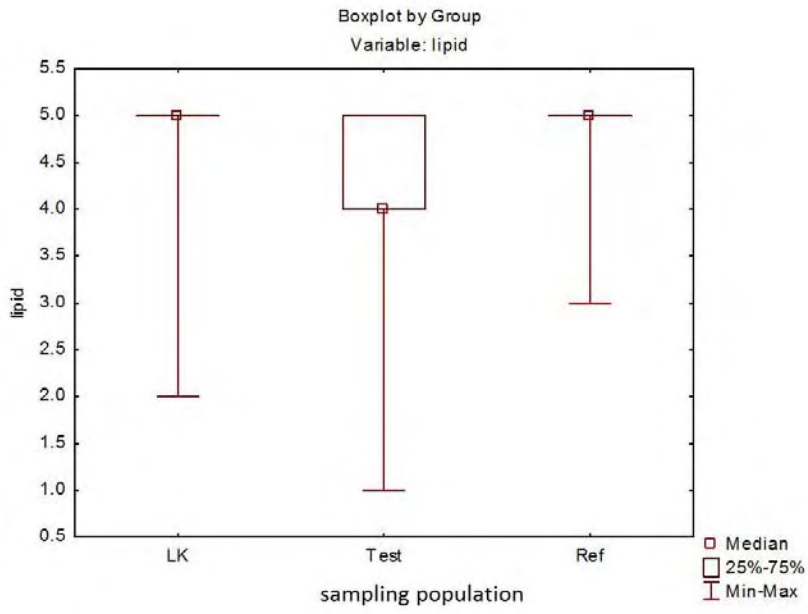

Figure 3.25: Comparison of hepatic lipidosis incidence between LK (positive reference site), Test (all OG sites) and Ref (RV & RVB negative reference site). Lipid=hepatic lipidosis on a scale of 1 to 5 with 1 representing severe lipidosis and 5 representing absence of lipidosis.

Within the Olifants Gorge population there was no significant difference in the amount of pigment (haemosiderin and ceroid) stored by hepatocytes between catfish with and without pansteatitis. In the livers of fish with pansteatitis, no association could be shown between pigment accumulation and age (Mann Whitney-U test, $p=0.89$), whereas in catfish from Reënvoël Dam, pigment accumulation in hepatocytes increased significantly with age (Mann Whitney-U test, $p<0.001$). Pigment accumulation in hepatocytes also increased significantly with age when healthy fish, from all sites sampled, were included in the analysis (Mann Whitney-U test $p<0.001$). A comparison of hepatocyte pigment accumulation in catfish between sites indicated a significantly lower pigment accumulation in catfish from Lunsklip Fisheries (Kruskal Wallis ANOVA by ranks, $p=0.006$) when compared with fish from the Olifants Gorge and Reënvoël Dam sites, whereas there was no overall difference between fish from the Olifants Gorge and Reënvoël Dam sites (Figure 3.26).

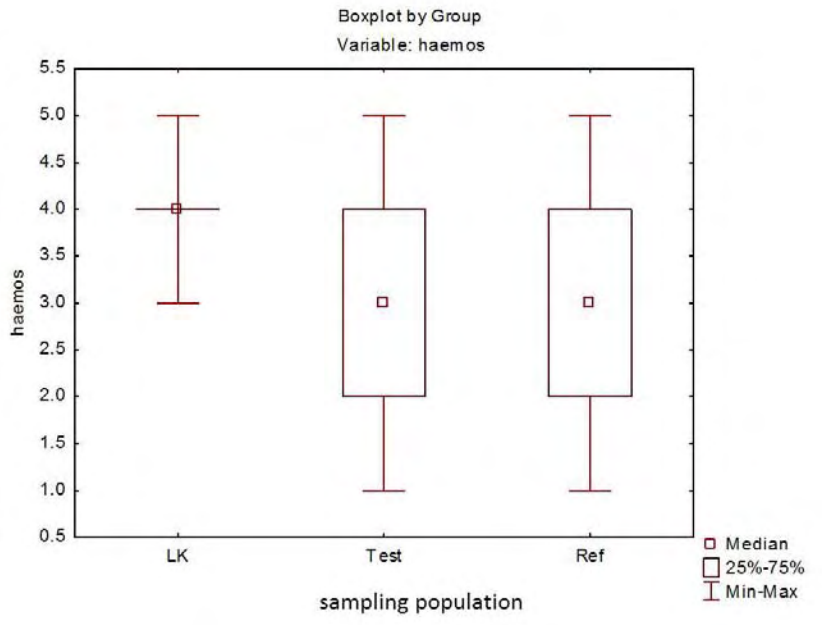

Figure 3.26: Comparison of level of pigment (haemosiderin and ceroid) stored by hepatocytes between LK (positive reference site), Test (all OG sites) and Ref (RV and RVB negative reference site). Haemos=hepatocyte pigment on a scale of 1 to 5 with 1 representing severe accumulation and 5 representing absence of pigment.

The degree of basophilia and compactness of hepatocytes as well as the presence of vacuoles and vesicles causing distension of hepatocytes was used to gauge the level of metabolic activity in the liver. There was no significant difference in the metabolic activity of hepatocytes between catfish with and without pansteatitis in the Olifants Gorge. A comparison between sites indicated significantly greater metabolic activity in the catfish from Lunsklip Fisheries (Kruskal-Wallis ANOVA by ranks, $p<0.001$). Some of the Olifants Gorge fish showed an equivalent degree of metabolic activity, although there were no significant differences between metabolic activity in the livers of fish from the Olifants Gorge and Reënvoël Dam (Figure 3.27).

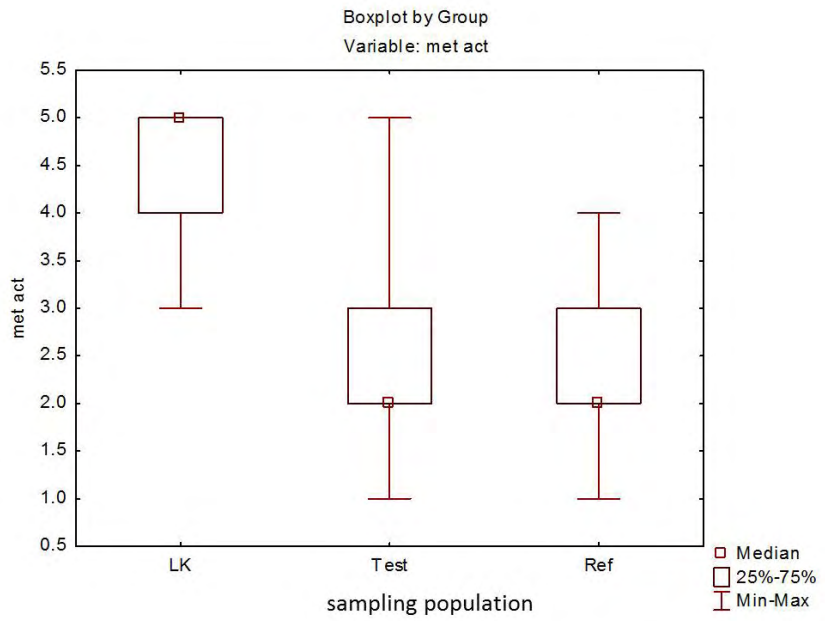

Figure 3.27: Comparison of metabolic activity of hepatocytes of catfish between LK (positive reference site), Test (all OG sites) and Ref (RV and RVB negative reference site). Met act=metabolic activity on a scale of 1 to 5 with 1 representing compact inactive hepatocytes and 5 representing distended vacuolated hepatocytes.

There were no significant differences in the median intensity of structural disorder of the livers when catfish from the Olifants Gorge were compared to catfish from Lunsklip Fisheries and Reënvoël Dam. Although not statistically significant, the worst degree of structural disorder of the liver was observed in a small number of catfish from Lunsklip Fisheries (Figure 3.28). There was no variation in bile duct fibroplasia between fish with and without pansteatitis or between sites.

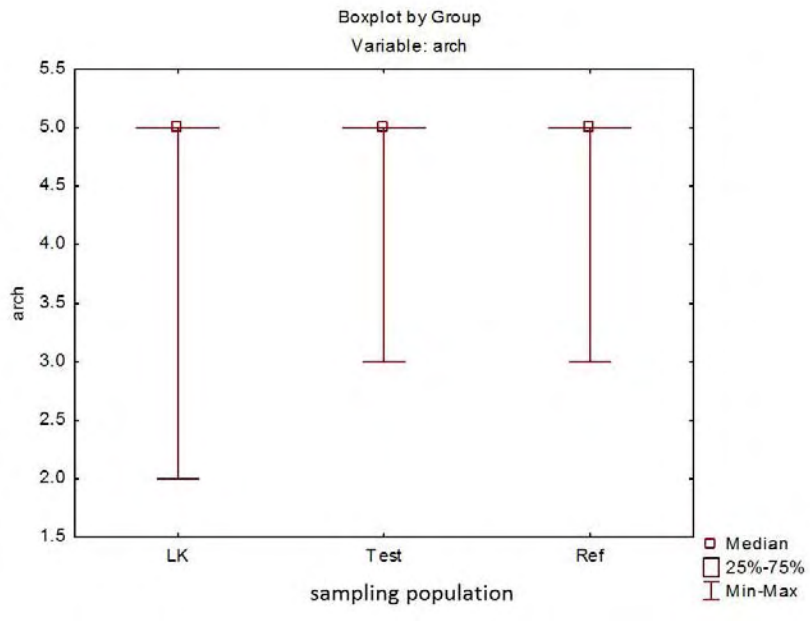

Figure 3.28: Comparison of structural disorder in the liver of catfish between LK (positive reference site), Test (all OG sites) and Ref (RV and RVB negative reference site). Arch=structural disorder on a scale of 1 to 5 with 1 representing a severe degree of disorder and 5 representing normal hepatic structure.

Livers from catfish from Lunsklip Fisheries showed the greatest number of vacuolated foci. A significant difference in intensity of vacuolated foci in the livers of fish from the Olifants Gorge with and without pansteatitis could not be detected. Although there was no significant difference in the means between the Olifants Gorge and Reënvoël Dam sites, there was a significant difference between the Olifants Gorge and Lunsklip Fisheries sites (Kruskal-Wallis ANOVA by ranks, $p<0.001$) (Figure 3.29).

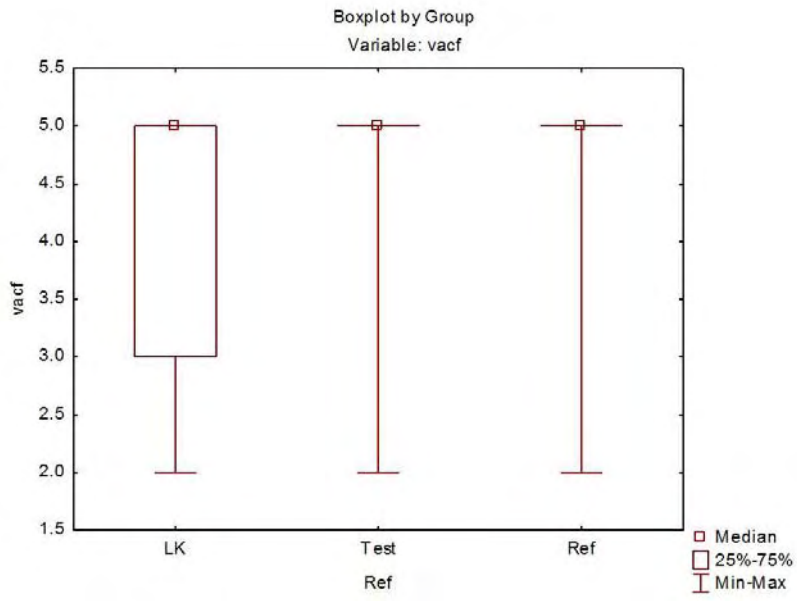

Figure 3.29: Comparison of vacuolated foci in the liver of catfish between LK (positive reference site), Test (all OG sites) and Ref (RV and RVB negative reference site). Vacf=presence of vacuolated foci on a scale of 1 to 5 with 1 representing numerous foci and 5 representing absence of foci.

There was no significant difference in intensity of melanomacrophage centres in the livers of catfish between the Olifants Gorge, Lunsklip Fisheries and Reënvoël Dam sites (Figure 3.30). Using the Mann-Whitney U test, there was also no significant variation in the intensity of melanomacrophages in the liver, spleen and gonad when catfish with and without pansteatitis were compared, although catfish with pansteatitis showed a negative correlation (p=0.003) between intensity of melanomacrophages in the kidney and presence of pansteatitis.

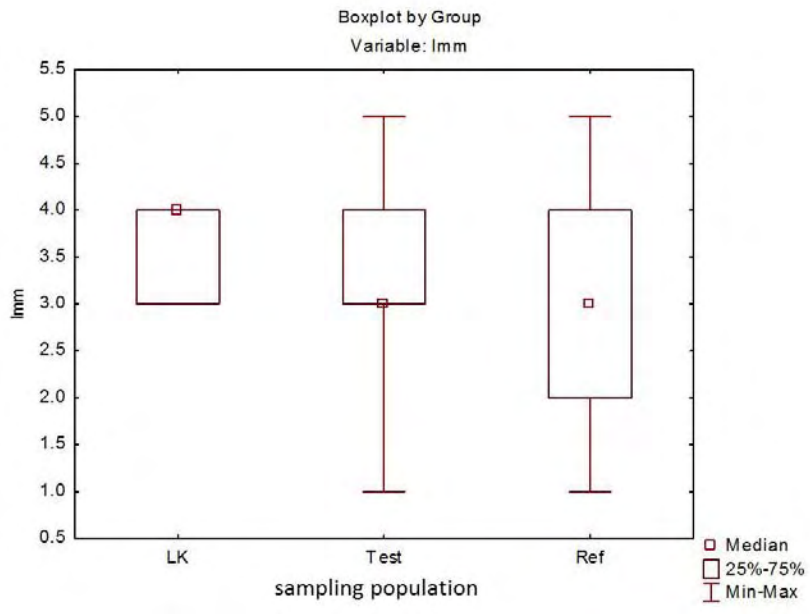

Figure 3.30: Comparison of melanomacrophage centre intensity in the liver of catfish between LK (positive reference site), Test (all OG sites) and Ref (RV and RVB negative reference sites). Lmm=melanomacrophage intensity on a scale of 1 to 5 with 1 representing numerous melanomacrophage centres and 5 representing absence of melanomacrophage centres.

Regression analysis showed a significant increase in the intensity of liver melanomacrophage centres with age ($p<0.001$) in the Reënvoël Dam reference catfish but not in the Olifants Gorge or Lunsklip Fisheries catfish, both populations with pansteatitis prevalence.

3.2.2.4. Discussion of descriptive pathology

In catfish from the Olifants Gorge, significant pathology was restricted to the adipose tissues, with the most intense and frequent lesions being in the mesenteric fat. Further changes observed in the liver and spleen appeared to be secondary to fat necrosis. Necrosis of the adipose tissue resulting in steatitis was repeatedly observed and was a consistent indicator of oxidative stress.

For many species the term “yellow fat disease” is used to describe pansteatitis. The fat of sharptooth catfish is distinct from that of other fish species in that a variation in colour of the mesenteric adipose tissues appears to be normal. Fat colour can thus not be used as an indication of lipid peroxidation as in other species. Pearson, Chinabut, Karnchanakharn and Somsiri (1994) have, however, described jaundice in sharptooth catfish hybrids associated

with lipoid liver degeneration, following on feeding of rancid chicken viscera, and this resulted in pale yellow discoloration of the body fat and gills. During the study, specific pathology relating to lipid autoxidation and pansteatitis was also observed in a captive population of sharptooth catfish suffering from known nutritionally-induced pansteatitis. In these fish severely affected fat took on a grey-brown colour rather than the yellow colour observed in wild catfish, and jaundice was not present.

Where pansteatitis was present in catfish, the mesenteric adipose tissues were most severely affected. Steatitis was only occasionally observed in other fat depots and this may relate to specific adipogenic and lipolytic factors associated with the differing functions of these fat tissues. The pectoral adipose tissue is embedded in a mesenchyme connective tissue matrix and appears to protect the lobes of the cranial kidney and liver where they lie outside of the abdominal cavity in a hypodermal position. The intracranial fat, also occasionally affected by steatitis, is a fluid filled loose tissue surrounding the brain. Large numbers of digenean trematode metacercariae were often in close proximity to this fat but appeared to have little effect on the tissue. The intermuscular fat was affected with steatitis in only a few fish in both the captive fish from Lunsklip Fisheries and the fish from the Olifants Gorge. The variable size of the mesenteric fat tissue suggests that this is the most labile and metabolically active fat depot in the body. It is not clear why this tissue is more prone to developing steatitis in catfish.

Catch methods imposed by the environment in the Olifants Gorge (presence of hippos and crocodiles) have limited most samplings to catching by baited hook and line. The most severely affected fish may not have taken bait and would have been easy prey for crocodiles. This favoured sampling of relatively healthy fish whereas the worst affected fish remained under-represented. Despite this, significant numbers of fish with pansteatitis were caught repeatedly in the Olifants Gorge and on the single occasion in the Sabiepoort.

Teleost hepatocytes can carry considerably more glycogen and lipid without showing nuclear degeneration than can mammalian hepatocytes (Stoskopf 1993), and fish have the capacity to store large amounts of lipid without ill effect. Hepatic lipid accumulation has been associated with exposure to toxicants and particularly with lipid peroxidation following on feeding of diets high in polyunsaturated lipids and/or with suppression of vitamin E (Wolf & Wolfe 2005). Various degrees of hepatic lipidosis and ceroidosis were observed in catfish with

severe pansteatitis; however, no correlation could be demonstrated between hepatic lipidosis and pansteatitis.

The degree of vacuolation in hepatocytes reflects imbalances in energy intake and expenditure, providing an indication of the metabolic state of the fish. During periods of starvation in fish, this may reflect preservation of hepatic glycogen (Wolf & Wolfe 2005). Exposure to organic toxicants resulting in sub-lethal injury to hepatocytes can also cause vacuolation of hepatocytes as a result of lipid-filled vacuoles (Hayes 2004). Presence of vacuolated foci in hepatic tissues of fish may indicate chronic hepatic injury in polluted waters, particularly in benthic fish (Gingerich 1982). The greater number of vacuolated foci observed in the livers of catfish from Lunsklip Fisheries may have been related to the severe degree of pansteatitis, and the obesity observed in the majority of these fish, caused by the excessive feeding of rancid, oil-rich fish waste.

Relative to cardiac output, the perfusion of the teleost liver is low, limiting exposure of the basal membrane of the hepatocyte to toxicants (Wolf & Wolfe 2005). Contributing to the stasis of chemicals and metabolites in the hepatobiliary system of fishes is the slow bile flow, which in rainbow trout is about 50 times slower than in mammals (Gingerich 1982). This stasis of chemical compounds and their metabolites in the liver and the propensity of chemicals excreted in the bile to become incorporated into enterohepatic cycling can cause prolonged hepatotoxic effects in fish (Gingerich 1982). Furthermore it is thought that the close association of the biliary system with hepatocytes in fish is responsible for the high incidence of peribiliary damage to hepatocytes and biliary epithelial cells with exposure to toxicants (Hinton & Laurén 1990). It is a well-established fact that many xenobiotics exert their harmful effects through oxidative damage to phospholipid structures in various organs and tissues. Exposure to such pollutants would be expected to result in detectable pathology particularly in the liver. There was, however, no significant correlation between pansteatitis incidence and hepatic lipidosis or peribiliary fibrosis within the Olifants Gorge population of catfish. When fish from the Olifants Gorge were compared to fish from Reënvoël Dam, a significantly higher proportion of the Olifants Gorge fish showed hepatic lipidosis ($p<0.001$), whereas there was no significant difference in hepatic lipidosis between fish from Lunsklip Fisheries and Reënvoël Dam. This may have reflected different feeding activity in the respective fish.

Melanomacrophage centres are groups of distinct pigment-containing cells that are a unique feature of the lymphomyeloid tissue of fish (Kennedy-Stoskopf 1993) and are generally found in the reticulo-endothelial supporting matrix of haemopoietic tissues (Agius & Roberts 2003). In teleosts they occur in both hepatic and extra-hepatic tissues and represent possible forerunners of the germinal centres in the spleen and lymph nodes of higher animals (Agius 1979). Melanomacrophage centres are known to increase in size and frequency with exposure to environmental stress and may increase with exposure to organic chemicals (Agius & Roberts 2003; Metcalfe 1999; Wolf & Wolfe 2005). Interpretation of such increases in melanomacrophages must be done with care as these increases also occur physiologically with age and during periods of starvation (Agius & Roberts 2003; Hinton & Laurén 1990; Wolf & Wolfe 2005). Melanomacrophages are thought to sequester and detoxify endogenous and exogenous substances. Apart from melanin and haemosiderin, they are known to carry lipogenic pigments (lipofuscin and ceroid) representing effete cellular components. These, as in the case of haemosiderin, may also increase during catabolic states such as starvation and with follicular atresia (Agius & Roberts 2003; Kennedy-Stoskopf 1993). In a comparison of catfish from the Olifants Gorge, Reënvoël Dam and Lunsklip Fisheries, no significant correlations were found between mean liver melanomacrophage intensity and site. The wide ranging standard deviation in the results from both the Olifants Gorge and Reënvoël Dam reflected a high degree of variation in intensity of melanomacrophages. Regression analysis showed significant correlation between age and intensity of liver melanomacrophage centres ($P<0.001$) in the Reënvoël Dam reference catfish but not in the Olifants Gorge or Lunsklip Fisheries catfish, both populations with pansteatitis prevalence.

Perl's Prussian blue stain demonstrates presence of ferric iron. Agius (1979) investigated the pattern of iron storage in the melanomacrophage centres in various organs of 14 different species of healthy and diseased fish. The spleen was the main organ of iron storage by melanomacrophages, whereas melanomacrophage centres in the liver and kidney were found to store insignificant amounts of iron. Whilst certain diseases including pansteatitis resulted in accumulation of iron in splenic macrophage centres, the same did not happen in hepatic and renal macrophage centres (Agius 1979). In contrast to the findings of Agius (1979), melanomacrophages from both the spleen and liver of fish suffering from pansteatitis in the Olifants Gorge appeared to be storing large amounts of iron, and even renal melanomacrophages contained obvious amounts of iron, albeit less than in the liver. Such ferric iron compounds may be derived predominantly from haemoglobin catabolism (Moccia

et al. 1984), in which case, bound to transferrin or sequestered as haemosiderin, the iron is well tolerated by the liver (Hayes 2004). In the Olifants Gorge catfish, statistical analysis showed no correlation between pigment accumulation by hepatocytes and incidence of pansteatitis. While adventitious macrophage aggregations in the ovaries contained large amounts of iron, macrophage aggregations associated with fat cell necrosis in the adipose tissues, and macrophage centres in the pancreas, testes and hypodermis did not contain iron. It is not clear whether the iron deposits represent increased iron storage as haemosiderin due to excessive haemolysis or whether they are indicative of abnormal uptake of iron from the environment. However, Baker, Martin and Davies (1997) have demonstrated heightened oxidative stress in *C. gariepinus* ingesting abnormally high levels of iron under experimental conditions. The clustering of haemosiderin around the perimeter of fat accumulation in livers of fish with pansteatitis is interesting in that redox cycling of iron has been implicated as a cause of iron-catalysed lipid peroxidation (Minotti & Aust 1992).

Splenomegaly was a consistent finding in fish with pansteatitis and was significantly correlated with presence of steatitis ($p=0.015$), and splenic haemosiderosis may have been indicative of increased haemoglobin catabolism. There was no correlation between pancreatic atrophy and pansteatitis incidence. Pancreatic atrophy may simply have reflected episodes of reduced feeding.

Myopathy, as described in association with pansteatitis and vitamin E deficiency in other species (Ginn *et al.* 2007; Murai & Andrews 1974; Roberts *et al*. 1979; van Vleet & Valentine 2007), was not observed in catfish with pansteatitis from either the Olifants Gorge or Lunsklip Fisheries. This may reflect adequate dietary intake of vitamin E, but may also have been influenced by the selenium status of the fish. Many fish with pansteatitis were in remarkably good condition. A few fish with pansteatitis did show muscle wasting, but on histological examination myopathy was not present.

Compared to control fish, catfish from most sampling sites carried heavy burdens of parasites. Frequent and varied pathology associated with parasites was observed in most of the wild caught fish and varied between sampling sites depending on parasite burdens and prevalence of specific parasites. Despite the associated pathology, presence of parasites appeared to be well tolerated by the fish. Fish from Reënvoël Dam, a population where pansteatitis could not be demonstrated, showed the heaviest parasite burdens. Focal steatitis

with minimal lipopigment formation was observed only infrequently in association with parasites and no correlation could be demonstrated between parasite burden and pansteatitis. The steatitis described in association with lipidosis and streptococcosis in cultured silver perch (Deng *et al*. 2012), was similarly characterised by an absence of ceroid within the necrotic lesions in fat deposits observed in various organs. It is interesting to note the presence of metacercariae of *Centrocestus formosanus* in gills of catfish from KNP, as spread of this zoonotic parasite has been associated with introduction of carp from Asia elsewhere (Vélez-Hernández, Constantino-Casas, García-Márquez, & Osorio-Sarabia 1998) and as far as the author is aware this is the first preliminary recording of the occurrence of this parasite in South Africa.

3.2.3. Haematology and blood chemistry in pansteatitis in catfish

3.2.3.1. Blood smear examinations

Examination of blood smears taken from catfish collected from the Olifants Gorge soon after the mass crocodile mortality in the winter of 2008 revealed an increase in numbers of immature erythrocytes and erythrocytes with irregular cell shapes and crenated cell membranes in many of the fish. Nuclear shapes were similarly irregular with a high prevalence of chromatin clumping visible within the nuclei in some blood smears. As the changes were not restricted to immature erythrocytes, these may have been an artefact of smear preparation. An increase in polychromatocytes was, however, still evident in blood smears taken during the November 2009 sampling (Table 3.7). Sharptooth catfishnormally have round nucleated erythrocytes which are distinct from the oval erythrocytes of many other fish species. Compared to mature erythrocytes, polychromatocytes were characterised by a more basophilic cytoplasm and a larger, granular appearing nucleus. Crenation of erythrocyte cell membranes was also present in some blood smears of fish sampled from the Olifants Gorge during November 2009.

Table 3.7: Comparison of percentage polychromatocytes in blood smears collected from catfish at three sampling sites during November 2009 (*standard deviation)

Sampling site	% polychromatocytes		
	mean ± SD*	range	number
Lunsklip Fisheries	0.75±0.60	0.00- 2.33	21
Olifants Gorge	3.26±2.73	0.67-12.33	19
Reënvoël Dam	1.95±1.65	0.33- 5.33	20

3.2.3.2. Haematocrit

Mean haematocrit values are presented in Table 3.8. There were no significant differences in haematocrit values between fish with and without pansteatitis at various sampling sites where pansteatitis was found to occur. In the Olifants Gorge, mean PCV values of fish ranged between 25% and 41% in apparently healthy fish and between 24% and 37% in fish with pansteatitis. The mean PCV value for fish sampled from Reënvoël Dam was 32.3% (n=13, standard deviation=6.1). Comparison of mean PCV values of fish sampled from the Olifants Gorge (32.6%) with Lunsklip Fisheries (39.4%) during November 2009 (Figure 3.31) indicated significantly lower haematocrit values in the Olifants Gorge fish ($p<0.05$). Haematocrit values were only available from fish sampled from Reënvoël Dam during January 2011; during the November 2009 sampling of catfish from Reënvoël Dam a field micro-centrifuge was not available. Analysis of variance ($p<0.001$) followed by post-hoc Tukey's HSD test showed significant differences between mean PCV values (39.4%) for fish from Lunsklip Fisheries (positive reference population, n=21) when compared to mean PCV values (30.3%) for all fish sampled from the Olifants Gorge (n=111), and to mean PCV values (32.3%) for fish from Reënvoël Dam (negative reference population, n=13).

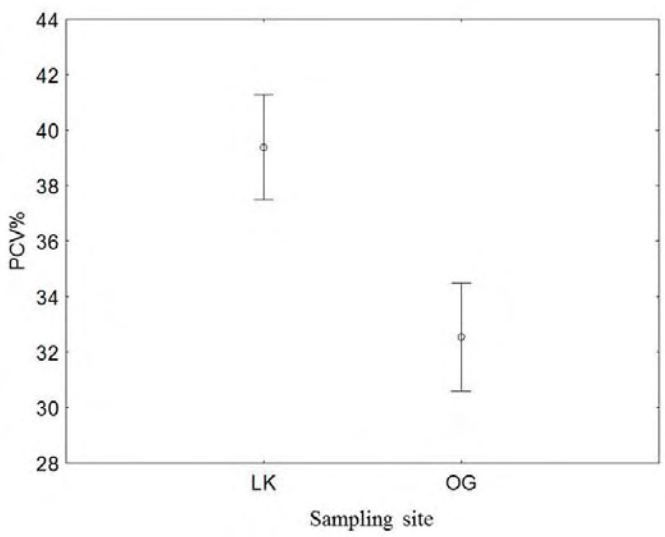

Figure 3.31: Comparison of mean haematocrit (PCV%) from catfish sampled from Lunsklip Fisheries (n 21) and the Olifants Gorge (n 20) during November 2009 (p=0.016, vertical bars denote +/- standard errors).

Table 3.8: Mean haematocrit values (packed cell volume=PCV) of blood collected from catfish from all sites (Olifants Gorge=GL, OGM, OG, OL, LOG, LOC; Lunsklip Fisheries=LK; Reënvoël Dam=RVB; Engelhard Dam=EH; van Ryssen Dam=FK; Mamba Weir=M; Levuvhu River=LUV; Crocodile River=CR) (*standard deviation)

Sample site	Date	Packed cell volume (%)		
		mean ± SD*	range	number
GL	Jun-09	26.33 ± 4.97	15-31	9
OGM	Aug-09	39.07 ± 6.75	30-50	14
LK	Nov-09	39.38 ± 7.60	22-50	21
OG	Nov-09	32.55 ± 9.71	10-50	20
EH	Jul-10	29.95 ± 5.96	19-40	19
OL	Jul-10	28.56 ± 6.92	15-38	25
M	Jul-10	27.75 ± 8.34	12-45	20
LOG	Jan-11	30.59 ± 6.04	14-39	22
RVB	Jan-11	32.31 ± 6.06	20-44	13
FK	Jan-11	32.20 ± 4.69	22-38	10
LUV	Jun-11	30.79 ± 5.82	21-40	14
LOC	Jun-11	25.67 ± 7.43	11-40	21

3.2.3.3. Blood haemoglobin

Mean blood haemoglobin concentrations for fish sampled from all sites are shown in Table 3.9 and were noticeably variable. Haemoglobin values (g/dl) did not differ significantly between fish with and without pansteatitis from the Olifants Gorge, with mean values of 10.1 and 9.7 respectively. Analysis of variance showed significant difference ($p<0.05$) in mean haemoglobin values of fish sampled during November 2009 from the Olifants Gorge, Lunsklip Fisheries and Reënvoël Dam (post-hoc Tukey's test) (Figure 3.32). The mean haemoglobin value of fish sampled from Reënvoël Dam during January 2011 was however, much lower than that of fish sampled from the same site during November 2009, with mean values being similar to those of catfish sampled from the Levuvhu and Crocodile rivers. The significance of the differences observed in the November results thus remains uncertain.

Table 3.9: Mean blood haemoglobin concentrations (g/dl) of blood collected from catfish from all sites (Olifants Gorge=GL, OGM, OG, OL, LOG, LOC; Lunsklip Fisheries=LK; Reënvoël Dam=RV, RVB; Engelhard Dam=EH; van Ryssen Dam=FK; Mamba Weir=M; Levuvhu River=LUV; Crocodile River=CR)(*standard deviation)

Sample site	Date	Haemoglobin		
		mean ± SD*	range	number
GL	Jun-09	12.32 ± 1.07	10.7-13.80	9
OGM	Aug-09	11.00 ± 1.11	8.93-13.00	14
LK	Nov-09	14.47 ± 3.06	9.53-23.00	21
OG	Nov-09	13.16 ± 3.84	6.65-21.00	20
RV	Nov-09	16.62 ± 3.39	10.8-21.30	14
EH	Jul-10	9.39 ± 3.57	3.82-16.90	20
OL	Jul-10	9.66 ± 4.29	3.24-21.30	25
M	Jul-10	8.04 ± 4.52	2.33-17.10	20
LOG	Jan-11	7.69 ± 3.05	0.10-13.70	22
RVB	Jan-11	8.05 ± 2.45	3.50-13.10	13
FK	Jan-11	13.83 ± 6.39	8.15-29.93	10
LUV	Jun-11	9.25 ± 1.68	6.40-12.20	14
LOC	Jun-11	7.90 ± 2.12	4.20-12.00	16
CR	Jun-11	7.89 ± 1.34	4.20-10.20	20

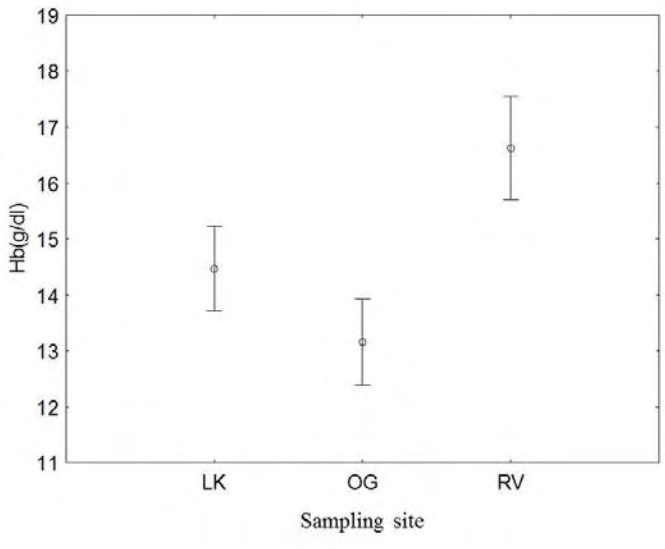

Figure 3.32: Comparison of mean blood haemoglobin concentrations (g/dl) of catfish sampled during November 2009. LK=Lunsklip Fisheries (n 21), OG=Olifants Gorge (n 20) and RV= Reënvoël Dam (n 14). (p=0.021, vertical bars denote +/- standard errors).

3.2.3.4. Serum vitamin E

Mean serum vitamin E values for catfish sampled from all sites are presented in Table 3.10. Vitamin E values did not differ significantly between fish with and without pansteatitis. Although some fish from the Olifants Gorge had very low serum vitamin E values, analysis of variance showed that there was no significant difference in mean serum vitamin E values between fish sampled from the Olifants Gorge and Reënvoël Dam during November 2009 whereas the values in fish sampled from Lunsklip Fisheries were significantly higher at this time (Figure 3.33).

Table 3.10: Mean serum vitamin E values of catfish from all sites (Olifants Gorge=OGM, OG, OL; Lunsklip Fisheries=LK; Reënvoël Dam=RV; Engelhard Dam=EH; Mamba Weir=M) (*standard deviation)

Sample site	Date	Vitamin E (mg/l)		
		mean ± SD*	range	number
OGM	Aug-09	4.67 ± 2.99	1.00-8.90	14
LK	Nov-09	4.60 ± 1.26	2.70-6.70	21
OG	Nov-09	3.13 ± 0.34	2.70-3.90	15
RV	Nov-09	3.06 ± 0.33	2.40-3.70	15
EH	Jul-10	3.33 ± 1.49	1.40-7.80	18
OL	Jul-10	2.75 ± 1.18	1.10-5.40	17
M	Jul-10	2.88 ± 1.40	0.80-5.70	16

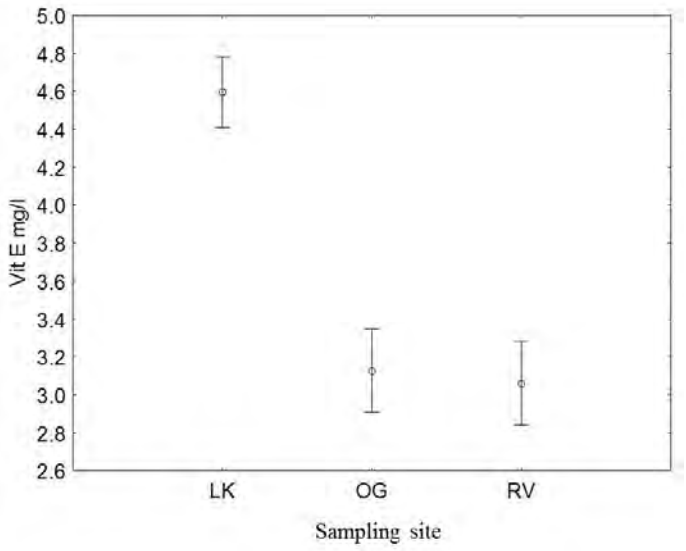

Figure 3.33: Comparison of mean serum vitamin E values (mg/l) of catfish sampled during November 2009. LK=Lunsklip Fisheries (n 20), OG=Olifants Gorge (n 15), and RV= Reënvoël Dam (n 15). ($p<0.001$, vertical bars denote +/- standard errors).

The lower fifth percentile of serum vitamin E values (2.7 mg/l) in healthy fish from Reënvoël Dam was used to identify fish with depressed vitamin E values and the data was subjected to chi-squared analysis ($p<0.05$). Whereas the percentage of fish with depressed serum vitamin

E values during the November 2009 sampling from the Olifants Gorge was similar to that of fish sampled from Reënvoël Dam and Lunsklip Fisheries in the same period, significantly higher percentages of fish with depressed serum vitamin E values were sampled from the Olifants Gorge and Mamba Weir during July 2010. Although at p values slightly above 0.05, numbers of fish with low serum vitamin E values sampled from the Olifants Gorge during August 2009 and from Engelhard Dam during July 2010 were indicative of a similar pattern of depression during these sampling episodes (Table 3.11).

Table 3.11: Percentage of catfish from the Olifants Gorge and other sites with serum vitamin E levels below the lower fifth percentile of values of healthy fish sampled from Reënvoël Dam. (Olifants Gorge=OGM, OG, OL; Lunsklip Fisheries=LK; Reënvoël Dam=RV; Engelhard Dam=EH; Mamba Weir=M)

Sampling site	Sampling date	% fish with vitamin E <2.7 mg/l	n
OGM	Aug-09	36	14
OG	Nov-09	7	15
RV	Nov-09	7	15
LK	Nov-09	10	21
OL	Jul-10	65	17
EH	Jul-10	33	18
M	Jul-10	50	16

3.2.3.5. Blood glutathione peroxidase

Exceptionally high erythrocyte glutathione peroxidase values were measured in catfish from three sampling sites during July 2010 (Figure 3.34). Fish with pansteatitis were found at all three of these sites. A comparison of erythrocyte glutathione peroxidase values measured in blood of catfish from the same sites in the Olifants Gorge on different sampling dates makes these high values appear suspicious and the results are inconclusive. No significant difference in erythrocyte glutathione peroxidase values could be demonstrated in fish sampled during November 2009 from the Olifants Gorge, Lunsklip Fisheries and Reënvoël Dam, and there was no significant difference in erythrocyte glutathione peroxidase values in fish with and without pansteatitis.

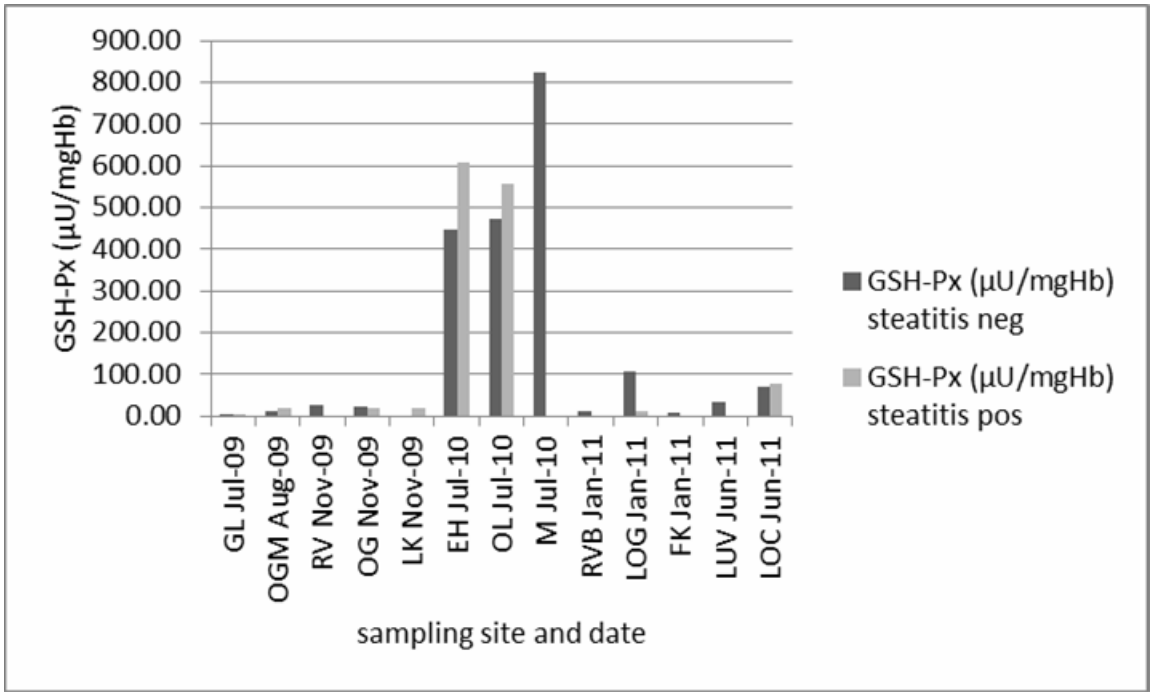

Figure 3.34: Mean glutathione peroxidase (GSH-Px) values (µU/mgHb) of catfish with and without pansteatitis sampled from all sampling sites.

3.2.3.6. Discussion of haematology and blood chemistry

In catfish with pansteatitis from both the Olifants Gorge and Lunsklip Fisheries the oxidative stress resulting in steatitis appears to have remained limited to the adipose tissues as reflected in the pathology. For the selected haematology and blood chemistry parameters no significant differences could be detected between fish with and without pansteatitis from both the Olifants Gorge and Lunsklip Fisheries. This may have been a reflection of the chronic nature of the condition in these fish. At sites in the Olifants Gorge with high pansteatitis prevalence, significant numbers of catfish had depressed serum vitamin E values. Yet catfish from Lunsklip Fisheries with severe chronic pansteatitis showed normal serum vitamin E levels.

The increased number of polychromatocytes in blood smears from catfish from the Olifants Gorge and Lunsklip Fisheries (Table 3.7) indicated a higher erythrocyte turnover rate in these fish, possibly a result of dietary oxidative stress. Lipid peroxidation may be enhanced in erythrocytes due to direct exposure of the polyunsaturated fatty acids in the erythrocyte cell membrane to molecular oxygen (Tappel 1973). An increase in polychromatocytes has been described in channel catfish fed diets deficient in vitamin E and containing oxidised fish

oil(Murai & Andrews 1974) and in Atlantic salmon, *Salmo salar* L., fed diets deficient in vitamin E or selenium (Poston *et al.* 1976). In rainbow trout fed diets containing oxidised fish oils and deficient in vitamin E, crenation of immature erythrocytes was observed (Moccia *et al*. 1984). Under experimental conditions, severe anaemia developed after 13 weeks in rainbow trout when fed diets containing rancid fish oils and deficient in vitamin E (Smith 1979). In channel catfish fed vitamin E free diets in the absence of oxidised fish oil, anaemia developed after 16 weeks (Murai & Andrews 1974). It is unlikely that in the natural environment of the Olifants Gorge such extreme dietary conditions would prevail for this long.Examination of blood smears may provide an indication of current oxidative stress and hypovitaminosis E, but in this study did not assist the diagnosis of pansteatitis.

Changes in fluid partitioning between blood and lymph can be rapid, variable and pronounced during stressful episodes, such as occur during sampling of wild fish. This is a reflection of the close association of blood and lymph in fish (Branson 1993). Haematocrit levels in fish from all sampling sites were variable, and no significant variation could be demonstrated in haematocrit values between fish with and without pansteatitis. Osmotic disruption was impossible to avoid when catching fish despite attempts to minimise sampling stress. Catfish sampled from the KNP sites were caught on hook and line with the added risk of haemorrhage, particularly where the hooks had been swallowed. These fish had lower mean haematocrit levels than catfish sampled by netting from Lunsklip Fisheries (Figure 3.31). The different factors resulting in stress during sampling would have impacted on haematocrit values of the fish, limiting the usefulness of this parameter in the study of pansteatitis in wild fish.

Blood haemoglobin values represent the haemoglobin released by erythrocyte lysis in the laboratory as well as plasma haemoglobin from *in vivo* lysis of erythrocytes, as might be expected with oxidative stress. Increased haemoglobin catabolism observed in vitamin E deficient rainbow trout, caused by the degeneration and failure of polychromatocytes to fully mature, resulted in splenic haemosiderosis (Moccia *et al.* 1984). During the November 2009 sampling, the blood haemoglobin concentrations of fish sampled from Lunsklip Fisheries, which had a high prevalence of pansteatitis, were significantly lower than those in fish from Reënvoël Dam (Figure 3.32). Haemoglobin values of fish from the Olifants Gorge were even lower than those of fish from Lunsklip Fisheries. This may indicate a more protracted, higher erythrocyte turnover in affected fish, a change consistent with oxidative stress and reflected

in the observed haemosiderin deposits within hepatocytes and splenic and hepatic macrophages (Table 3.5). The wide range and the large differences in mean values in the fish from Reënvoël Dam measured during two sampling occasions (Table 3.9) indicated that factors other than pansteatitis were influencing the values. In experiments with blue tilapia, *Oreochromis aureus* (Steindachner), Roem, Kohler and Stickney (1990) found that haemoglobin concentrations remained unaffected by varying levels of dietary lipid and vitamin E. Under field conditions the interpretation of blood haemoglobin values in relation to pansteatitis may not be straightforward, limiting the usefulness of this test.

From the pathology in catfish from Lunsklip Fisheries it is evident that pansteatitis in these fish was chronic. The oxidative stress caused by prolonged ingestion of rancid dietary fats in the slaughter-house waste was the most likely inciting cause of the pansteatitis in these fish. During this study, only serum vitamin E levels were measured,and were normal in the catfish from Lunsklip Fisheries. Kelly *et al.* (1998) also ascribe a lipid protective role to ascorbic acid which acts through regeneration of tocopherol. Catfish from Lunsklip Fisheries might have benefited indirectly from the high vitamin E and ascorbic acid inclusion in the commercial diet fed to the trout, as some of the waste consumed by the catfish would have been fresh. Nevertheless, peroxidation of lipids in the adipose tissues of these fish leading to pansteatitis occurred. Serum vitamin E values may, therefore, not always reflect the increased consumption of vitamin E by the oxidative processes taking place in the adipose tissues.

Dietary sources of vitamin E in the natural aquatic habitat are numerous. Presence of steatitis lesions did not correlate with low serum vitamin E in catfish from the Olifants Gorge, but significant numbers of catfish with depressed vitamin E levels (Table 3.11) may have indicated a recent episode of dietary oxidative stress. Inconsistent results limited the usefulness of serum vitamin E as a monitoring tool for pansteatitis.

In aquatic systems oxidative stress studies have centred on depletion and induction of various antioxidant defences. In fish the antioxidant protective enzyme glutathione peroxidase shows higher basal activity than the enzymes superoxide dismutase and catalase when compared to other vertebrate systems, and it has been proposed that glutathione peroxidase is a suitable biomarker of oxidative damage in fish (Kelly *et al*. 1998). Measurement of blood values of glutathione peroxidase showed no statistical difference between catfish with and without pansteatitis from the Olifants Gorge. Differences in erythrocyte glutathione peroxidase values

were detected between sampling sites, but the significance of these occasional very high values is uncertain. There was no significant difference in mean erythrocyte glutathione peroxidase values between fish sampled from Lunsklip Fisheries and Reënvoël Dam, questioning the usefulness of this test for monitoring pansteatitis under field conditions.

The focus of this study was to determine whether some haematological and biochemical parameters in the blood and serum collected from live fish might serve as a monitoring tool for presence of pansteatitis in the fish of the KNP. Serum vitamin E and erythrocyte glutathione peroxidase measurements, shown to be useful in studying the acute manifestations of pansteatitis in cats (Fytianou *et al.* 2006), were of limited use for monitoring pansteatitis in catfish in the Olifants Gorge. The diagnosis of oxidative stress in live fish from the Olifants Gorge is complicated by the possible episodic exposure and the protracted chronic nature of the pansteatitis. More work needs to be done before these or similar tests can be used to monitor the status of fish non-lethally in these rivers. Determining malondialdehyde levels in the lipid fraction of serum by use of the thiobarbituric acid reactive substances assay still needs to be evaluated as a monitoring tool. Further markers of lipid peroxidation include the measure by mass spectrometry of F2-isoprostanes, prostanoids resulting from the *in vivo* free-radical-catalysed peroxidation of arachidonic acid (Awad *et al.* 1994). As free F2-isoprostanes in plasma reflect whole body lipid peroxidation these prostanoids may also be of value as a pro-oxidant marker in fish. The measure of F2-isoprostanes is used in the racehorse industry in South Africa but the cost of these tests was beyond the budget of this study. Although not a direct measure of lipid peroxidation, the associated damage to DNA structures provides a further opportunity for biomonitoring, and the comet assay has been suggested as a rapid, sensitive and inexpensive method of measuring DNA oxidation (Collins 2009; Klaude, Eriksson, Nygren & Ahnström 1996; Lee & Steinert 2003) and has been used to measure the effects of dietary antioxidants in human disease (Collins 2009).

3.2.4. Pathology in other fish species

Mozambique tilapia were difficult to catch in the Olifants Gorge. However a few specimens caught from the Letaba River at the confluence with the Olifants River at the entrance to the gorge appeared thin, despite presence of moderate mesenteric fat reserves. Distinctly demarcated pale areas of discolouration were noted in the livers of some of these fish. These were confirmed by histology to be zones of fat accumulation within hepatocytes. Such zones

are not uncommon in farmed tilapia. Gills of Mozambique tilapiaspecimens appeared normal. Mesenteric fat showed no evidence of steatitis. However, on histology one fish showed small amounts of lipopigment within macrophages associated with mesenteric adipose tissue. Distinct from catfish, Mozambique tilapiaspecimens showed no ceroid or haemosiderin deposition in the livers. All other organs appeared histologically normal.

Only one large specimen of the purple labeo, *Labeo congoro* Peters,was collected in the gorge. The gills of this fish manifested with an unusual severe fusion of the distal ends of the primary lamellae, a change that was not observed in either catfish or Mozambique tilapia. Similar gill lesions have been noted, with presence of cell bodies of the dinoflagellate *Ceratium* spp. trapped amongst the gill filaments, in rednose labeo from Lake Loskop during a *Ceratium* spp. bloom (J. Steyl, Faculty of Veterinary Science, University of Pretoria, pers. comm. 2012). Several smaller purple labeo specimens caught in the Olifants Gorge and the lower Letaba River did not show this gill fusion.

Pansteatitis could not be demonstrated in 5 tigerfish collected from the Olifants Gorge during September 2008 or in 21 tigerfish collected from the Olifants Gorge during June 2011. The fish were all in good condition with distinctly white mesenteric fat reserves. Fish sampled during June 2011 ranged in age from 1 to 10 years and both male and female fish were represented. All fish carried low numbers of larval nematodes in the peritoneal cavity; however, digenean trematode cysts were absent in all but one fish.

CHAPTER FOUR: PERSISTENCE OF PANSTEATITIS AFTER REMOVAL OF THE INCITING DIETARY CAUSE

4.1. Introduction

Pansteatitis has been identified in African sharptooth catfish in the Olifants River Gorge in the same area where crocodile deaths due to pansteatitis have occurred (see Chapter 3). An increasing prevalence of pansteatitis in catfish from the Olifants Gorge has been shown with repeated samplings from 2009 to 2011(see Chapter 3). In some instances both old and recent lesions have been observed in the same fish. There is no published information on how long pansteatitis lesions persist after initiation of the lesions and whether the increasing prevalence was the result of increasing pro-oxidant challenge or whether it was the cumulative effect of repeated challenge at a lower level.

During investigation of pansteatitis in catfish the author identified a positive control group of fish suffering from pansteatitis, unrelated to developments in the Olifants River Gorge (see Chapter 3). A high proportion of catfish from this farm suffered from pansteatitis as a result of poor feeding practices. These fish provided a valuable control group to which the pathology of the KNP fish was compared; they also provided a unique opportunity to study the regression or progression of pathological lesions in the fat over time once the diet had been corrected. As far as the author is aware this has not been studied in fish before. In addition this information provides an insight to what has been happening in the Olifants Gorge crocodiles since the large die-off of crocodiles in 2008 and sheds light on whether the inciting cause of pansteatitis in the Olifants Gorge is on-going.

4.2. Hypothesis

It is hypothesised that pansteatitis may change over time once the inciting cause has been removed; acute lesions will not continue to spread but lesions that have progressed to the chronic stage are unlikely to heal and will remain detectable in the fat of affected fish after prolonged periods. Furthermore, it is hypothesised that mobilisation of lipids from pansteatitis affected adipose tissues is likely to be compromised.

4.3. Objective

The objective of this study was to study the regression/progression of nutritionally-induced pansteatitis lesions in catfish over time once affected fish had been removed from the inciting nutritional cause.

4.4. Materials and Method

4.4.1. Experimental design

Sharptooth catfishfrom a population of farmed fish suffering from pansteatitis, belonging to Lunsklip Fisheries (LK) (25°23′08,9″S 30°15′35″E), Mpumalanga Province, South Africa, were donated to the study. The fish were obese and weak as a result of the debilitating effects of pansteatitis, and experience from the farmer had shown that the survival of such fish was poor. The trial was started with 12 study fish with the anticipation that some might die as a consequence of the advanced pansteatitis. Ten control fish were sourced from Reënvoël Dam (RV) (23°58′37.2″S 31°19′38.4″E) in the KNP. The catfish in Reënvoël Dam have been identified as a reliable negative reference population (see Chapter 3). The mesenteric fat of LK fish was examined by laparotomy for gross lesions of steatitis, and a biopsy was taken for histological confirmation of the lesions. Both the control fish from Reënvoël Dam and the LK fish were kept in the same recirculated pond, mimicking a natural water body, for 11 months through the winter and into the next summer. At the end of this period both the LK and RV fish were euthanized, and post-mortem examinations were performed to establish whether pansteatitis was present. Mesenteric fat samples were collected and histological sections prepared by standard histological technique. Sections were stained with haematoxylin eosin and examined under a standard compound microscope to confirm presence of steatitis in both the biopsy and post-mortem sections of adipose tissue.

4.4.2. Experimental facility

The author's private recirculated fish facility was used for the experiment. This consisted of a 5 m^3 portable holding pond (Minurphy Tarpaulins, Pietermaritzburg) connected to a mechanical high pressure swimming pool filter filled with coarse sand, and a trickle tower filled with high surface area plastic media. The experimental pond consisted of a 150 m^3 concrete pool connected to a vortex chamber filled with nylon brushes, an artificial wetland and a mechanical gravel filter. Water was continuously recirculated through both systems. Water lost through daily flushing of the filters and through evaporation was added to the system from a borehole through a fine garden sprinkler to allow supersaturated gasses from the borehole water to escape. The experimental pond was in full sun and had a natural growth of phytoplankton. In addition to the experimental fish, the pond was stocked with koi carp, *Cyprinus carpio* L. and Mozambique tilapia. All fish were fed a commercial fish feed (Avi Feeds trout pellet), containing a standard vitamin premix including vitamin E, on a daily basis. The catfish were also able to feed *ad lib* on Mozambique tilapias that were breeding

prolifically in the pond, providing a further natural food source. After 6 of the LK fish escaped from the pond 23 days from the start of the trial, the pond was fenced in with a 60 cm high chicken mesh wire fence.

4.4.3. Experimental animal procedures

Fish were caught live by scoop net and transported from Lunsklip Fisheries to the author's facility in a water-filled, 500 L fish transport container. The fish from Reënvoël Dam were caught by baited hook and line and were transported in a water-filled 250 L fish transport water container. Sharptooth catfish, being air-breathing fish, were transported without additional aeration of the transport water. The LK fish were initially held in the 5 m^3 holding pond. On the 1st and 2nd February 2011 the fish were individually anaesthetised in an anaesthetic bath containing 30 ppm benzocaine (Kyron Laboratories, Johannesburg). Once the fish had been anaesthetised they were weighed and the length measured, and the condition score determined. The condition score was assigned on a scale from 1 (emaciated) to 5 (obese). The anaesthetised fish were then placed into a wet cradle on their backs, and the head and gills covered by a clean wet towel. The surgical area along the ventral midline was prepared for surgery by disinfection with F10 Surgical Wound Preparation (Health and Hygiene, Johannesburg). The surgical field was covered by a window drape, and a 50 mm long midline incision was made through the skin with a scalpel, ending approximately 25-50 mm cranial to the pelvic bones (50-75 mm cranial to the cloaca) (Figure 4.1). The incision was extended through the skin, linea alba and peritoneum to reveal the underlying mesenteric fat (Figure 4.2).

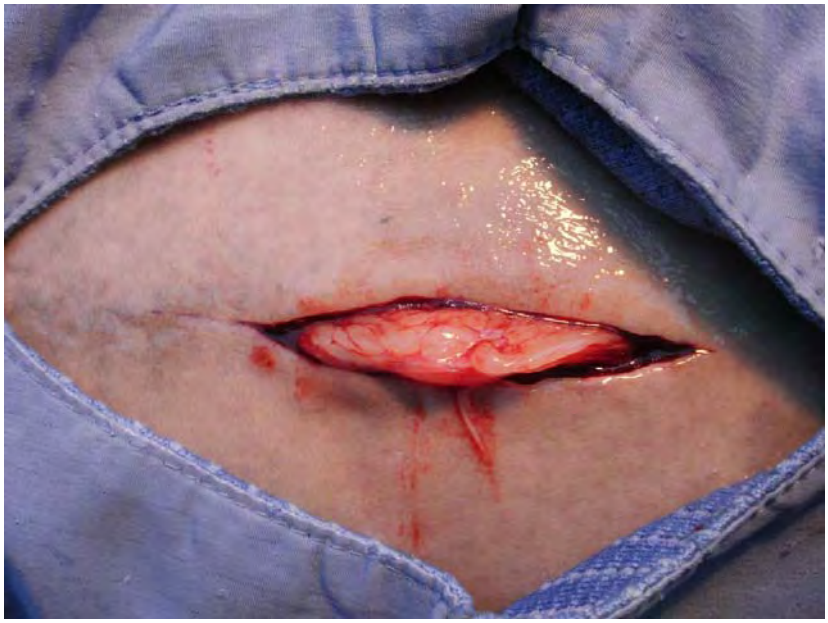

Figure 4.1: Laparotomy incision along the ventral midline of the abdomen of a test fish with mesenteric fat protruding from the incision.

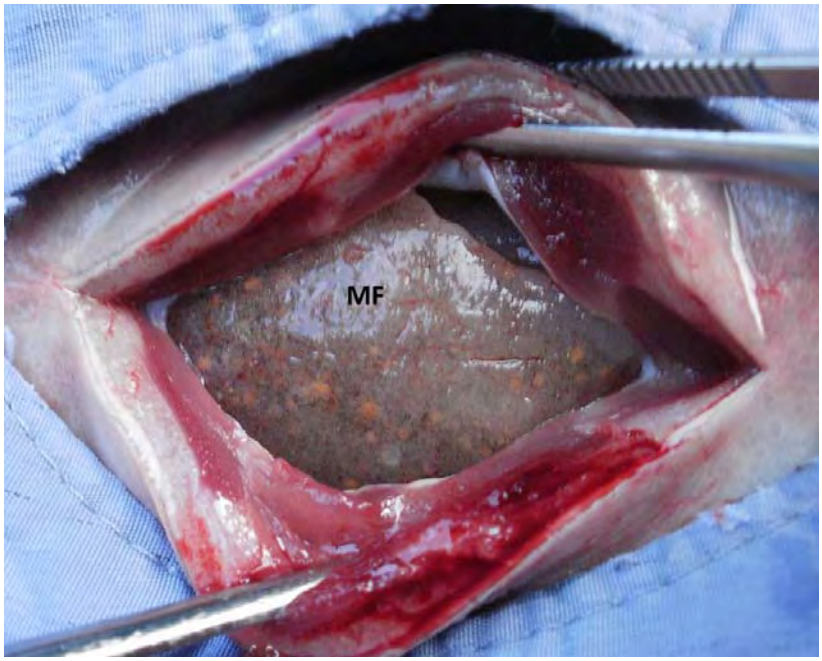

Figure 4.2: Incision through skin, linea alba and parietal peritoneum of a catfish to access the underlying mesenteric fat (MF). Note severe steatitis of the mesenteric fat evidenced by the grey-brown colour and presence of granulomata visible beneath the surface of the fat.

The underlying mesenteric fat was inspected through the incision for signs of steatitis and a biopsy of affected adipose tissue, measuring approximately 10x10x15 mm, was removed and placed into 10% buffered formalin (Figure 4.3).

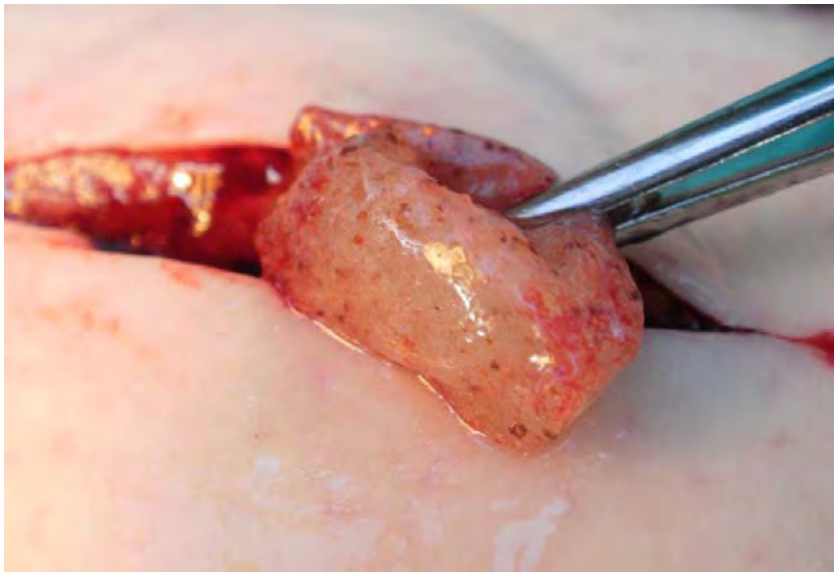

Figure 4.3: Removal of a fat biopsy through a ventral midline abdominal incision in a test fish. Note the obvious signs of steatitis in the fat biopsy.

Once the biopsy had been taken, enrofloxacin (Baytril 5% injectable, Bayer) at a dose of 5 mg/kg was injected into the peritoneal cavity. The wound was closed by tightly placed, simple interrupted sutures using 3/0 nylon with an atraumatic needle, each suture passing through skin, linea alba and peritoneum. The wound was sealed with Wound Gel Powder (in-house proprietary formulation for aquatic animals, Sterkspruit Veterinary Clinic, Lydenburg). Nylon mattress sutures (1/0) were placed into the soft tissue of the pelvic fins between the fin rays to identify individual fish numerically. The fish were then placed into the 150 m^3 recirculated fish pond and allowed to recover.

Control fish from Reënvoël Dam were received on 24 March 2011, 50 days after the laparotomies had been performed on the LK fish. They were initially placed into the 5 m^3 holding facility. Anaesthesia took place in an anaesthetic bath containing 30 ppm benzocaine. Once anaesthetised, the fish were weighed and measured, and condition scores were determined. No laparotomies were performed on the control fish, and the fish were not given

identification marks. The control fish were released into the same recirculated 150 m^3 pond with the trial fish.

At the end of the trial, from the 6th to 10th of January 2012, both the LK and RV catfish were euthanized by an overdose of clove oil. For euthanasia, fish were held in the anaesthetic bath until all life signs had ceased. All fish were again weighed and measured, and a detailed post-mortem examination was performed on each fish. The amount of mesenteric and pectoral fat was recorded as a score of 1 (negligible fat) to 5 (large amount of fat). The mesenteric fat was then dissected away from the organs and weighed. The various fat depots were evaluated for gross lesions of steatitis. Data sheets were completed for all gross observations and measurements. Samples of mesenteric fat, pectoral fat, intermuscular fat, liver, spleen and caudal kidney were collected from each fish and fixed in 10% buffered formalin. Five micron sections were prepared by standard histological technique, stained with haematoxylin eosin and examined under a standard light microscope. Data sheets were completed for all histological observations.

4.5. Results

At the beginning of the trial the LK fish were very much larger than the fish from Reënvoël Dam and carried considerable amounts of fat (Table 4.1 & 4.3). The laparotomies were performed on the 1st and 2nd February 2011. Fish took from 5 to 10 minutes to reach full surgical anaesthesia in the benzocaine anaesthetic bath. Surgical anaesthesia was indicated by a reduction in opercular movement, loss of voluntary movement and loss of the righting reflex when the fish were turned onto their backs. The anaesthetic level attained in the benzocaine bath was sufficient to last for the 10 to 15 minute duration of the surgical procedure. The laparotomy procedure through the abdominal midline required no haemostasis. Full recovery from anaesthesia took 10 to 20 minutes and was uneventful in all operated fish. Water temperature in the experimental pond varied from 24 to 26°C during February.

Table 4.1: Measurements of test fish at the start of the trial

Sample no.	Sex	Body mass (g)	Length (mm)	Body condition
LT1	M	8740	1050	5
LT2	M	8580	1100	3
LT3	F	9500	940	5
LT4	M	7000	980	4
LT5	M	6940	960	4
LT6	M	6500	900	4
LT7	F	9100	1080	4
LT8	F	6400	890	4
LT9	M	7800	960	5
LT10	M	10000	1200	2
LT11	M	6100	980	2
LT12	F	4780	890	3

Table 4.2: Measurements of surviving test fish at the end of the trial

Sample no.	Sex	Body mass (g)	Length (mm)	Body condition	Mesenteric fat mass (g)	Mesenteric fat score	Pectoral fat score
LT2	M	7000	1100	3	178	4	2
LT4	M	7000	980	5	652	5	3
LT5	M	6000	920	5	1110	5	3
LT7	F	7500	1080	4	42	2	3
LT8	F	7000	920	5	566	5	3
LT12	F	4000	860	3	240	4	3

Table 4.3: Measurements of control fish at the start of the trial

Sample no.	Sex	Body mass (g)	Length (mm)	Body condition
LC1	M	4660	820	3.0
LC2	F	380	400	3.0
LC3	M	440	540	2.5
LC4	F	380	400	2.0
LC5	M	400	410	2.5
LC6	M	500	400	3.0
LC7	F	460	390	2.5
LC8	F	560	420	3.0
LC9	M	480	390	3.0
LC10	F	380	400	3.0

Table 4.4: Measurements of control fish at the end of the trial

Sample no.	Sex	Body mass (g)	Length (mm)	Body condition	Mesenteric fat mass (g)	Mesenteric fat score	Pectoral fat score
LC1	M	3580	830	2	10	2.0	2
LC2	F	1006	470	4	22	3.0	3
LC3	M	1800	610	4	14	2.5	3
LC4	F	1100	615	4	8	2.0	3
LC5	M	1300	550	4	26	3.0	3
LC6	M	1100	540	4	12	3.0	3
LC7	F	1600	570	4	40	4.0	3
LC8	F	1100	500	4	8	2.0	3
LC9	M	1300	570	3	12	3.0	3
LC10	F	1050	520	3	30	4.0	3

The laparotomies in all 12 test fish revealed prominent mesenteric fat stores (Figure 4.1) with clear signs of pansteatitis consisting of a greyish discolouration of the fat and presence of numerous focally-disseminated to coalescing small (1 to 5 mm diameter) brown and white granulomata throughout the fat (Figure 4.2). The affected adipose tissue was rubbery in consistency. Removal of a biopsy from the mesenteric fat (Figure 4.3) was possible with

minimal bleeding and required no suturing of the mesenteric adipose tissue. Several of the female fish had large gravid ovaries that were visible through the laparotomy incision. Measurements of test and control fish are given in Table 1 and 3 respectively

A heavy rainstorm 23 days after the start of the trial enabled 6 of the LK fish to migrate out of the pond during the night. When found the next day they were still alive, however all six died over the next few days. The laparotomy wounds had healed completely and the sutures were still in place. Autopsies on these fish indicated that the abdominal incisions had healed without complications. Lesions of pansteatitis had remained unchanged over the 23 day period. Enclosure of the pond with a chicken mesh wire fence prevented further escapes.

During the first 6 months of the trial none of the LK fish were observed to take feed. They were shy to come to the surface other than to gulp air and, when seen, showed slow swimming movements. By comparison, the RV fish were observed to take commercial feed when fed at night within a few days of being released into the experimental pond. During the winter months of May, June, July and August the pond water reached a minimum temperature of 12^{o}C, measured one meter below the surface, and neither the LK nor the RV fish were observed to take feed during this period. From the end of August onwards both LK and RV fish started to take commercial feed actively. The LK fish were, however, slower in their movements than the RV fish, and one particular fish was often observed hanging near the surface belly up as if dead. Whereas the RV fish would actively search for floating fish pellets on the pond surface using their feelers, the LK fish would appear at the feeding spot hanging vertically in the water, slowly rising to where their feelers would detect pellets, which would then be sucked into their mouths. Swimming movements of the LK fish remained sluggish. The RV fish in contrast made strong dashing movements and were quick to be startled. Both groups of fish could only be observed after dark using a torch. It was not possible to establish whether the catfish were feeding on the Mozambique tilapia; active hunting of these fish was not observed.

Both the test and control fish were euthanized after the 11 month trial period. The measurements of the test and control fish at the end of the trial are given in Table 4.2 and 4.4 respectively. Four of the surviving test fish had lost weight over the trial period; one fish maintained the same weight and one fish gained in weight. Over the same period all of the test fish either maintained the same body condition or gained condition. A 30 to 40 mm

length reduction was measured in two of the test fish at the end of the trial. With the exception of the largest of the control fish, all the control fish increased in weight, the majority more than doubling in mass over the trial period.

At the end of the trial all 3 female test fish had mature gonads and were gravid with eggs, despite the severe degree of pansteatitis present. The 3 male fish showed only moderate testicular development. Of the control fish 3 of the 5 female fish had mature gonads and were gravid with eggs, one female had a moderately mature ovary and one female had an immature ovary. As in the case of the test fish, the gonads of the five male control fish showed only moderate testicular development.

The laparotomy wounds of the test fish had healed completely leaving barely discernible scars after the 11 month trial period (Figure 4.4a). All but one fish had rejected the nylon sutures used to close the wounds (Figure 4.4b).

Figure 4.4a: Male test fish showing the scar of the laparotomy incision in the ventral abdominal skin, at the end of the 11 month trial period. This fish had rejected all of the original nylon sutures.

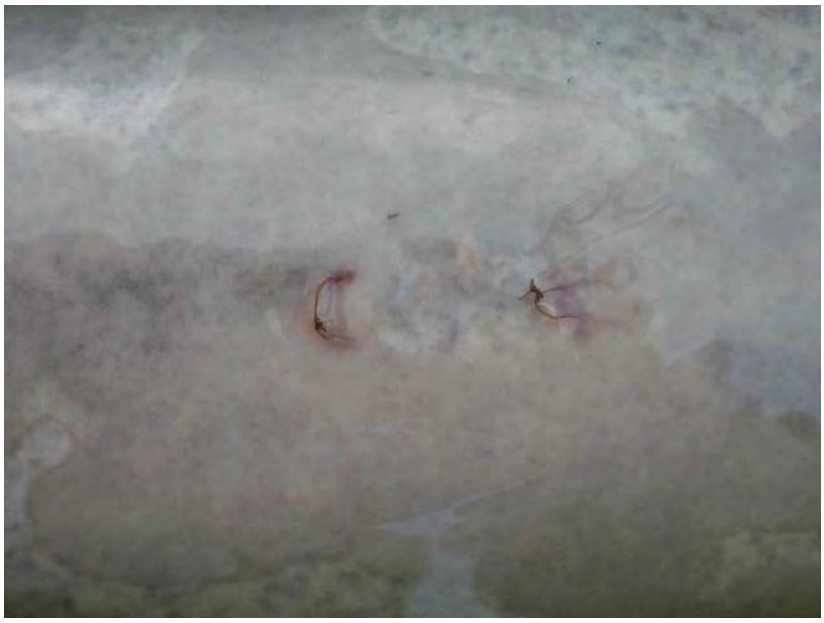

Figure 4.4b: Laparotomy scar in the abdominal skin of a test fish after the 11 month trial period showing good healing despite retention of 2 nylon sutures.

The majority of sutures placed in the pelvic fins to identify the fish had been rejected, but it was possible to identify individual fish from their sex and length measurements. At post-mortem examination no adhesions were observed between the parietal peritoneum covering the laparotomy scar and the visceral peritoneum covering the adjacent mesenteric adipose tissue. A barely discernible fibrous scar could be observed at the site from which the biopsy had been taken (Figure 4.5). Re-examination of the adipose tissues of the test fish, 11 months after the original biopsies were taken, confirmed that a severe degree of steatitis was still present throughout the adipose tissues of the fish (Figures 4.6a & 4.6b).

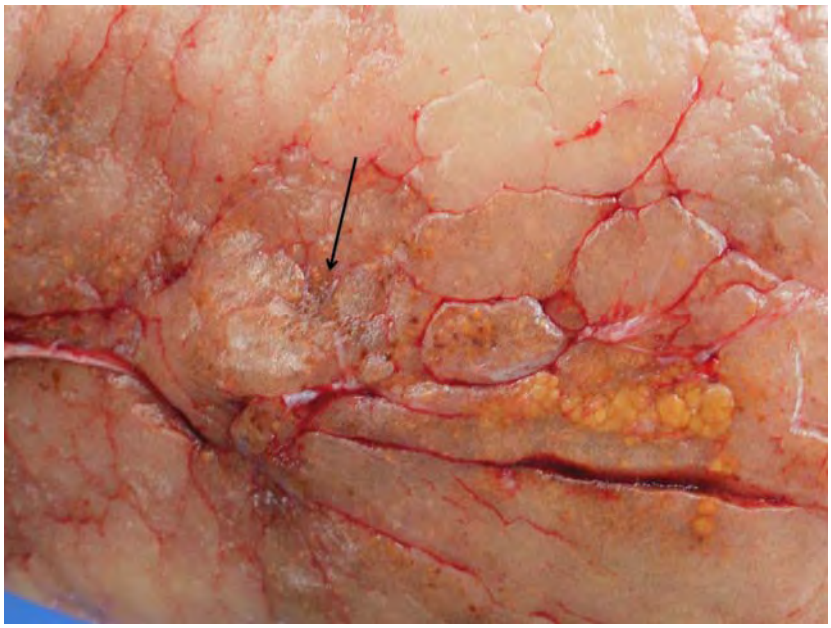

Figure 4.5: Surface of mesenteric fat body of a test fish after the 11 month trial period showing minimal scar formation where the biopsy had been taken (arrow).

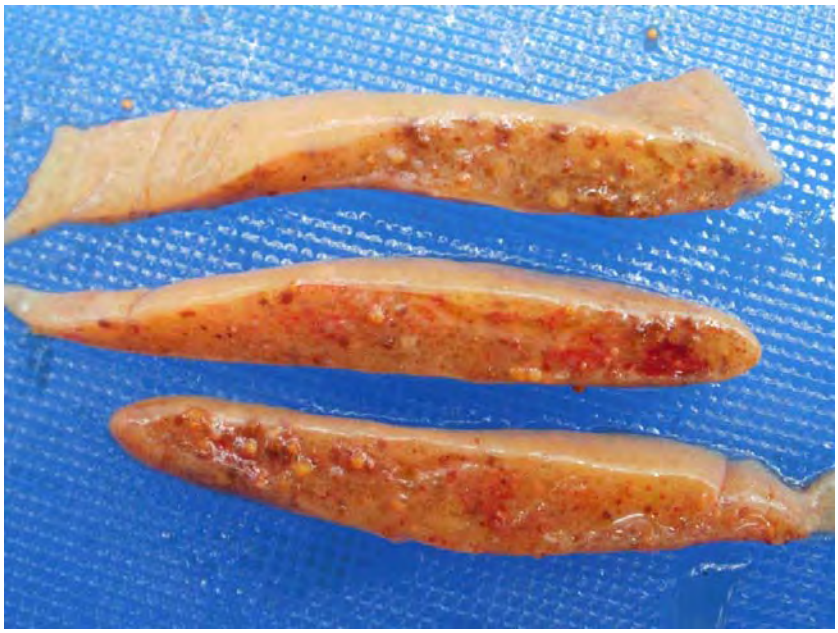

Figure 4.6a: Cross section from the edge of mesenteric fat of a test fish at the end of the 11 month trial period showing distinct yellow and brown granulomata and smaller brown spots dispersed throughout the fat.

The mesenteric fat bodies had not reduced in size visibly since the start of the trial and retained the rubbery texture noticed at the outset of the trial. Numerous focally disseminate to coalescing small brown granulomata (1-5 mm diameter) were dispersed throughout the mesenteric fat, and in some fish there was a clear demarcation of areas of fat with varying severity of steatitis (Figure 4.6b).

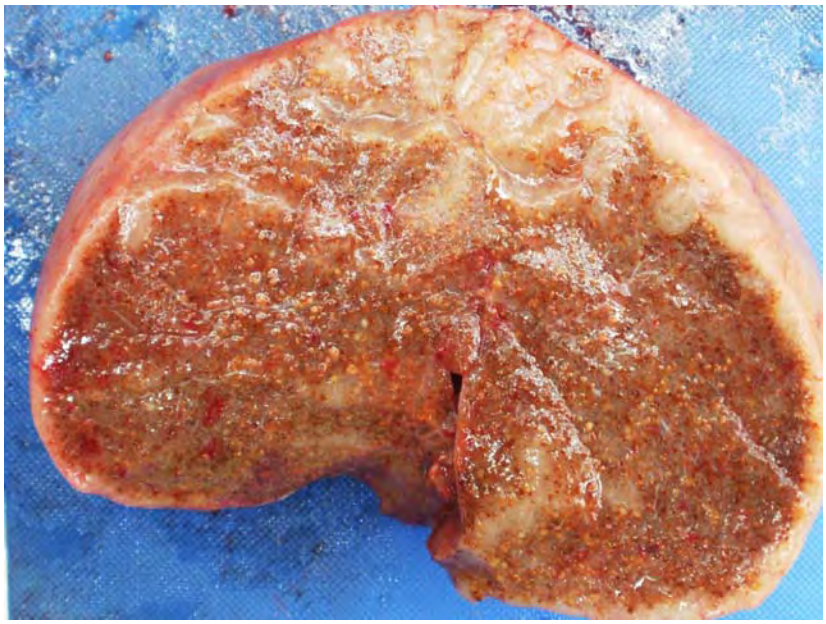

Figure 4.6b: Cross section of mesenteric fat body retained after the 11 month trial period. Note more recently deposited white fat on the surface of the fat body, whereas the bulk of the fat body shows intense granulomata formation imparting the brown colour.

This was particularly evident in one fish that died after escaping from the pond 23 days after the laparotomies had been performed (Figure 4.7). Mesenteric fat, severely affected by steatitis, was greyish brown in this fish, whereas adjacent mildly affected fat appeared almost white with only a few widely dispersed small brown spots and may have been deposited more recently. The whiter fat had the typical oily feel of fish fat, whereas the grey brown fat had the rubbery consistency typical of the fat of catfish with severe pansteatitis. At the end of the trial one fish showed deposition of what appeared to be new white fat without granulomata in the mesentery between the liver and the main body of pansteatitis-affected fat in the caudal mesentery (Figure 4.8).

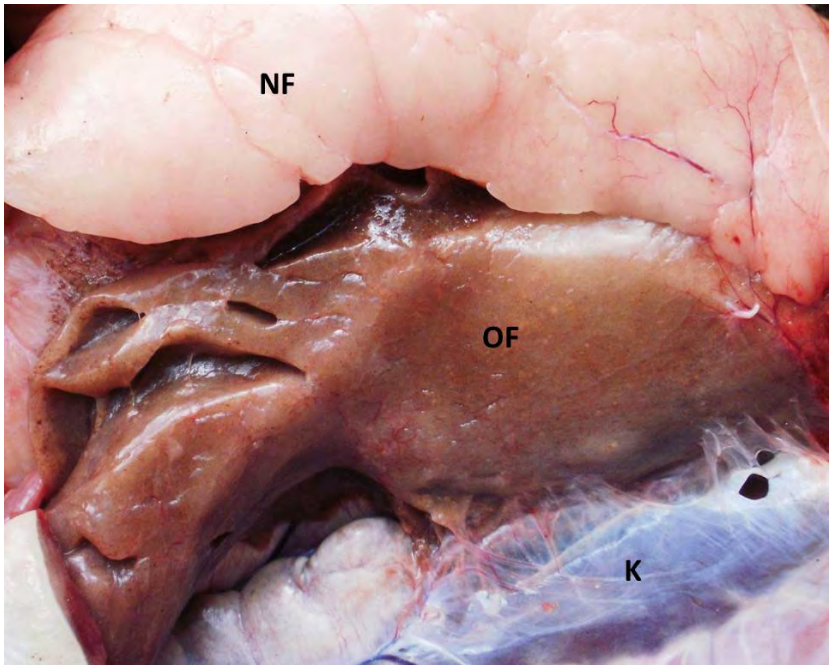

Figure 4.7: Mesenteric fat of a test fish that died 23 days after the start of the trial. Note the severely affected older fat (OF) deposited in the mesentery closest to the kidney (K) that has taken on a diffuse brown colour due to the intensity of small granulomata in the fat, and the white more recently deposited fat (NF).

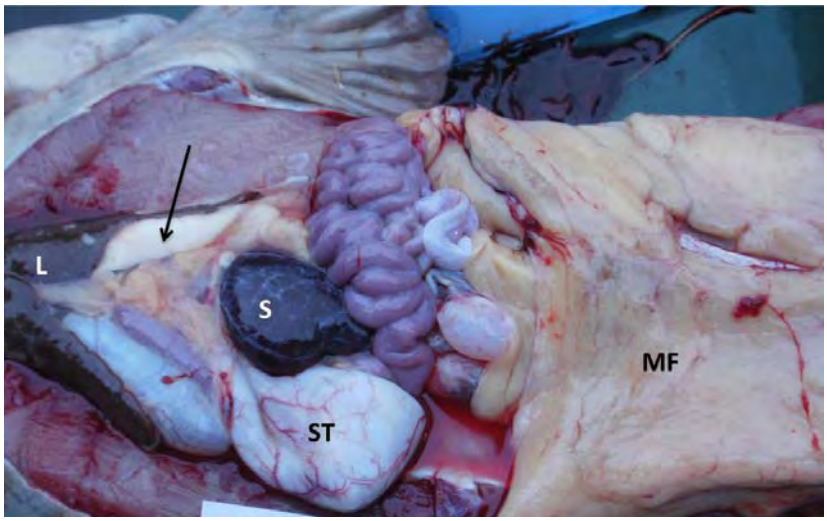

Figure 4.8: Ventro-dorsal view of the abdominal cavity of a dissected test fish at the end of the 11 month trial. Note the large amount of mesenteric fat (MF). Recently deposited white fat (arrow) can be observed in the mesentery caudal to the liver (L). Also note the large rounded spleen (S). Stomach (ST).

Steatitis was not observed in the pectoral fat of any of the test fish, however, several test fish showed presence of steatitis in the intermuscular fat. This was particularly evident in the fat adjacent to the pterygiophores (Figure 4.9).

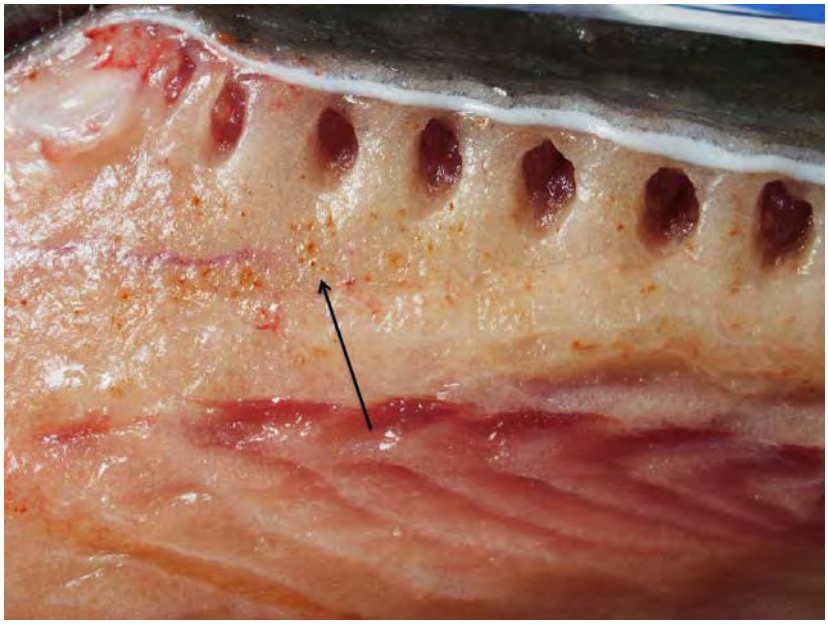

Figure 4.9: Steatitis of the intermuscular fat in the region of the pterygiophores of a test fish at the end of the 11 month trial period. Note focally disseminated small brown spots in the adipose tissue (arrow).

Histological examination of sections of mesenteric fat from the start and end of the trial revealed the same type of lesions. These consisted of focal to coalescing, roughly circular, areas of lipopigment surrounded by a prominent macrophage reaction with presence of Langhans giant cells. Affected foci were variably infiltrated with fibroblasts and showed some deposition of connective tissue. On histological examination the mesenteric fat from the control fish showed no abnormality. Adipocyte cell walls were well defined, the cell content appearing as empty space due to the solvent extraction of the fat content of the cells, and a narrow nucleus could be observed on the margin of the cells. No inflammatory cells could be detected, and the sections were devoid of fibroblasts and connective tissue. Sections of pectoral fat of both the test and control fish showed no sign ofsteatitis. Whereas some test fish showed steatitis in the intermuscular fat, control fish showed no lesions.

4.6. Discussion

Visual assessment of the mesenteric fat reserves of the test fish, through the laparotomy incision at the start of the trial, indicated that these fish carried a large amount of fat in the mesenteries. Histological examination of biopsies of this fat indicated that the fat was severely affected by pansteatitis. At the end of the 11 month trial, the test fish, having survived the winter and losing body weight, had all retained large fat reserves, affected severely by pansteatitis. Utilization of fat reserves is dependant on lypolysis of the adipose tissues. In non-feeding fish, concomitant with a loss in body weight, the mesenteric fat reserves would be expected to reduce visibly after the winter. In the test fish, these fat reserves, evaluated subjectively, appeared to remain unchanged. This lends support to the observations of Danse and Verschuren (1978b) that, in rats, lipolysis, although unaffected in early stages of steatitis, is blocked in the more advanced stages of the disease. The decrease in hormone-stimulated lypolysis was found to be proportional to the number of degenerated fat cells, most likely as a result of membrane damage in the affected fat cells preventing activation of lipase (Danse & Verschuren 1978b).

Histological examination of biopsies taken from the fat at the start and end of the trial confirmed that the histological appearance of the lesions in the fat had not changed. A reduction in body mass of the test fish compared to the weight gains of the control fish over the trial period appeared to support the observation that the test fish were markedly compromised by the pansteatitis affecting their adipose tissues. The reduction in body length noted in two of the test fish at the end of the trial was a result of erosion and shortening of the tail fin. Most of the control fish were much smaller and probably younger than the test fish. Age related factors may also have contributed to the reduction in body mass observed in the test fish, as the only very large control fish also lost weight during the trial. This may have been a result of senescence, as the fish was very large and showed no other signs of pathology.

During the last 4 months of the trial the test fish fed actively, and when euthanized at the end of the trial small amounts of healthy fat were found adjacent to the pansteatitis-affected mesenteric fat in a few fish. Persistence of pansteatitis-affected fat, while new healthy fat appears to have been deposited, supports the argument that, in catfish, fat tissues severely affected by pansteatitis are irreversibly damaged. Once the initial incitement of fat necrosis by oxidative processes has passed or the intake of vitamin E has improved, newly deposited

fat appears to remain unaffected by the inflammatory processes attempting to deal with the fat necrosis in the adjacent disturbed tissues. In cats, early cases of pansteatitis are treatable, and recovery occurs after administration of vitamin E and corticosteroids and correction of the diet (Niza*et al.* 2003). In older cats with severe chronic pansteatitis, the prognosis for recovery is poor despite treatment (Niza *et al.* 2003; White 2000).

During the first two months of the trial, which were warm, the test fish refused to take feed, nor did they feed during the subsequent winter months. Most poikilothermic animals will reduce feed intake or stop feeding when ambient temperatures drop too low. Sharptooth catfish have a wide distribution throughout the whole of Africa and are more temperature tolerant than many indigenous fish species, yet they will stop feeding during the winter at the temperatures experienced in Lydenburg. The fish were released into the experimental pond during February when water temperature was still between 24 and 26°C. Water temperatures only dropped below 20°C from April onwards, reaching 12°C by June. When the water warmed up after the winter the test fish started to feed for the first time. This was 6 months from the start of the trial. The failure to feed during the first 2 months may have had several reasons. It was not possible to establish whether the test fish were feeding at the time when they were caught at Lunsklip Fisheries and moved to the experimental facility. As they had been caught by scoop net, it can be presumed that they would have been amongst the weaker fish in the population. The debilitating effects of advanced pansteatitis, evidenced by the observed weak swimming movements and abnormal bouyancy, may have initially discouraged the test fish from actively seeking feed. These fish, previously fed exclusively on trout slaughterhouse waste, may have become conditioned to this type of diet and needed time to become accustomed to a commercial pelleted diet. The control fish, which were smaller and probably much younger and used to a diverse natural diet, may have been more adaptable, as they were keenly taking the commercial pellets within days of the start of the trial. Tilapia sleep at night, and the numerous fry and fingerlings in the pond were easy prey for the nocturnally feeding catfish. Limited visibility in the water, due to phytoplankton growth, restricted feeding observations to the surface of the pond, and the extent to which the fish were preying on tilapia could not be established.

Even though they were severely affected by pansteatitis the condition was not rapidly fatal in the test catfish. Steatitis has been reported as an incidental finding at slaughter from apparently healthy farmed channel catfish (Goodwin 2006) and American alligators (Larsen

et al. 1983). Pansteatitis is a painful condition in cats (Fytianou *et al.* 2006; Niza *et al.* 2003) and horses (de Bruijn *et al*. 2006), and probably also in other species. In contrast to crocodiles, where the intermuscular fat was most severely affected in cases of pansteatitis (F. Huchzermeyer, Faculty of Veterinary Science, University of Pretoria, pers. comm. 2009), the intermuscular fat of catfish was often unaffected or only mildly affected, even in individuals where steatitis of the mesenteric fat reserves had progressed to an advanced stage. This difference in susceptibility of the different adipose reserves of the body of catfish may provide the explanation why these animals remain reasonably mobile even when severely affected by pansteatitis.

4.7. Conclusion

After the diet had been changed, pansteatitis lesions persisted in the adipose tissues of the test fish virtually unaltered over the 11 month period of the trial, and control fish fed the same diet remained healthy. The results suggest that pansteatitis in catfish is not rapidly fatal, and the presence of chronic pansteatitis lesions does not imply recent exposure.Lesions may accumulate over time, with periodic or seasonal exposure to dietary oxidative stress. The retention of large mesenteric fat stores, affected by pansteatitis, after prolonged starvation, suggests a reduction in lipolysis in pansteatitis-affected adipose tissue. Deposition of healthy fat adjacent to pansteatitis-affected fat indicated that, despite the severe degree of pansteatitis, adipogenisis was not compromised in the test fish. The cold-tolerance of the test fish appeared unaffected by the severe degree of pansteatitis in the fish. Compared to control fish, test fish showed weaker swimming movements and some were unable to regulate bouyancy.

CHAPTER FIVE: EXPOSURE OF FISH TO SEDIMENTS FROM SITES WHERE PANSTEATITIS HAS OCCURRED

5.1. Introduction

Pansteatitis was found to be the cause of death of large crocodiles in the Olifants Gorge in the KNP in 2008 (Ferreira & Pienaar 2011) and to a lesser extent in the Sabie River in the Sabiepoort of the KNP in 2009 (D. Pienaar and D. Govender, SANParks, Skukuza, pers. comm. 2009). Pansteatitis was identified in African sharptooth catfish inhabiting the Olifants Gorge in the same area where crocodile mortalities occurred. Large dams in the Olifants River catchment act as traps for sediments, nitrates, phosphates and heavy metals and are regarded as the epicentre of recent mortalities of fish and crocodiles (Heath *et al.* 2010). Over time, changing water quality may cause sediment-bound contaminants to become bio-available and result in bio-accumulation in fish tissues.

When the pansteatitis deaths amongst the KNP crocodiles were first noticed in 2008, SANParks opened a docket with the South African Police (SAP) in the belief that the crocodiles might have been poisoned. A pollution-related aetiology was suspected stemming from the known high levels of pollution impacting the Olifants River catchment (Botha *et al.* 2011; Coetzee, du Preez & van Vuuren 2002; de Villiers &Mkwelo 2009; Heath *et al.* 2010; Oberholster *et al.* 2011). As a result the SAP Forensics Laboratory became involved in the on-going investigation. The forensics team was working in association with Prof H. Bouwman from the University of the North West to carry out toxicological evaluation of the sediments from the Olifants Gorge and Sabiepoort and tissues of fish and crocodiles.

During a sampling trip along the Olifants River gorge from 3 to 7 August 2009 the team of researchers, including the author, was given the opportunity to inspect and collect samples from the normally inaccessible section of the gorge that lies on the Mozambique border and opens into the inlet to Lake Massingir. It is in this area that the river deposits a large part of its load of silt since the raising of the Massingir dam wall in 2007 (Figure 5.1). On 8th July 2009, for the first time, a large scale fish die-off involving almost exclusively large sharptooth catfish was noticed over this area (D. Pienaar and D. Govender, SANParks, Skukuza, pers. comm. 2009). The fish kill remained localized in space and time. Unfortunately the fish carcasses were already in an advanced stage of autolysis when detected and cause of death could not be established. It was however noted that the catfish

carcasses were obese. This portion of the river is remote, and periodic fish kills may have gone unnoticed in the past. Large crocodiles favour the same area due to the presence of mud banks that provide suitable basking spots. During the period that the team worked in this area the carcasses of four large crocodiles were found that had succumbed from the effects of pansteatitis. There was a suspicion that sediments in this area of the Olifants Gorge might contain toxic pollutants and the opportunity was taken to collect samples for a bio-assay trial.

Figure 5.1: Silt deposits in the Olifants Gorge at the inlet to Lake Massingir on the South African-Mozambique border in KNP.

5.2. Objective

The objective of this experiment was to establish whether or not pansteatitis or other pathology could be induced in juvenile catfish held in water over sediments from the Olifants Gorge and the Sabie River.

5.3. Materials and Method

Sediments were collected on 6 August 2009, using a sediment grabber manufactured by the SAP Forensics Laboratory. Samples were collected from several random sites of differing depth at the inlet of Lake Massingir where the Olifants Gorge opens into the lake on the Mozambique border (S:23°57′48″ E:031°52′97″). The collected samples were pooled and transferred wet into 20 L plastic containers for transportation. Control sediments were

collected from a pool in the Bangu River, a tributary of the Olifants River in KNP that is regarded as relatively unpolluted.

Two days later SANParks collected further sediment samples from the Sabie River where this river flows into Lake Corumana in the Sabiepoort on the South African-Mozambique border of the KNP. The sediment samples were all transported wet to Lydenburg where an experimental facility was put in place.

Three duplicate pairs of trial tanks were set up for the respective sediments from the Olifants Gorge, Bangu River and Sabie River on 9th August 2009 (Table 5.1). The tanks were cylindrical and manufactured of polyethylene (Sinvac Plastics, Pretoria). A further 3 glass aquaria of 26 L volume were set up containing a portion of the same sediments.

Table 5.1: Respective experimental tank and sediment volumes used for the bio-assay trial

Tank number	Sediment source	Tank volume (L)	Sediment volume (L)
OG1	Olifants Gorge	262	86
OG2	Olifants Gorge	159	29
B1	Bangu River	262	49
B2	Bangu River	146	29
S1	Sabiepoort	262	45
S2	Sabiepoort	108	20

The tanks were housed in a plastic-covered greenhouse and were used as a stagnant water system. Being intended for use with catfish, an air-breathing species, no provision was made for aeration or filtration of the water, and bacteria from the sediments, suspended in the water column, were expected to supply sufficient biological filtration capacity for the relatively small fish biomass. The respective sediments were added to each tank (Table 5.1) and borehole water was added to fill the tanks. A sample of approximately 250 ml of each sediment was retained and frozen for future toxicological analysis. Sediment made up between 18% and 32% of the trial tank volume (Table 5.1). The sediments were mixed thoroughly with the water and then left to stand. Suitably sized catfish fingerlings were not available until 3rd January 2010, when 23 fingerlings (25-45 mm body length) were

transferred into each of the larger tanks (OG1, B1 and S1) containing the respective Olifants Gorge, Bangu and Sabie River sediments. The smaller tanks (OG2, B2 and S2) were each stocked with 35 Mozambique tilapiafingerlings (20-25 mm body length). Further tilapia fingerlings were added to tanks OG1, B1 and S1 as an extra food source for the catfish. The fish were all fed a commercial trout diet (Aquanutro).

The aquaria were placed indoors on a sunny window sill and stocked with *Daphnia* spp. (an aquatic filter-feeding crustacean). *Daphnia* in the aquaria were observed from 10th August until 26th August, after which algal growth on the tank sides made observations difficult.

The fish were kept in the trial tanks until 3 March 2011 and were not fed for 24 hours before the tanks were drained and the fish removed and euthanized with an overdose of benzocaine. The catfish were dissected and examined for gross pathology. Tissue samples from the mesenteric and pectoral adipose tissues, the liver, pancreas, spleen, kidney, heart, gonad (where sufficiently developed), muscle, skin and gills were collected and immediately fixed in 10% buffered formalin. The remainder of the carcasses were frozen for future toxicological analysis. Mozambique tilapia that had survived in the trial tanks were not examined.

Formalin fixed tissue samples were prepared by standard histological technique and 5 µm sections were stained with haematoxylin and eosin and examined by standard light microscopy.

5.4. Results

During the 16 day period during which the *Daphnia* were observed they appeared to thrive in all three aquaria and no adverse effects were observed.

Water temperature in the tanks at the start of the trial was 16°C. The temperature dropped to 12°C during the winter and reached a high of 26°C during the summer months. Within 24 hours of stirring up the sediments in the experimental tanks at the outset of the trial the colloidal suspended clay particles started to settle out, and the water began to clear. Once the catfish fry were added to the tanks the water increased in turbidity within days, as the catfish began to stir up the sediments. The tanks remained turbid due to suspended sediment for the duration of the trial. Except for 2 catfish that had jumped out of the B1 tank, on 21st October 2010, no mortalities were observed amongst the catfish during the trial period. The catfish

were observed to feed actively, mainly at night. In the turbid water it was not possible to monitor the actual numbers of fish in the tanks. Some catfish appeared to grow rapidly and were most often observed at the surface during feeding times. The tilapia fingerlings remained deep in the tanks and even at feeding times were seldom seen. Between 25th December 2010 and 14th March 2011 the tilapia died in the S2 tank as a result of oxygen depletion. No deaths were noted in the S1 tank. On 14th March 2011, 7 and 8 tilapia also died of oxygen depletion in the OG2 and B2 tanks respectively.

Measurements of the surviving catfish at the end of the 14 month trial are presented in Table 5.2. From this table it can be seen that of the original 23 catfish stocked into each trial tank 17% survived up to the end of the trial in the Olifants Gorge sediment tank and ranged in mass from 36 to 260 gram and in length from 175 to 330 mm. In the Sabiepoort sediment tank, 52% of the catfish survived and ranged in mass from 12 to 72 gram and in length from 130 to 230 mm. In the Bangu sediment tank, 30% survived with a range in body mass from 14 to 80 gram and a length of 135 to 315 mm.

Table 5.2: Measurements of surviving catfish at the end of the 14 month trial period (OGST1-OGST4=Olifants Gorge sediment; SST1-SST12= Sabiepoort sediment; BST1-BST7=Bangu sediment)

Fish no.	Sex	Mass (g)	Length (mm)	Body condition score	Mesenteric fat score
OGST1	M	260	330	3	3
OGST2	M	80	200	3	4
OGST3	F	86	222	3	4
OGST4	F	36	175	3	3
SST1	M	38	183	3	3
SST2	F	72	216	3	3
SST3	F	22	149	3	4
SST4	F	12	130	2	1
SST5	F	22	155	3	4
SST6	M	70	230	3	2
SST7	F	44	190	3	3
SST8	M	36	180	3	3
SST9	F	28	160	3	3
SST10	F	50	200	3	4
SST11	M	44	190	3	4
SST12	F	24	153	3	3
BST1	F	50	205	3	3
BST2	F	64	210	3	4
BST3	M	80	235	3	2
BST4	F	44	190	4	4
BST5	F	78	315	4	4
BST6	F	18	140	3	3
BST7	F	14	135	2	2

Dissection of the organs revealed white to pale cream mesenteric fat in all the fish. Livers of fish from all three sediment tanks were a pale ochre colour. No abnormalities were discernible in the other organs and tissues. The fish from all three tanks showed no signs of parasitosis. Sand grains were found in the stomachs of 2 of the 4 OGST fish, and sediment

detritus and clay was found in the intestines of all the OGST fish. Sediment detritus was found in the stomach content of 3 of the 12 SST fish, and 5 SST fish had sediment detritus and clay in the intestines. All the BST fish had empty stomachs, and 5 had sediment detritus and clay in the intestines. Despite the relatively small size, two female SST fish and one female OGST fish had almost fully mature ovaries; the gonads of the remaining fish showed mild to moderate development. Histological examination revealed no abnormalities in the organs and tissues examined. Steatitis could not be detected in the adipose tissues of any of the trial fish.

The Mozambique tilapia had grown slowly throughout the trial, and because of the small size, it was felt that nothing would be gained from dissecting them. No mortalities other than those caused by oxygen depletion were noticed in the OG2, S2 and B2 tanks. Survival of tilapia in the OG1, S1 and B1 tanks where they were stocked together with the catfish was poor, with 3 and 4 fish surviving to the end of the trial in the OG2 and B2 tanks respectively.

5.5. Discussion

As a result of the *ad hoc* decision to utilize sediments for a bio-assay, a makeshift experimental facility was put up at very short notice. The benthic habits of the catfish became evident within days of stocking the fish into the experimental tanks, and for the duration of the trial the fish actively kept sediment suspended in the water column, confirming the close association of this species with the sediments. Examination of stomach and intestinal content at the conclusion of the trial confirmed that the catfish were ingesting sediment prior to being euthanized. The results of this trial showed that the catfish thrived normally in the tanks containing sediments from the Olifants Gorge, the Sabiepoort and the Bangu River over the 14 month trial period. No differences could be detected in the gross and microscopic appearance of the organs of the three groups of fish, and pansteatitis was not detected in any of the fish. *Daphnia* survived for a full 16 days during which they were exposed to the respective sediments, suggesting that the sediments were not acutely toxic to this invertebrate filter feeder.

Mozambique tilapia fingerlings used in the duplicate trial tanks were not the ideal target fish for the trial and were found to grow slowly under the experimental conditions. During the later stages of the trial the duplicate sediment tanks, OG2, S2 and B2 proved to be too small for the tilapia biomass in the tanks, and tilapia died as a result of oxygen depletion. Tilapias

are known to sleep at night, resting near the bottom of waters, where they become easy prey for catfish that are nocturnally active (Bruton 1979). In the OG1, S1 and B1 tanks most of the tilapias were consumed by the catfish, tilapia being a favoured prey species of catfish (Bruton 1979). Catfish are also renowned for their cannibalistic tendencies when young, and have been observed to prey on other catfish up to half their own length (Groenewald 1964). In the confined space of the experimental tanks it was obvious that the catfish had preyed on each other. This was most noticeable in the OG1 tank where one fish reached a size of 260 gram over the trial period. This represented a 722% size increase over the smallest surviving fish in this tank.

Thisstudy attempted to address the question of whether possible bio-accumulation of contaminants released from sediments resulted in pathology in fish exposed to such sediments.Results of the field study (see Chapter 3.2.1.1) indicated that, in the Olifants Gorge, pansteatitis was not present in catfish under 3 years of age and the 14 month sediment-exposure study may have been too short to demonstrate development of pansteatitis. Although not conclusive, the results of this trial suggest that the Olifants Gorge and Sabiepoort sediments were not significantly toxic to sharptooth catfish fingerlings, and attempts to induce pansteatitis or other pathology by exposure to these sediments was unsuccessful in these fish within the limited scope of the trial.Chemical analysis of the sediments and tissue samples,by other researchers, was beyond the scope of the study and was still on-going at the time of completion of the study.

CHAPTER 6: GENERAL DISCUSSION

6.1. Introduction

When crocodiles were found to die of pansteatitis in the KNP in 2008, the author suggested to SANParks that, as pansteatitis was believed to be a nutritional disease, and crocodiles in the Olifants Gorge were likely to feed on fish, similar pathology might be found in fish inhabiting the waters of the gorge. The author subsequently discovered pansteatitis in wild African sharptooth catfish in the Olifants Gorge and several other localities in the KNP, in particular the Sabiepoort on the Sabie River where crocodiles have also died from pansteatitis. As far as the author is aware pansteatitis has not been described before from wild African sharptooth catfish in their natural habitat. This finding adds important information to the scientific knowledge on pansteatitis and dietary oxidative stress.

The objectives of this study were met in that the pathology of pansteatitis in wild catfish was described and compared to the pathology of known nutritionally-induced pansteatitis in farmed catfish. During the study an increasing prevalence of pansteatitis was found in catfish in the Olifants Gorge, and a lack of regression of lesions over time was demonstrated in captive pansteatitis-affected catfish. By showing that lesions do not regress, the author has been able to explain how lesions in wild fish are able to accumulate over time with repeated exposure to oxidative stress. The author has demonstrated that pansteatitis in catfish in the KNP was associated with damming of rivers. The author and co-workers have been able to show through comparative analysis of stomach contents, stable isotopes and analysis of fatty acids, in healthy and pansteatitis-affected catfish from the KNP, that consumption of phytoplankton-feeding fish, by both catfish and crocodiles, at certain localities in the KNP, was the most likely aetiology of the pansteatitis in these animals. The author has also demonstrated that certain haematological and blood chemistry parameters known to reflect oxidative stress are not suitable for the study of pansteatitis in wild catfish, particularly where exposure to oxidative stress is likely to have been intermittent, as the lesions in catfish can be chronic and persist long after the exposure occurred. With this study the author has contributed significant knowledge to help SANParks and South Africa's authorities to better conserve keystone aquatic species such as the Nile crocodile. The information from this study has also provided a valuable perspective on the far reaching consequences of anthropogenic impacts on our country's water catchments.

6.2. Prevalence of Pansteatitis

The results of this project provide the first insight into a serious and increasing condition in wild sharptooth catfish in some rivers in the KNP. Circumstantially the pansteatitis diagnosed in the catfish appears to be linked to outbreaks of pansteatitis in Nile crocodiles inhabiting the same stretches of these rivers. During the two year period of field sampling, the prevalence of pansteatitis in catfish steadily increased from 33% to 67% in the Olifants Gorge. Prevalence during the summer samplings was slightly lower than during the preceding winter samplings, but overall an increasing prevalence was evident in both winter and summer samplings. The author's study on persistence of pansteatitis lesions in catfish indicated that the condition was not rapidly fatal, but that pansteatitis-affected catfish were weakened by the disease. Furthermore, as lipolysis in pansteatitis-affected adipose tissue appears to be suppressed by the condition, such fat is retained, and pansteatitis lesions persist in the affected fat over long periods of time. In the wild, affected fish are likely to be more prone to predation by crocodiles. This may explain the lower prevalence of pansteatitis observed in the catfish of the Olifants Gorge during the summer, when crocodiles are feeding actively, when compared to the previous winter. Similarly, the higher prevalence during the following winter may indicate recruitment of new cases on a seasonal basis. This is supported by the pathology observed in some catfish from the Olifants Gorge that showed presence of both new and old lesions in the adipose tissues. Observations on captive catfish suffering from long-standing pansteatitis revealed the presence of pansteatitis-affected adipose tissue adjacent to more recently deposited healthy fat, reflecting periodic episodes of nutritional oxidative stress.

Pansteatitis was also identified in catfish sampled from the Sabiepoort on the Sabie River, an area topographically similar to the Olifants Gorge. Prevalence of pansteatitis in these fish was of a similar high magnitude to that recorded from the Olifants Gorge. Crocodiles have also died from pansteatitis in the Sabiepoort (D. Govender, SANParks, Skukuza, pers. comm. 2010), and, as in the case of the Olifants Gorge, a large man-made lake extends from Mozambique into the gorge of the Sabiepoort. Compared to the catchment area of the Olifants River, which is heavily impacted by a multitude of human activities such as mining, industrial, forestry, agricultural and urban developments, and is subject to extensive erosion resulting in heavy silt loads, the catchment of the Sabie River lies on the Drakensberg escarpment in areas that are extensively afforested (Anon 2001). The river passes through

some areas of agricultural development and receives municipal discharges from the town of Sabie (Thaba Chweu Municipality) before entering conservation areas and the KNP.

Pansteatitis was present at a much lower level at two further sites in the KNP, Engelhard Dam and Mamba Weir. Engelhard Dam is situated on the Letaba River just above the gorge through which the Letaba River passes before its confluence with the Olifants River in the Olifants Gorge. To the north of the Olifants River catchment, the rivers feeding the Letaba River arise in the Great Escarpment Mountains in an area dominated by afforestation, and have been dammed in many places. In the middle reaches the Letaba River flows through subtropical fruit plantations and rural settlements and agriculture before entering the KNP (Anon 2001). The low prevalence of pansteatitis identified in fish from Engelhard Dam may be explained by upstream migration of pansteatitis-affected catfish from the Olifants Gorge. Mamba Weir is a small gauging barrier across the Olifants River near the western entry point of the Olifants River into the KNP. The low prevalence of pansteatitis in catfish from this site may also be explained by upstream movement of pansteatitis-affected fish from the Olifants Gorge. The section of river around Mamba Weir differs from the Olifants Gorge in that riparian vegetation along this section of the Olifants River includes sycomore fig trees. Compared to the predominantly piscivorous diet of catfish from the Olifants Gorge, fruit of the sycomore fig trees was the most common constituent of the stomach content of catfish sampled from Mamba Weir, reflecting a clear dietary difference at this site.

No pansteatitis was found in catfish examined from several other sites in and on the outskirts of the KNP. The Crocodile River is not dammed in or near the KNP but has a similar catchment area to the Sabie River, with extensive afforestation and industrial and waste water discharges from the city of Nelspruit (Mbombela Municipality). Catfish from the Crocodile River were however healthy. The Levuvhu River and its tributaries arising in the Soutpansberg are perennial. As in the case of the Letaba River the upper catchment of the Levuvhu River is dominated by forestry. In the middle reaches of the catchment the Levuvhu River and its tributaries flow through subtropical fruit plantations and areas of rural settlement and subsistence agriculture before entering the KNP (Anon 2001). Catfish from the Levuvhu River were found to be healthy. Reënvoël Dam, an entirely rain-fed water body in the KNP represented a further reference site. Catfish from this site were found to be healthy, as were catfish from a dam at the phosphate mine on the outskirts of the town of Phalaborwa on the western side of the KNP.

Comparing the prevalence of pansteatitis in catfish in the Olifants, Sabie and Crocodile rivers in the KNP, the similarity between the Sabie and Crocodile River catchment areas and the widely different anthropogenic impacts in the Olifants River catchment provide an argument against a primary pollution-related aetiology.

The high levels of erosion in sections of the Olifants river catchment result in large amounts of sediment being deposited where the river enters Lake Massingir. The crocodile deaths of 2008 coincided with the rising level of the lake brought about by completion of therehabilitationof the dam wall in late 2006. Since then the lake extends into the Olifants Gorge, and the sediments carried by the river are now deposited in the gorge where they have drastically altered the aquatic habitat (Ferreira & Pienaar 2011). Upstream of Mamba Weir the Phalaborwa Barrage dams the Olifants River near the town of Phalaborwa. In anticipation of arriving flood waters the sluices of the Phalaborwa Barrage are from time to time opened to remove sediment and create space for incoming water. This in itself is problematic for the KNP, as low dissolved oxygen levels associated with the high oxygen demand of the stirred up sediments have occasionally resulted in the deaths of oxygen sensitive species downstream in the KNP (J. Venter, SANParks, Skukuza, pers. comm. 2012).During such episodes, for example in February 1999 and January 2004, large numbers of silver carp, were identified amongst the dead fish in the Olifants River within KNP, confirming the presence of this species during these months (J. Venter, SANParks, Skukuza, pers. comm. 2012).Such episodes, however, did not coincide with the crocodile deaths in 2008 or the pansteatitis prevalence in catfish since then.

Presence of pansteatitis in the Olifants Gorge was positively correlated with age, weight and length of the catfish. No pansteatitis was observed in fish under 3 years of age. The age, weight and length correlations with pansteatitis incidence may indicate a size-associated nutritional preference or selection. This is supported by the analysis of stomach contents of catfish sampled from the Olifants Gorge and other reference sites in and around KNP. Stomach content may only reveal the most recent food ingested by a fish, but repeated samplings from the Olifants Gorge, and other sites where pansteatitis was found, indicated a high prevalence of fish in the diet of catfish with pansteatitis. This suggests a strong link between consumption of fish and the development of pansteatitis and supports the findings of many researchers who have studied pansteatitis in various poikilothermic and homothermic animals. The condition has repeatedly been linked to high intake of polyunsaturated fats,

usually of fish origin, and the relative hypovitaminosis E that is often caused by this type of diet (Danse & Verschuren 1978a;Davis & Gorham 1954; Fytianou*et al.*2006; Goodwin 2006; Niza*et al*. 2003;Roberts & Agius 2008; Roberts & Rodger 2001; Wallach & Hoessle 1968; White 2000).

In the Olifants Gorge the causes of pansteatitis may be multi-factorial (see section 6.5). Although unlikely, if bio-accumulation of one or more xenobiotics had been involved as inciting agent in the aetiology of the pansteatitis, this process might have needed time and could have been reflected by the absence of pansteatitis in fish under 3 years of age. While the action of many xenobiotics is through a process of oxidative stress exerted on the lipids of biological membranes similar to the lipid peroxidation taking place in adipose tissues of pansteatitis-affected animals, there is little evidence in the literature linking xenobiotic bio-accumulation to the aetiology of pansteatitis. Furthermore, Coetzee*et al*. (2002) found that in the Klein Olifants River, in the heavily polluted upper catchment of the Olifants River, bio-accumulation of metals in catfish and other fish was size-related, with higher metal concentrations being found in smaller fish.

The high prevalence of pansteatitis in captive catfish at Lunsklip Fisheries could be ascribed to the excessive intake of trout slaughterhouse waste which was likely to contain rancid fats and was observed rotting in the catfish pond. Under such nutritional conditions development of pansteatitis is not surprising. The debilitating effects of pansteatitis were clearly observed in these fish, both on the farm when they were caught and during the subsequent trial to establish the persistence of the pansteatitis lesions (see Chapter 4). Such debilitating effects were also observed in pansteatitis-affected crocodiles in the Olifants Gorge and have been reported in other species suffering from pansteatitis (de Bruijn *et al*. 2006; Ginn *et al.* 2007; Niza *et al*. 2003). This is the first time that the debilitating effects of pansteatitis have been reported in sharptooth catfish.

6.3. Pathology

Necrosis of the adipose tissue resulting in pansteatitis was the main pathological change repeatedly observed in catfish from the Olifants Gorge and was a consistent indicator of oxidative stress. The fat of sharptooth catfish is distinct from that of other fish species in that a variation in colour of the mesenteric adipose tissues appears to be normal, and yellow discolouration of the fat cannot be used as an indication of lipid peroxidation as in other

species. In moderate to severely affected catfish, the lesions may be extensive and easily recognized. Histological confirmation is based on demonstrating ceroid-containing macrophages and giant cells in the extensive inflammatory reaction surrounding foci of fat necrosis.

During the study, specific pathology relating to lipid autoxidation and pansteatitis was also observed in a captive population of sharptooth catfish suffering from known nutritionally-induced pansteatitis. Observation of these fish indicated that even in severely affected fish, though debilitating, the condition was not rapidly fatal. Results of the trial done by the author to establish the fate of pansteatitis lesions confirmed persistence of pansteatitis in catfish from Lunsklip Fisheries after an 11 month period. During this period the fish were kept in a recirculated facility on a combination of live natural as well as commercial trout food. Despite a 6 month period through the winter during which the fish refused to feed and lost body condition, there was no reduction in the amount of stored mesenteric fat nor in the degree of steatitis in the fat. Similar observations were made in channel catfish (Goodwin 2006) and in captive alligators (Larsen *et al.* 1983).

Various degrees of hepatic lipidosis and ceroidosis were observed in fish with severe pansteatitis. The clustering of haemosiderin around the perimeter of fat accumulation in livers of fish with pansteatitis is interesting in that redox cycling of iron has been implicated as a cause of iron-catalysed lipid peroxidation (Kibanova *et al.* 2009; Minotti & Aust 1992). Ferric iron compounds may, however, be derived predominantly from haemoglobin catabolism (Moccia *et al.* 1984) in which case, bound to transferrin or sequestered as haemosiderin, the iron is well tolerated by the liver (Hayes 2004). Splenomegaly was a consistent finding in fish with pansteatitis, as was splenic haemosiderosis, indicative of increased haemoglobin catabolism. Reduced feed intake by fish suffering from pansteatitis is a possible cause of the observed atrophy of the pancreatic acinar tissues.

Nutritional myopathy as described in association with vitamin E deficiency and pansteatitis in some species of fish (Cowey *et al.*1984; Helder 1979; Murai & Andrews 1974;Poston *et al.* 1976; Roberts *et al.* 1979) was not observed in fish with pansteatitis from either the Olifants Gorge or Lunsklip Fisheries. This may reflect adequate dietary intake of vitamin E and selenium in these fish. Although vitamin E levels are known to deplete with acute pro-oxidant exposure, dietary vitamin E deficiency, rather than lipid peroxidation, has been

implicated as the cause of myopathy observed in various species (Ginn *et al.* 2007; van Vleet & Valentine 2007). Although an integral part of the aetiology of pansteatitis, from the results of this study a primary vitamin E deficiency appears unlikely.

Compared to catfish from Lunsklip Fisheries, catfish from most other sampling sites carried heavy burdens of parasites. Frequent and varied pathology associated with parasites was observed in most of the wild-caught fish. This varied between sampling sites depending on parasite burdens and prevalence of specific parasites. Despite the associated pathology, presence of parasites appeared to be well tolerated by the fish. Fish from Reënvoël Dam, a population where pansteatitis could not be demonstrated, showed the heaviest parasite burdens. Focal steatitis with minimal lipopigment formation was observed only infrequently in association with parasites and no correlation could be demonstrated between parasite burden and pansteatitis. The steatitis described in association with lipidosis and streptococcosis in cultured silver perch (Deng *et al*. 2012), was similarly characterised by an absence of ceroid within the necrotic lesions in fat deposits in various organs. It is interesting to note the presence of metacercariae of *Centrocestus formosanus* in gills of catfish from KNP. Spread of this zoonotic parasite,that causes marked deformity of the gill cartilage, has been associated elsewhere with introduction of carp from Asia (Velez-Hernandez *et al.* 1998).

6.4. Haematology, Blood Chemistry and Bio-monitoring

The variable intensity of pansteatitis observed in the catfish from the Olifants Gorge, and the chronic nature of the disease in these fish,provided challenges for the interpretation of the haematology and blood chemistry results. The unpredictable response of anti-oxidant defences, showing either induction or depletion, and their ability to adapt to chronic oxidant exposure further complicate interpretation (Di Giulio *et al.* 1989). However, in the Olifants Gorge, significant numbers of catfish did have low serum vitamin E values at certain times. Under field conditions in the Olifants Gorge, where exposure to oxidative stress may have been episodic, it was not possible to establish whether or not oxidative stress was present at the time when fish were sampled. In contrast to pathological changes that remained present long after the oxidative insult had occurred, the selected haematology and blood chemistry parameters that were used in this study appeared in many instances to reflect normal variation. These tests, shown to be suitable indicators of oxidative stress under experimental

conditions in the laboratory (Bell *et al*. 1985; Fytianou *et al*.2006; Moccia *et al*. 1984; Smith 1979), were not suitable under the conditions being experienced by catfish in the Olifants Gorge. Other more sensitive tests suitable for non-sacrificial monitoring of fish in the KNP warrant further investigation but were beyond the scope of this study.

Tests that merit investigation for field monitoring of oxidative stress in catfish in the KNP include determining malondialdehyde levels in the lipid fraction of serum, by use of the thiobarbituric acid reactive substances assay, and the measure by mass spectrometry of F2-isoprostanes, which are prostanoids resulting from the *in vivo* free-radical-catalysed peroxidation of arachidonic acid (Awad *et al.* 1994). As free F2-isoprostanes in plasma reflect whole body lipid peroxidation, these prostanoids may also be of value as a pro-oxidant marker in fish. The measure of F2-isoprostanes is used in the racehorse industry in South Africa but the cost of these tests was beyond the budget of this study. Although not a direct measure of lipid peroxidation, the associated damage to DNA structures provides a further opportunity for bio-monitoring, and the comet assay has been suggested as a rapid, sensitive and inexpensive method of measuring DNA oxidation (Collins 2009; Klaude *et al.* 1996;Lee & Steinert 2003) and has been used to measure the effects of dietary antioxidants in human disease (Collins 2009).

Catfish are an abundant and ubiquitous, benthic feeding species, with a relatively long life span. This study has shown that they are an ideal monitoring species in the rivers of the KNP, and may be used to monitor, indirectly, oxidant exposure in crocodiles inhabiting the same waters. Pansteatitis is not necessarily lethal in catfish, and lesions in the adipose tissues indicative of oxidant exposure persist over long periods. This allows monitoring at times of the year when access to and conditions along rivers in the KNP are conducive to sampling fish.

6.5. Xenobiotics as Possible Cause of Pansteatitis

Concern about the KNP Olifants River crocodile demise stems from the possible influence of anthropogenic effects on the Olifants River catchment, which covers some 74 500 km^2 and is home to about 8% of South Africa's population (Ashton 2010). Approximately 90% of the country's saleable coal is mined in this catchment and is used to generate 55% of South Africa's electricity, resulting in serious pollution concerns (Coetzee*et al*. 2002; de Villiers & Mkwelo 2009). The area contains numerous dams, including 38 major dams as well as the

country's second largest irrigation scheme (Anon 2001). In addition, large areas of the landscape have been changed by afforestation and agriculture. Huge increases in urban wastewater discharge and on-going high nutrient run-off from agricultural practices raise added concerns of eutrophication (Heath *et al.* 2010). A large phosphate mine is situated near the town of Phalaborwa just west of the KNP near the entry point of the Olifants River into the KNP. For a number of years prior to 2004, and once in 2008, abnormally high phosphate levels were recorded in the Olifants River within the KNP (J. Venter, SANParks, Skukuza, pers. comm. 2012). These were ascribed to the discharge of tailings from the phosphate mine into the Selati River, a tributary of the Olifants River, and to municipal sewerage discharges from the town of Phalaborwa. Phosphate discharges would have added to the inorganic nutrient load trapped in sediments of Lake Massingir and have contributed to the eutrophication of the lake (Mussagy 2008). Dissolved phosphate is often the limiting nutrient governing phytoplankton growth in fresh water, and phosphates released from sediments will continue to drive the nutrient cycle of the lake. The high levels of phosphate reaching Lake Massingir may have been a significant stimulus for phytoplankton growth resulting in the blooms observed in 2008 (J. Myburgh, Faculty of Veterinary Science, University of Pretoria, pers. comm. 2008) and may have contributed to an increasing biomass of fish in the lake.

Contamination of surface waters in the catchment, with accumulation of heavy metals within sediments through adsorption and precipitation processes, has long been recognised as a serious pollution concern. Site specific bio-accumulation of metals has been demonstrated in sharptooth catfish in the upper catchment of the Olifants River (Coetzee *et al.* 2002), and Oberholster *et al.* (2011) have suggested that aluminium and iron bio-accumulation by Mozambique tilapia in Lake Loskop may have induced their yellow fat at that site. Large dams in the catchment act as traps for sediments, nitrates, phosphates and heavy metals and are regarded as the epicentre of recent mortalities of fish and crocodiles (Heath *et al.* 2010). Over time, changing water quality may cause sediment-bound contaminants to become bio-available and result in bio-accumulation in fish tissues. Certain species of phytoplankton have been shown to bio-accumulate high concentrations of aluminium and iron from the environment in Lake Loskop in the polluted upper catchment of the Olifants River (Oberholster *et al.*2011). A similar process in Lake Massingir, though speculative, may contribute to metal bio-accumulation in fish entering the Olifants Gorge and may contribute to a multifactorial aetiology of the pansteatitis in catfish at this site. Juvenile catfish held under experimental conditions over sediments collected from the Olifants Gorge as part of

this study grew normally and did not develop pansteatitis or other pathology (see Chapter 5). This part of the study could not, however, reflect all the factors that may have been present in the Olifants Gorge, nor the role of phytoplankton in bio-accumulation of metals.

Redox cycling of iron is known to be an initiator of lipid peroxidation (Baker*et al.*1997; Demopoulos 1973; Minotti & Aust 1992; Tappel 1973), and depletion of tissue vitamin E levels by high dietary iron intake may render polyunsaturated fats in the tissues of the fish vulnerable to peroxidation (Baker *et al.* 1997). Avenant-Oldewage and Marx (2000) demonstrated bio-accumulation of various metals including iron in catfish in the Olifants River within the KNP. However numerous abiotic and biotic factors appeared to influence the degree of bio-accumulation in individual fish, and metal levels in fat tissues were not examined by these authors. Low bio-concentration factors of a number of metals, including iron, in various tissues, indicated low bioavailability of these metals in tigerfish from the Olifants River in the KNP in a study done by du Preez and Steyn (1992). Contrary to the findings of Oberholster *et al*. (2011) that indicated high levels of iron and aluminium bio-accumulation in the yellow fat of Mozambique tilapia in Lake Loskop, du Preez and Steyn (1992) found that mean iron levels in the fat of tigerfish were much lower than in the liver and gills. It has not been established to what extent iron bio-accumulates in the adipose tissues of catfish and whether such bio-accumulation has played a role in the initiation of the pansteatitis observed in the catfish sampled from the Olifants Gorge.

Pansteatitis-affected catfish caught during September of 2008 and from January to November of 2009 in the lower Letaba and Olifants Rivers were shown by special histological staining to have accumulated large amounts of iron in the form of haemosiderin in the melanomacrophages of the liver, spleen, ovary and to a lesser extent in the kidney (see Chapter 3). However, haemosiderin was not detected in the intense macrophage reaction associated with steatitis in the adipose tissues. Blood smears of many of these fish showed an abundance of immature erythrocytes as well as irregular erythrocyte shapes as described in cases of vitamin E deficiency in fish (Murai & Andrews 1974; Smith 1979;Stewart 1993), and increased erythrocyte turnover may have been the source for the increased haemosiderin carried by macrophages. Stomach contents of fish caught in the gorge at the confluence yielded mainly remnants of large fish, whereas specimens caught directly over the silt deposits on the Mozambique border only contained detritus and silt. These findings suggested bio-accumulated iron as an additional oxidative trigger, possibly ingested in polluted

sediment. Baker *et al*. (1997) have, however, proposed that African catfish efficiently regulate iron status and are able to prevent tissue assimilation of dietary iron intake. This is an important adaptation to their benthic habitat, in which they are likely to consume sediment-burrowing organisms with inadvertent ingestion of sediment. Results from a preliminary study indicated that hepatic iron levels in fish from the Olifants Gorge were lower than in fish from Lunsklip Fisheries (Dixon, Huchzermeyer, Espach& Huchzermeyer 2011). This, and the absence of haemosiderin in macrophages associated with steatitis in the adipose tissues of catfish from the Olifants Gorge, suggests that the role of iron in the aetiology of pansteatitis in catfish in the Olifants Gorge remains uncertainand needs further investigation.

Water-borne pollutants or bio-accumulated xenobiotics moving up the food chain would be expected to exert similar pathology in fish feeding at the same trophic level. Isotopic studies of the lotic food web in the Olifants Gorge have indicated that catfish from this locality had changed their dietary niche to a trophic level similar to that of tigerfish, an obligate piscivore. Both tigerfish and catfish occupy a higher trophic level in the Olifants Gorge than in other river systems in the KNP (Woodborne *et al.*2012) [see Appendix A.3], yet tigerfish in the Olifants Gorge do not develop pansteatitis. Measurement of metallothionins, acetylcholinesterase and ethoxyresorufin-O-deethylase, biomarkers respectively of metal, organophosphate and carbamate, and organochlorine exposure, have been proposed for monitoring exposure of tigerfish to these pollutants in the KNP (van Vuuren, Wepener, Smit & Vlok 2012) and may be found suitable for future monitoring of catfish.

Many xenobiotics exert their harmful effects through oxidative damage to phospholipid structures in tissues. Exposure to such pollutants would be expected to result in detectable pathology in various organs. In catfish from the Olifants Gorge, significant pathology was restricted to the adipose tissues, with the most intense and frequent lesions being present in the mesenteric fat. Changes in the liver, spleen and pancreas were secondary to the pansteatitis. Further pathology that might have indicated xenobiotic exposure or bio-accumulation was not evident.

6.6. Dietary Change and Pansteatitis in the KNP

The sharptooth catfish is a benthic opportunistic scavenger, feeds indiscriminately, and is known to hunt actively, and predation on other fish is part of the natural feeding habit of this species (Bruton 1979; Groenewald 1964; Skelton 2001; Spataru, Viveen & Gophen1987; Willoughby & Tweddle 1978). Food source varied distinctly between sampling sites, and prevalence of fish in the diet correlated with presence of pansteatitis in catfish from the Olifants Gorge and the Sabiepoort. Fish remnants observed in the stomach content of catfish from the Olifants Gorge, often in an advanced stage of digestion, frequently consisted of bones and scales of noticeably large unidentified fish. In the Olifants Gorge, both crocodiles and catfish have been observed feeding off the carcasses of dead crocodiles (D. Pienaar, SANParks, Skukuza, pers. comm. 2009), and crocodile fat afflicted with steatitis was found in the stomach contents of some catfish sampled from the Sabiepoort. In contrast, stomach content of catfish from van Ryssen Dam contained only Mozambique tilapia. These catfish showed no signs of pansteatitis.

The extension of Lake Massingir into the KNP has caused a habitat change in the Olifants Gorge that may have favoured a change in access to certain species of fish not normally consumed in large numbers by crocodiles and catfish. This could have exposed these animals to levels of polyunsaturated fatty acids in the diet to which they are not adapted. An increase in dietary polyunsaturated fat intake has been reported to result in pansteatitis in various animals. Wallach and Hoessle (1968) concluded that a change in diet from smelt (6.7% fat) to mackerel (29.9% fat) was the precipitating cause of pansteatitis in captive American alligators. Goodwin (2006) stressed the dangers of using diets high in fish oils for inappropriate species, and a change from Baltic and Mediterranean clupeids to Moroccan Atlantic pilchards was suspected to have been the cause of pansteatitis in northern bluefin tuna reported by Roberts and Agius (2008). Similarly pansteatitis is known to be induced in cats by feeding oil rich fish-based diets (Fytianou *et al*. 2006).

Examination of fatty acid ratios sheds important light on the possible aetiology of pansteatitis in the Olifants Gorge (Huchzermeyer *et al.* in press) [see Appendix A.4]. The n-6 and n-3 fatty acids derived from linoleic and α-linolenic acids respectively are essential fatty acids that cannot be synthesized by animals (Steffens 1997). The relative abundance of these fatty acids in the diet of animals is reflected in the composition of their fat tissues (Hoffman & Prinsloo 1995; Steffens 1997). The fatty acid composition of marine fish oils, and in

particular the high n-3 to n-6 ratio of polyunsaturated fatty acids contained in these oils, is a reflection of the fatty acid composition of marine phytoplankton (Steffens 1997). The ratio of total n-3 to n-6 fatty acids in marine fish oils typically lies between 5 and more than 10, whereas that of freshwater fish is much lower, ranging from 1 to 4 (Steffens 1997). In freshwater fish, as in marine fish, these fatty acid ratios are influenced by the composition of the diet. In nutrition trials the n-3 to n-6 fatty acid ratio in muscle lipid of sharptooth catfish could be manipulated from 0.1 in fish on a sunflower oil diet to 1.8 in fish on a cod liver oil diet (Hoffman & Prinsloo 1995). The fat of captive farmed crocodiles, receiving a diet of chicken, beef and horse meat, had an n-3 to n-6 fatty acid ratio of 0.08 (Osthoff, Hugo, Bouwman, Buss, Govender, Joubert&Swarts 2010). By contrast the n-3 to n-6 ratio of fatty acids in the fat of wild crocodiles suffering from pansteatitis from the Olifants and lower Letaba Rivers was found to be 2 (Osthoff *et al.* 2010). Compared to the fat of farmed crocodiles, this reflected a much higher intake of n-3 fatty acids by crocodiles in the Olifants Gorge. Mean ratios of n-3 to n-6 fatty acids in catfish with mild or no steatitis sampled from Lunsklip Fisheries, Reënvoël Dam and the Olifants Gorge in November 2009 were 0.8, 1.32 and 0.96 respectively (Huchzermeyer *et al.*in press) [see Appendix A.4]. There appeared to be no significant difference in n-3 to n-6 ratio between fish from Lunsklip Fisheries with varying degree of severity of pansteatitis. The fish with severe pansteatitis sampled from the Olifants Gorge, however, had an n-3 to n-6 fatty acid ratio of 2.87 (Huchzermeyer *et al.*in press) [see Appendix A.4]. From these results it can be inferred that rancidity rather than high polyunsaturated fatty acid intake was the cause of the pansteatitis observed in catfish from Lunsklip Fisheries. By extension of this argument it would seem unlikely that rancidity associated with intake of dead rotting fish could have been the cause of pansteatitis in the Olifants Gorge catfish and crocodiles.

Silver carp, an invasive species outside of its home range in East Asia (Kolar, Chapman, Courtenay, Housel, Williams& Jennings2005), were introduced into Mozambique from Cuba and are known to occur in Lake Massingir (Skelton 2001). Silver carp are also known to have escaped into the Olifants River in South Africa and may have spread downstream (P. Skelton, South African Institute of Aquatic Biodiversity, Grahamstown, pers. comm. 2012). This fish is a specialised plankton feeder that by preference feeds off phytoplankton and is an important consumer of cyanobacterial blooms, with *Microcystis* constituting 20-98% of the food bolus during some seasons (Kolar *et al.* 2005). Such blooms have been observed near the inlet to Lake Massingir (D. Pienaar, SANParks, Skukuza, pers. comm. 2009).

Phytoplankton naturally contain large quantities of α-linolenic acid and other n-3 polyunsaturated fatty acids, in particular EPA and DHA(Steffens 1997). Intake of these fatty acids is reflected in the adipose tissues of silver carp, with these two fatty acids, in one study, making up to 5.28% and 3.4% of body fat triacylglycerols respectively (Buchtová & Ježek 2011). As a result of the high levels of C20 and C22 fatty acids, consumption of the fat of silver carp has been proposed to have health benefits to humans equivalent to those of oil-rich marine fish (Buchtová & Ježek 2011; Steffens 1997).

A significant proportion of the essential fatty acids derived from the diet are stored in the adipose tissues of animals, and of these DHA is deposited into the adipose tissues preferentially over EPA (Lin & Connor 1990). Although the polyunsaturated fatty acids are mobilised more rapidly from the adipose tissues than saturated fats, DHA, the most polyunsaturated fatty acid, has been shown to be poorly mobilised (Connor, Lin & Colvis 1996). The higher levels of DHA found in the mesenteric fat of catfish with pansteatitis from the Olifants Gorge (11.06%) compared to mesenteric fat of those without pansteatitis (5.09%) strongly points to a higher intake of DHA in the diet of those fish that developed pansteatitis at this site (Huchzermeyer *et al*.in press) [see Appendix A.4]. A similar differentiation was not observed in the mesenteric fat of catfish with mild and severe pansteatitis from Lunsklip Fisheries, supporting the argument for a different dietary aetiology, most likely associated with rancidity of fats in the slaughterhouse waste fed to these fish.

Pansteatitis was not present in tigerfish sampled from the Olifants Gorge. Tigerfish, having evolved as obligate piscivores, may have developed anti-oxidant protective mechanisms better enabling them to cope with the consumption of higher levels of dietary polyunsaturated fats than the omnivorous catfish. Differences in prey preference and size may provide a further reason why this species has remained healthy in the Olifants Gorge.

Catfish are concentrated feeders, utilising specific food sources almost exclusively at times when these are abundant (Bruton 1979), and, although euryphagous, the role of fish in the diet of sharptooth catfish can be substantial. When circumstances allow easy access to prey, the most common species of suitable size is taken most frequently (Willoughby & Tweddle 1978). Catfish are stalking rather than active predators and, despite their generally sluggish movements, can be powerful and fast swimmers when hunting in rapids (Bell-Cross 1976). Silver carp, a schooling species, seasonally migrate upstream into rivers from the still waters

of lakes to spawn (Skelton 2001). Spawning is associated with an increase in suspended alluvium and a rise in water level of the river and occurs over an 8 to 10 week period (Kolar *et al.* 2005). The spawning migration takes place during early to midsummer, and in the Olifants Gorge this mass migration may account for intense dietary exposure of crocodiles and catfish to this species and the consequential intake of excessive polyunsaturated fats during a short period each year. This may explain the increase in crocodile mortality during the subsequent autumn and winter as observed in 2008 and to a lesser extent in the following years. In the Olifants Gorge, fish surveys are conducted by KNP scientists during the winter months when the river can be safely accessed and the waters of the river become clearer (A. Deacon, SANParks, Skukuza, pers. comm. 2012). The migratory movement of silver carp into the Olifants Gorge may thus easily have been over-looked.

The unnatural habitat in the Olifants Gorge is compounded by the presence of silver carp. In the Sabiepoort the natural habitat has been disturbed by Lake Corumana, which also acts as an inorganic nutrient trap. This has likely consequences for phytoplankton composition and growth, and plankton-feeding fish in this lake may also carry high levels of polyunsaturated fats. The similarity in habitat to the Olifants Gorge points to consumption of fish rich in polyunsaturated fats as the cause of pansteatitis at both sites, but it is not clear whether silver carp have been introduced into Lake Corumana, and other dietary factors, that have not yet been elucidated, may be involved in the Sabiepoort. Alternatively, large scale fish die-off episodes could account for a high intake of rancid fats by both catfish and crocodiles leading to pansteatitis. Both gorges are remote and difficult to access from the KNP. From the Mozambique side local fishermen frequent the gorges on the border with South Africa to set nets, and fish die-offs of the scale that caused the pansteatitis deaths of crocodiles in Lake Loskopshould have alerted Mozambican fishermen. As such die-offs have seldom been reported, the possibility of rancid fat ingestion remains unlikely.

Further investigation is needed to confirm the role of dietary fatty acid composition in the development of pansteatitis in catfish in the Olifants Gorge. The influence of phytoplankton composition on fatty acid assimilation by plankton-feeding fish species, that are likely to be preyed on by catfish, also needs to be researched. The seasonal migratory movements of plankton-feeding fish species out of lakes Massingir and Corumana into the Olifants and Sabie rivers respectively needs to be studied to confirm, in particular, the role of silver carp in the diets of catfish and crocodiles.

6.7. Pansteatitis in Catfish and the Crocodile Mortality

The discovery of pansteatitis in catfish at sites in the KNP where crocodiles have died of this disease has raised several questions. Was the pansteatitis in crocodiles linked to consumption of catfish suffering from pansteatitis or was there a common inciting factor for the disease in both species; why were crocodile and catfish deaths in the Olifants Gorge restricted to the winter months; why did surviving crocodiles become emaciated; and why have crocodile deaths declined while prevalence in catfish has increased? These questions and other points are addressed in the following paragraphs.

It is unlikely that consumption of pansteatitis-affected catfish could have led to pansteatitis in crocodiles, and the likelihood of a common nutritional cause has been discussed in the preceding point 6.6 of this chapter and in two publications (Huchzermeyer *et al.* in press, [see Appendix A.4]; Woodborne *et al*. 2012 [see Appendix A.3]). Crocodiles would have had to ingest a large number of affected catfish over a short period to consume sufficient polyunsaturated fatty acids to deplete the antioxidant mechanisms that limit the oxidative stress associated with such a diet. Pansteatitis-affected catfish from the Olifants Gorge did have higher levels of n-3 fatty acids, but the total fat content of the adipose tissues was lower than that of healthy catfish (Huchzermeyer *et al*. in press) [see Appendix A.4]. Except for occasional pack-hunting and spawning migration, catfish are mostly solitary benthic fish. Whether sporadic concentration of numbers could lead to sufficiently increased consumption by crocodiles to cause pansteatitis remains unknown.

There are several possible reasons why crocodile deaths from pansteatitis in the Olifants Gorge were restricted to winter (Ferreira & Pienaar 2011). In many aquatic poikilotherms, such as fish and crocodiles, acclimation to colder water temperatures in winter involves an increase in membrane polyunsaturated fatty acids, in particular DHA (Hazel 1979; Hulbert 2003; Seebacher, Murray & Else 2009). The greater number of double bonds in these fatty acids increases the risk of damage by reactive oxygen species released by energy-regulating metabolic pathways used during cold acclimation (Seebacher *et al.* 2009). This may be exacerbated where antioxidant mechanisms, particularly vitamin E, are depleted through oxidative stress, such as that following concentrated dietary polyunsaturated fat intake (Niki, Yamamoto, Takahashi, Komuru & Miyama 1989).

Steatitis is largely a foreign-body type reaction following on breakdown of fat cells, and presence of the lesion may imply either continuous or preceding oxidative stress. Where oxidation of lipids is not currently taking place in the adipose tissues, vitamin E levels may return to normal, as appeared to be the case in catfish from the Olifants Gorge. Depletion of vitamin E may also exert a negative effect on membrane lipids in other tissues. The only observed catfish mortality in the Olifants Gorge occurred during winter (see Chapter 1 section 1.1.2), and, although the cause of death was not established, live catfish sampled around this time were affected by pansteatitis. Under experimental conditions, catfish severely affected by pansteatitis survived water temperatures down to 12°C, well below the lowest water temperature (18°C during the study period) experienced in the Olifants Gorge (see Chapter 4). Yet, despite the severe degree of pansteatitis and the long period of fasting, female test fish had either retained or developed newly gravid ovaries by the end of the trial (see Chapter 4). It is likely that bio-membranes in critical tissues were not impaired in these fish and hence their adaptation to cold remained uncompromised.

The effect of pansteatitis on cold adaptation in crocodiles remains speculative. Crocodiles regulate their energy metabolism during cold acclimation by increased basking (Seebacher*et al*. 2009). A reduction in basking space may have occurred following the flooding of the Olifants Gorge by Lake Massingir. This may have necessitated greater reliance on cold-acclimated energy metabolism, possibly at an increased cost of oxidative stress. The reduced mobility observed in pansteatitis-affected crocodiles in the Olifants Gorge may also have impaired the basking behaviour of the animals, further compromising cold adaptation. It is not known whether hypovitaminosis E occurred in the crocodiles prior to death.

Although poikilothermic animals do not need energy to maintain homeothermy, the energy requirements needed to maintain basal metabolic processes will lead to eventual starvation if the animals stop feeding. When feeding is interrupted by crocodiles during the winter,available fat reserves provide energy. Once fat reserves become depleted or are no longer accessible, amino-acids from muscles are metabolised as a source of energy. As in the case of rats, where Danse and Verschuren (1978b) have shown that stimulated lipolysis was reduced in adipose tissues affected by steatitis, pansteatitis-affected catfish, and probably also crocodiles, are unable to fully access fat reserves damaged by pansteatitis. The catabolic processes needed to meet energy requirements explain the emaciation observed in both crocodiles (D. Govender, SANParks, Skukuza, pers. com. 2009) and catfish (see Chapter 3

section 3.2.1.1.) chronically affected by pansteatitis. Typical of pansteatitis-affected crocodiles in the Olifants Gorge (D. Govender and D. Pienaar, SANParks, Skukuza, pers. comm. 2008; personal observation 2009) was their inability to move effectively. Reduced mobility was also observed in pansteatitis-affected farmed catfish (Chapter 4). In the wild, inability to hunt prey would have exacerbated loss of condition in pansteatitis-affected animals.

Since 2008 crocodile deaths in the Olifants Gorge have declined, indicating a decreasing problem. Yet the prevalence of pansteatitis in catfish has increased. This apparent paradox can be explained by the better survival rate of catfish with pansteatitis. At the time of the first crocodile mortalities most of the dead crocodiles were large and noticeably obese (D. Govender and D. Pienaar, SANParks, Skukuza, pers. comm. 2008), as were catfish sampled at this time. The level of obesity has declined in the catfish during the study period, and SANParks scientists have observed a greater number of lean and wasted crocodiles since 2008. These crocodiles have also been diagnosed with pansteatitis (D. Govender and D. Pienaar, SANParks, Skukuza, pers. comm. 2010). Similar wasting of catfish, suffering from pansteatitis, has been observed in the Olifants Gorge, but was not present in farmed catfish with pansteatitis. This suggests that the observed muscle atrophy was an indirect consequence of the pansteatitis, most likely due to an inability to find or catch food. The trial on persistence of nutritionally induced pansteatitis (see Chapter 4) showed that lesions in the fat of catfish remained unchanged over time and that even severely affected fish survived protracted periods provided they found food. In the wild, recruitment of new cases may have contributed to the rising prevalence in the Olifants Gorge. The 2011 aerial crocodile survey by SANParks has indicated that more recently smaller crocodiles have moved into the Olifants Gorge (D. Pienaar, SANParks, Skukuza, pers. comm. 2011). Time will tell whether these animals will develop pansteatitis as they grow larger.

Catfish have been shown to be a suitable monitoring species for possible pansteatitis in crocodiles as they appear to show similar sensitivity to pansteatitis within their overlapping habitat, and a common dietary factor appears to be involved in the aetiology of the condition. Whereas the Nile crocodile is classed as endangered, the sharptooth catfish is an abundant species that in the Olifants Gorge and Sabiepoort is relatively easy to sample.

6.8. Conclusion and Recommendations

The objective of this study was to investigate the occurrence of pansteatitis in catfish inhabiting the waters in the KNP where crocodiles had died of pansteatitis, and to establish the probable causes of the pansteatitis. Catfish were sampled repeatedly over a two year period from the Olifants Gorge and from other sites in and around KNP.This study has shown that sharptooth catfish in the Olifants Gorge and several other sites in the KNP, most notably the Sabiepoort on the Sabie River, are affected withpansteatitis. During the study period an increasing prevalence of pansteatitis was recorded in catfish from the Olifants Gorge.Co-existence of old and recent lesions indicated an on-going incitement of pansteatitis in the catfish. In both the Olifants Gorge and the Sabiepoort, development of pansteatitis in both catfish and crocodiles appears to be linked to dam building and drastic alteration of the aquatic habitat of the respective gorges.

The pathology of pansteatitis in catfish in the Olifants Gorge and from other sites in the KNP is similar to that described in other animals. The pathology differed only in degree of severity from pansteatitis in catfish from a farmed population suffering from nutritionally-induced pansteatitis. Catfish, with pansteatitis, from the farmed population were found to retain lesions in the adipose tissues, unaltered, 11 months after the dietary factors had been corrected. Furthermore, these fish were shown to retain pansteatitis-affected mesenteric fat stores during a protracted period of starvation, supporting the finding of other workers that pansteatitis prevents use of fat reserves as a source of energy.

A number of haematological and blood chemistry parameters were examined to determine whether pansteatitis could be confirmed in live fish. Whilst suitable for experimental studies of pansteatitis under laboratory conditions, these tests were not appropriate for field evaluation of pansteatitis. Other tests that might be suitable, such as the thiobarbituric acid reactive substances test, the measure of F2-isoprostanes and the comet assay, need to be evaluated before non-sacrificial monitoring of pansteatitis in catfish in the KNP can be recommended.

Several explanations for the cause of pansteatitis in crocodiles and fish in the Olifants Gorge have been proposed. Bio-accumulation of one or more xenobiotics resulting from upstream pollution cannot be ruled out; yet juvenile catfish, held under experimental conditions over sediments collected from the Olifants Gorge, grew normally and did not develop pansteatitis

or other pathology.Lack of recognisable pollutant-related pathology in organs other than adipose tissue in catfish from the Olifants Gorge, and the fact that pansteatitis was found in catfish in the Sabiepoort, which has a different pollution profile, makes this aetiology seem unlikely.It is unclear whether a pathway of bio-accumulation of iron via phytoplankton similar to that in Lake Loskop exists in Lakes Massingir and Corumana, and this question provides an area for further study.

The consumption of large quantities of dead rotting fish, containing rancid fats,also seems unlikely as an inciting factor, as mass fish mortality has not been a consistent finding at the sites where pansteatitis was observed, and the fatty acid profile of farmed catfish fed rancid fish fats differed from that of catfish suffering from pansteatitis in the Olifants Gorge. The possibility cannot be precluded that consumption of catfish suffering from pansteatitis by itself could have precipitated the pansteatitis outbreak in the crocodiles, but seems unlikely.

This study raises the possibility that seasonal abundance of other fish species rich in n-3 polyunsaturated fats in the diet of catfish and crocodiles in the Olifants Gorge may have resulted in development of pansteatitis in these two species. The habitat change brought about by damming of rivers extending into KNP likely influenced access to such fish. The increasing prevalence of pansteatitis in catfish in the Olifants Gorge, and the accumulation of lesions over time, points to episodic or seasonal exposure to dietary oxidative stress. The presence of large schools of the invasive alien silver carp, benefiting from the nutrient-rich raised water level of Lake Massingir, and known for its high content of n-3 polyunsaturated fatty acids, likely formed much of the summer diet of the catfish and crocodiles, either alive or as dead remains, and is thus proposed as a cause of the obesity and pansteatitis in these animals. It is not yet clear whether silver carp occur in Lake Corumana. A study of the diet of crocodiles and catfish during the summer when migratory fish are likely to be present in the Olifants Gorge and the Sabiepoort may verify these proposals.

The study emphasizes the ecological importance and complexity of oxidative stress in a disturbed aquatic environment. The association between nutrient pollution of the aquatic environment, eutrophication, and the influence of phytoplankton on fatty acid composition of fish consuming such phytoplankton needs further study. The role of phosphate discharges into the Olifants River, the impact of dam building and subsequent silt and nutrient entrapment on relative fish species abundance, and particularly the presence of alien silver

carp within the KNP need to be researched. Pansteatitis in wild catfish is a unique finding, and although work is being done on fish from Lake Loskop (J. Myburgh and J. Steyl, Faculty of Veterinary Science, University of Pretoria, pers. comm. 2009), further work needs to be done to establish the extent to which pansteatitis may be present in fish in other artificial impoundments in polluted catchments. It is recommended that the distribution of alien fish species within rivers traversing the KNP is investigated, and that in dams within KNP and elsewhere in South Africa the long-term effects of hydrodynamic change and nutrient entrapment on the aquatic food chain are monitored, with particular reference to the health of top aquatic predators such as crocodiles.

REFERENCES

Adams S.M., Brown A.M. & Goede R.W. (1993) A quantitative health assessment index for rapid evaluation of fish condition in the field. *Transactions of the American Fisheries Society***122,** 63-73.

Agius C. (1979)The role of melano-macrophage centres in iron storage in normal and diseased fish. *Journal of Fish Diseases***2**, 337-343.

Agius C. & Agbede S.A. (1984) An electron microscopical study on the genesis of lipofuscin, melanin and haemosiderin in the haemopoietic tissues of fish. *Journal of Fish Biology***24**, 471-488.

Agius C. & Roberts R.J. (2003) Melanomacrophage centres and their role in fish pathology.*Journal of Fish Diseases***26**, 499-509.

Agresti A. & Franklin C. (2007) *Statistics: The Art and Science of Learning from Data*. Pearson Prentice Hall, Pearson Education, Inc., New Jersey, pp. 693.

Åkerman G., Amcoff P., Tjärnlund U., Fogelberg K., Torrissen O. & Balk L. (2002) Paraquat and menadione exposure of rainbow trout (*Oncorhynchus mykiss*)–Studies of effects on pentose-phosphate shunt and thiamine levels in liver and kidney. *Chemico-Biological Interactions***142**, 269-283.

Anon (2001) State of the Rivers Report: Crocodile, Sabie-Sand and Olifants River Systems, 2001, viewed 2011 from http://www.dwaf.gov.za/iwqs/rhp/state_of_rivers/crocsabieolif_01_toc.html

Ashton P. J.(2010)The demise of the Nile crocodile (*Crocodylus niloticus*) as a keystone species for aquatic ecosystem conservation in South Africa: The case of the Olifants River. *Aquatic Conservation: Marine and Freshwater Ecosystems***20**, 489-493.

Avenant-Oldewage A. & Marx H.M. (2000) Bioaccumulation of chromium, copper and iron in the organs and tissues of *Clarias gariepinus* in the Olifants River, Kruger National Park. *Water SA***26**, 569-582.

Awad J.A., Morrow J.D., Hill K.E., Roberts L.J. & Burk R.F.(1994) Detection and localization of lipid peroxidation in selenium- and vitamin E-deficient rats using F_2-isoprostanes. *Journal of Nutrition***124**, 810-816.

Bachowski S., Xu Y., Stevenson D.E., Walborg Jr. E.F. & Klaunig J.E. (1998) Role of oxidative stress in the selective toxicity of dieldrin in the mouse liver. *Toxicology and Applied Pharmacology* **150**, 301-309.

Bainy A.C.D., Saito E., Carvalho P.S.M. & Junqueira V.B.C.(1996) Oxidative stress in gill, erythrocytes, liver and kidney of Nile tilapia (*Oreochromis niloticus*) from a polluted site. *Aquatic Toxicology***34,** 151-162.

Baker R.T.M. & Davies S.J.(1996a) Changes in tissue α-tocopherol status and degree of lipid peroxidation with varying α-tocopherol acetate inclusion in diets for the African catfish. *Aquaculture Nutrition***2**, 71-79.

Baker R.T.M. & Davies S.J. (1996b) Oxidative nutritional stress associated with feeding rancid oils to African catfish, *Clarias gariepinus* (Burchell) and the protective role of α-tocopherol. *Aquaculture Research***27**, 795-803.

Baker R.T.M. & Davies S.J. (1997a) Modulation of tissue α-tocopherol in African catfish, *Clarias gariepinus* (Burchell), fed oxidized oils, and the compensatory effect of supplemental dietary vitamin E. *Aquaculture Nutrition***3**, 91-97.

Baker R.T.M. & Davies S.J.(1997b) Muscle and hepatic fatty acid profiles and α-tocopherol status in African catfish (*Clarias gariepinus*) given diets varying in oxidative state and vitamin E inclusion level.*Animal Science***64**, 187-195.

Baker R.T.M., Martin P. & Davies S.J. (1997) Ingestion of sub-lethal levels of iron sulphate by African catfish affects growth and tissue lipid peroxidation. *Aquatic Toxicology***40,** 51-61.

Begg G.S., Bruno D.W. & McVicar A.H. (2000) The histopathology and ultrastructure of steatitis affecting common dab *Limanda limanda*. *Diseases of Aquatic Organisms***41**, 123-133.

Bell J.G., Cowey C.B., Adron J.W. & Shanks A.M. (1985) Some effects of vitamin E and selenium deprivation on tissue enzyme levels and indices of tissue peroxidation in rainbow trout (*Salmo gairdneri*). *British Journal of Nutrition***53,** 149-157.

Bell-Cross G. (1976) *The Fishes of Rhodesia*. National Museums and Monuments, Harare, Zimbabwe, pp. 268.

Bernet D., Schmidt H., Meier W., Burkhardt-Holm P. & Wahli T (1999) Histopathology in fish: proposal for a protocol to assess aquatic pollution. *Journal of Fish Diseases***22**, 25-34.

Bonar C.J. & Wagner R.A. (2003) A third report of “golf ball disease” in Amazon River dolphin (*Inia geoffrensis*) associated with *Streptococcus iniae*. *Journal of Zoo and Wildlife Medicine* **34,** 296-301.

Botha H., van Hoven W. & GuilletteJr. L.J.(2011) The decline of the Nile crocodile population in Loskop Dam, Olifants River, South Africa.*Water SA***37**, 103-108.

Branson E. (1993) Basic anatomy and physiology. In: *Aquaculture for Veterinarians: Fish Husbandry and Medicine* (ed. by L. Brown), pp. 1-30. Pergamon Press, Oxford, New York, Seoul, Tokyo.

Bricknell I.R., Bruno D.W., Bowden T.J. & Smith P. (1996) Fat cell necrosis syndrome in Atlantic halibut *Hippoglossus hypoglossus* L. *Aquaculture***144**, 65-69.

Bruton M.N. (1979) The food and feeding behaviour of *Clarias gariepinus* (Pisces: Clariidae) in Lake Sibaya, South Africa, with emphasis on its role as a predator of cichlids. *Transactions of the Zoological Society of London***35**, 47-114.

Buchtová H. & Ježek F. (2011) A new look at the assessment of silver carp (*Hypophthalmichthys molitrix* Val.) as a food fish. *Czech Journal of Food Science***29**, 487-497.

Burton G.W.(1994) Vitamin E: molecular and biological function. *Proceedings of the Nutrition Society***53**, 251-262.

Bury N. & Grosell M.(2003) Iron acquisition by teleost fish. *Comparative Biochemistry and Physiology Part C* **135,** 97-105

Bus J.S., Aust S.D. & Gibson J.E. (1976) Paraquat toxicity: proposed mechanism of action involving lipid peroxidation. *Environmental Health Perspectives***16,** 139-146.

Case L.P., Carey D.P. & Hirakawa D.A. (1995) Vitamin deficiencies and excesses. In: *Canine and Feline Nutrition. A Resource for Companion Animal Professionals* (ed. by L.L. Duncan), pp. 303–308. Mosby-Year Book, St. Louis.

Coetzee L., du Preez H.H. & van Vuuren J.H.J. (2002) Metal concentrations in *Clarias gariepinus* and *Labeo umbratus* from the Olifants and Klein Olifants River, Mpumalanga, South Africa: Zinc, copper, manganese, lead, chromium, nickel, aluminium and iron. *Water SA***28,** 433-448.

Collins A.R. (2009) Investigating oxidative DNA damage and its repair using the comet assay.*Mutation Research***681**, 24-32.

Connor W.E., Lin D.S. & Colvis C. (1996) Differential mobilization of fatty acids from adipose tissue.*Journal of Lipid Research***37**, 290-298.

Cowey C.B., Degener E., Tacon A.G.J., Youngson A. & Bell J.G. (1984) the effect of vitamin E and oxidized fish oil on the nutrition of rainbow trout (*Salmo gairdneri*) grown at natural varying temperatures. *British Journal of Nutrition***51**, 443-451.

Danse L.H.J.C.& Verschuren P.M. (1978a) Fish oil-induced yellow fat disease in rats. I. Histological changes. *Veterinary Pathology***15**, 114-124.

Danse L.H.J.C.& Verschuren PM (1978b) Fish oil-induced yellow fat disease in rats. III. Lipolysis in affected adipose tissue. *Veterinary Pathology***15**, 544-548.

Davis C.L. & Gorham J.R. (1954) The pathology of experimental and natural cases of "yellow fat disease" in swine. *American Journal of Veterinary Research*.**15**, 55-59.

de Bruijn C.M., Veldhuis Kroeze E.J.B. & Sloet van Oldruitenborgh-Oosterbaan M.M. (2006) Yellow fat disease in equids. *Equine Veterinary Education***18**, 38-44.

Demopoulos H.B. (1973) Control of free radicals in biologic systems.*Federation Proceedings***32**, 1903-1908.

Deng C.Y., Peng J.H., Chen M.M., Chen M.H. & Chang P.H. (2012) Lipoidosis, steatitis, and streptococcosis in mariculture of silver perch (*Bidyanus bidyanus*). *Bulletin of the European Association of Fish Pathologists***32**, 49-55.

de Villiers S. & Mkwelo S.T.(2009) Has monitoring failed the Olifants River, Mpumalanga? *Water SA***35**, 671-676.

Di Giulio R.T., Washburn P.C., Wenning R.J., Winston G.W. & Jewell C.S. (1989) Biochemical responses in aquatic animals: a review of determinants of oxidative stress. *Environmental Toxicology and Chemistry* **8**, 1103-1123.

Diplock A.T., Green J., Bunyan J., Mchale D. & Muthy I.R. (1967) Vitamin E and stress. 3. The metabolism of D-α-tocopherol in the rat under dietary stress with silver. *British Journal of Nutrition***21**, 115-125.

Dixon R., Huchzermeyer D., Espach H. & Huchzermeyer F. (2011) Baseline metal levels in *Clarias gariepinus* and *Crocodylus niloticus* in the Kruger National Park in relation to pansteatitis. South African National Parks 9th Annual Savannah Science Network Meeting. 13-18 March 2011.Skukuza.

Draper H.H., James M.F. & Johnson B.C.(1952) Tri-o-cresyl phosphate as a vitamin E antagonist for the rat and lamb. *Journal of Nutrition***47**, 583-599.

Driescher A.C. (2007) A water quality study of Loskop Dam and the upper catchment of the Olifants River. MSc thesis.University of the Free State.

Dubey J.P. & Hartley W.J. (1992) Steatitis in a red kangaroo (*Macropus rufus*) associated with a coccidia-like protozoon. *Journal of Veterinary Diagnostic Investigation***4**, 93-96.

du Preez H.H. & Steyn G.J. (1992) A preliminary investigation of the concentration of selected metals in the tissues and organs of the tigerfish (*Hydrocynus vittatus*) from the Olifants River, Kruger National Park, South Africa. *Water SA***18**, 131-136.

Elleder M. (1991) Primary extracellular ceroid type lipopigment.A histochemical and ultrastructural study.*Histochemical Journal***23**, 247-258.

Ferreira S.M. & Pienaar D.(2011) Degradation of the crocodile population in the Olifants River Gorge of Kruger National Park, South Africa.*Aquatic Conservation: Marine and Freshwater Ecosystems* **21**,155-164.

Frye F.L. & Schelling S.H. (1973) Steatitis in a captive caiman.*Veterinary Medicine / Small Animal Clinician***68**, 143-145.

Fytianou A., Koutinas A.F., Saridomichelakis M. N. &Koutinas C. K.(2006) Blood α-tocopherol, selenium, and glutathione peroxidase changes and adipose tissue fatty acid changes in kittens with experimental steatitis (yellow fat disease). A comparative study between the domestic shorthaired and Siamese breed.*Biological Trace Element Research***112**, 131-143.

Gallagher M.L. (1993) Nutrition and nutritional diseases of temperate freshwater fishes.In: *Fish Medicine* (ed. by M.K. Stoskopf), pp. 247-250. W.B. Saunders Company. Harcourt Brace Javanovich, Inc., Philadelphia.

Geraci J.R. & St. Aubin D.J. (1980) Nutritional disorders of captive fish-eating animals. In: *The Comparative Pathology of Zoo Animals* (ed. by R.J. Montali and G. Migaki),pp. 41-49. The Symposia of the National Zoological Park.Smithsonian Institution Press. Washington DC.

Gingerich W.H. (1982) Hepatic toxicology of fishes. In: *Aquatic Toxicology* (ed. by L.J. Weber), pp. 55-105. Plenum Press, New York,

Ginn P.E., Mansell J.E.K. & Rakich P.M. (2007) Skin and appendages. In: *Jubb, Kennedy and Palmer's Pathology of Domestic Animals*, 5th edn (ed. by M.G. Maxie), pp. 553-781. Elsevier Saunders, London, New York, Oxford, Philadelphia, St. Louis, Sydney, Toronto.

Gonzalez M.J., Gray J.I., Schemmel R.A., Dugan Jr. L. & Welsch C.W. (1992) Lipid peroxidation products are elevated in fish oil diets even in the presence of added antioxidants. *Journal of Nutrition* **122**, 2190-2195.

Goodman G.C., Bunting H. & Melnick J.L. (1952) The histopathology of coxsackie virus infection in mice. I. Morphologic observations with four different viral types. *American Journal of Pathology***28**, 223-257.

Goodwin A.E.(2006) Steatitis, fin loss and skin ulcers of channel catfish, *Ictalurus punctatus* (Rafinesque), fingerlings fed salmonid diets. *Journal of Fish Diseases***29**, 61-64.

Groenewald A.A. van J.(1964) Observations on the food habits of *Clarias gariepinus* Burchell, the South African freshwater barbel (Pisces: Clariidae) in Transvaal. *Hydrobiologia***28**, 287-291.

Guarda F., Bertoja G., Zoccarato I., Tartari E. & Biolatti B. (1997) Spontaneous steatitis of epicardial fat in farmed white sturgeon (*Acipenser transmontanus*). *Aquaculture***158**, 167-177.

Gutteridge J.M.C., Paterson S.K., Segal A.W. & Halliwell B. (1981) Inhibition of lipid peroxidation by the iron-binding protein lactoferrin.*Biochemistry Journal***199**, 259-261.

Halare A.V., Seiler T-B. & Hollert H.(2011)The versatile, changing, and advancing roles of fish in sediment toxicity assessment–a review. *Journal of Soils and Sediments***11**, 141-173.

Hassoun E., Bagchi M., Bagchi D. & Stohs S.J. (1993) Comparative studies on lipid peroxidation and DNA-single strand breaks induced by lindane, DDT, chlordane and endrin in rats. *Comparative Biochemistry and Physiology***104C(3)**, 427-431.

Hayes A.M. (2004) Pathophysiology of the liver. In: *Veterinary Pathophysiology*, 1st edn (ed. by R.H. Dunlop and C-H. Malbert), pp. 371-399. Blackwell Publishing Professional, Ames Iowa, USA; Carlton, Victoria, Australia.

Hazel J.R.(1979) Influence of thermal acclimation on membrane lipid composition of rainbow trout liver.*American Journal of Physiology – Regulatory, Integrative and Comparative Physiology***236**, 91-101.

Heath R., Coleman T. & Engelbrecht J.(2010) Water quality overview and literature review of the ecology of the Olifants River *WRC Report No. TT452/10*.

Heath R., du Preez H., Genthe B. & Avenant-Oldewage A.(2004)Freshwater Fish and Human Health Reference Guide.A report to the Water Research Commission*WRC Report No TT212/04*.

Helder T.H.(1979) Myopathy and steatitis in the common guppy, *Poecilia* (*Lebistes*) *reticulata* (Peters).*Laboratory Animals***13**, 225-226.

Herman R.L. & Kircheis F.W.(1985) Steatitis in Sunapee trout, *Salvelinus alpinus oquassa* Girard.*Journal of Fish Diseases***8**, 237-239.

Hinchcliff K.W. & Piercy R.J. (2000) Oxidant stress, oxidant damage, and antioxidants: Review and studies in Alaskan sled dogs. In: *Recent Advances in Canine and Feline Nutrition,Volume III, 2000 IAMS Nutrition Symposium Proceedings*, (ed. by G.A. Reinhart and D.P. Carey), pp. 517-529. Orange Frazer Press, Wilmington, Ohio.

Hinton D.E. &Laurén D.J. (1990) Integrative histopathological approaches to detecting effects of environmental stressors on fishes.*American Fisheries Society Symposium***8**, 51-66.

Hoffman L.C. & Prinsloo J.F. (1995) Genetic and nutritional influence on the total lipid fatty acid profile of *Clarias gariepinus* muscle. *Aquatic Living Resources***8**, 415-421.

Hove E.L. (1955) Anti-vitamin E stress factors as related to lipid peroxides. *American Journal of Clinical Nutrition***3**, 328-336.

Huchzermeyer F.W.(2003)*Crocodiles.Biology, Husbandry and Diseases.*CABI Publishing Wallingford, Cambridge, pp. 337.

Huchzermeyer K.D.A.(2012) Prevalence of pansteatitis in African sharptooth catfish, *Clarias gariepinus* (Burchell), in the Kruger National Park, South Africa.*Journal of the South African Veterinary Association*83(1) Art.#916,9 pages. http://dx.doi.org/ 10.4102/jsava.v83i1.916

Huchzermeyer K.D.A., Govender D., Pienaar D.J. & Deacon A.R.(2011) Steatitis in wild sharptooth catfish, *Clarias gariepinus* (Burchell), in the Olifants and Lower Letaba Rivers in the Kruger National Park, South Africa. *Journal of Fish Diseases***34**, 489-498.

Huchzermeyer K.D.A., Osthoff G., Hugo A. & Govender D. (in press) Comparison of the lipid properties of healthy and pansteatitis-affected African sharptooth catfish, *Clarias gariepinus* (Burchell), and the role of diet in pansteatitis outbreaks in the Olifants River in the Kruger National Park, South Africa. *Journal of Fish Diseases*.

Hulbert A.J.(2003) Life, death and membrane bilayers.*Journal of Experimental Biology***206**, 2303-2311.

Hung S.S.O., Cho C.Y. & Slinger S.J. (1980) Measurement of oxidation in fish oil and its effect on vitamin E nutrition of rainbow trout (*Salmo gairdneri*). *Canadian Journal of Fisheries and Aquatic Sciences* **37**, 1248-1253.

Jolly R.D. & Dalefield R.R.(1990)Lipopigments in veterinary pathology: pathogenesis and terminology. In: *3rd International Symposium on Lipofuscin and Ceroid Pigments, 1989, Wailea, Maui, Hawai*(ed.by E.A. Porta), pp. 157-168. Plenum Press, New York.

Jones D., Howard A.N. & Gresham G.A. (1969) Aetiology of “yellow fat disease” (pansteatitis) in the wild rabbit.*Journal of Comparative Pathology***79**, 329-337.

Juan-Sallés C., Prats N., Resendes A., Domingo M., Hilton D., Ruiz J.M., Garner M.M., Valls X. & Marco A.J. (2003) Anemia, myopathy, and pansteatitis in vitamin E-deficient captive marmosets (*Callithrix* spp.). *Veterinary Pathology***40,** 540–547.

Kelly S.A., Havrilla C.M., Brady T.C., Abramo K.H. & Levin E.D. (1998) Oxidative stress in toxicology: established mammalian and emerging piscine model systems. *Environmental Health Perspectives***106**, 375-384.

Kennedy-Stoskopf S. (1993) Immunology. In: *Fish Medicine* (ed. by M.K. Stoskopf), pp. 149-159. W.B. Saunders Company, Harcourt Brace Jovanovich, Inc., Philadelphia.

Kibanova D., Nieto-Camacho A. & Cervini-Silva J. (2009) Lipid peroxidation induced by expandable clay minerals.*Environmental Science and Technology***43**, 7550-7555.

Klaude M., Eriksson S., Nygren J. & Ahnström G.(1996) The comet assay: mechanisms and technical considerations. *Mutation Research***363**, 89-96.

Kolar C.S., Chapman D.C., Courtenay Jr. W.R., Housel C.M., Williams J.D. & Jennings D.P.(2005) Asian carps of the genus *Hypophthalmychthys* (Pisces, Cyprinidae) –A biological synopsis and environmental risk assessment. *Report to US Fish and Wildlife Service per Interagency Agreement 94400-3-0128* pp.175.

Kumar V., Cotran R.S. & Robbins S.L. (1997) Environmental diseases. In: *Basic Pathology*, 6th edn. pp. 221-262.W.B. Saunders, Philadelphia, London, Toronto, Montreal, Sydney, Tokyo.

Ladds P.W., Mangunwirjo H., Sebayang D. & Daniels P.W. (1995) Diseases in young farmed crocodiles in Irian Jaya.*Veterinary Record***136**, 121-124.

Langham R.F., Zydek F.A. & Bennet R.R.(1971) Steatitis in a captive Marley garter snake.*Journal of the American Veterinary Medical Association*.**159**, 640-641.

Larsen R.E., Buergelt C., Cardeilhac P.T. & Jacobson E.R. (1983) Steatitis and fat necrosis in captive alligators. *Journal of the American Veterinary Medical Association***11**, 1202-1204.

Lee R.F. & Steinert S. (2003) Use of the single cell gel electrophoresis/comet assay for detecting DNA damage in aquatic (marine and freshwater) animals. *Mutation Research***544**, 43-64.

Levander O.A. & Beck M.A. (1997) Interacting nutritional and infectious etiologies of Keshan disease.*Biological Trace Element Research***56**, 5-21.

Lin D.S. & Connor W.E. (1990) Are the n-3 fatty acids from dietary fish oil deposited in the triglyceride stores of adipose tissue? *American Journal of Clinical Nutrition***51**, 535-539.

Lübcker N. (2011) Potential distribution of silver carp (*Hypophthalmichthys molitrix*) in South Africa.B.Sc. Honours Thesis, University of Pretoria.

Metcalfe C.D. (1998) Toxicopathic responses to organic compounds. In: *Fish Diseases and Disorders. Volume 2: Non-infectious Disorders* (ed. by J.F. Leatherhead and T.K. Woo), pp. 133-162. CABI Publishing, Wallingford, UK; New York, USA.

Minotti G. & Aust S.D. (1992) Redox cycling of iron and lipid peroxidation.*Lipids***27**, 219-226.

Moccia R.D., Hung S.S.O., Slinger S.J. & Ferguson H.W. (1984) Effect of oxidized fish oil, vitamin E and ethoxyquin on the histopathology and haematology of rainbow trout, *Salmo gairdneri* Richardson.*Journal of Fish Diseases*7, 269-282.

Murai T. & Andrews J.W.(1974) Interactions of dietary α-tocopherol, oxidized menhaden oil and ethoxyquin on channel catfish (*Ictalurus punctatus*).*Journal of Nutrition***104**, 1416-1431.

Mussagy A. (2008) Plankton monitoring program MIDSAR DP04, annual report.Massingir Dam and small holder agricultural rehabilitation project.National Directorate of Water.Ministry of Public Works and Housing. Mozambique.

Neagari Y., Arii S., Udagawa M., Onuma M., Odaya Y., Kawasaki T., Tenpaku M., Hayama H., Harada K., Mizukami M. & Murata K.(2011) Steatitis in egrets and herons from Japan.*Journal of Wildlife Diseases***47**, 49-55.

Nichols D.K., Campbell V.L. & Montali R.J.(1986) Pansteatitis in great blue herons.*Journal of the American Veterinary Medical Association***189**, 1110-1112.

Niki E., Yamamoto Y., Takahashi M., Komuru E. & Miyama Y.(1989) Inhibition of oxidation of biomembranes by tocopherol. In: *Vitamin E: Biochemistry and Health Implications* (ed. byA.T. Diplock, L.J. Machlin, L. Packer&W.A. Pryor) *Annals of the New York academy of Science***570**, 23-31.

Niza M.M.R.E., Vilela C.L. & Ferreira L.M.A. (2003) Feline pansteatitis revisited: hazards of unbalanced home-made diets. *Journal of Feline Medicine and Surgery***5**, 271-277.

Oberholster P.J., Myburgh J.G., Ashton P.J., Coetzee J.J.& Botha A-M.(2011) Bioaccumulation of aluminium and iron in the food chain of Lake Loskop, South Africa.*Ecotoxicology and Environmental Safety***75**, 134-141.

Osthoff G., Hugo A., Bouwman H., Buss P., Govender D., Joubert C.C. & Swarts J.C. (2010) Comparison of the lipid properties of captive, healthy wild, and pansteatitis-affected wild Nile crocodiles (*Crocodylus niloticus*). *Comparative Biochemistry and Physiology* A. **155**, 64-69.

Parvez S. & Raisuddin S. (2006) Effects of paraquat on freshwater fish *Channa punctata* (Bloch): Non-enzymatic antioxidants as biomarkers of exposure. *Archives of Environmental Contamination and Toxicology***50**, 392-397.

Pearson M.D., Chinabut S., Karnchanakharn S. & Somsiri T. (1994) Jaundice disease in the farmed catfish hybrid, *Clarias macrocephalus* (Gunther) x *C. gariepinus* (Burchell), in Thailand. *Journal of Fish Diseases***17**, 325-336.

Pollock C.G., Sleeman J.M. Houle, C.D. & Ramsay E.C. (1999) Vitamin E deficiency and pansteatitis in juvenile boat-billed herons (*Cochlearius cochlearius*). *Journal of Zoo and Wildlife Medicine***30**, 297–300.

Porter N.A. (1989) Autoxidation of polyunsaturated fatty acids: initiation, propagation, and product distribution (basic chemistry). In: *Free Radicals in Biology and Medicine*. 2nd edn (ed. by B. Halliwell and J.M.C Gutteridge), pp. 34-62. Clarendon Press. Oxford.

Post G.W. (1993) Nutrition and nutritional diseases of salmonids. In: *Fish Medicine* (ed. by M.K. Stoskopf), pp. 343-358. W.B. Saunders Company. Harcourt Brace Javanovich, Inc., Philadelphia.

Poston H.A., Combs Jr. G.F. & Leibovitz L. (1976) Vitamin E and selenium interrelations in the diet of Atlantic Salmon (*Salmo salar*): Gross, histological and biochemical deficiency signs. *Journal of Nutrition***106**, 892-904.

Puertollano M.A., Puertollano E., Álvarez de Cienfuegos G. & de Pablo M.A. (2011) Dietary anti-oxidants: immunity and host defence.*Current Topics in Medicinal Chemistry***11**, 1752-1766.

Raynard R.S., McVicar A.H., Bell J.G., Youngson A., Knox D. & Fraser C.O. (1991) Nutritional aspects of pancreas disease of Atlantic salmon: the effects of dietary vitamin E and polyunsaturated fatty acids. *Comparative Biochemistry and Physiology***98A(1)**, 125-131.

Roberts R.J. & Agius C. (2003) Melanomacrophage centres and their role in fish pathology. *Journal of Fish Diseases***26**, 499-509.

Roberts R.J. & Agius C.(2008) Pan-steatitis in farmed northern bluefin tuna, *Thunnus thynnus* (L.), in the eastern Adriatic.*Journal of Fish Diseases***31**, 83-88.

Roberts R.J., Richards R.H. & Bullock A.M.(1979) Pansteatitis in rainbow trout *Salmo gairdneri* Richardson: a clinical and histopathological study. *Journal of Fish Diseases***2**, 85-92.

Roberts R.J. & Rodger H.D.(2001)The pathophysiology and systemic pathology of teleosts. In: *Fish Pathology*, 3rdedn (ed. by R.J. Roberts), pp. 55-132. W.B. Saunders, London, Edinburgh, New York, Philadelphia, St. Louis, Sydney, Toronto.

Roem A.J., Kohler C.C. & Stickney R.R. (1990) Vitamin E requirements of the blue tilapia, *Oreochromis aureus* (Steindachner), in relation to dietary lipid level. *Aquaculture***87**, 155-164.

Seebacher F., Murray S.A. & Else P.L. (2009) Thermal acclimation and regulation of metabolism in a reptile (*Crocodylus porosus*): The importance of transcriptional mechanisms and membrane composition. *Physiological and Biochemical Zoology***82(6)**, 766–775.

Skelton P. 2001 *A Complete Guide to the Freshwater Fishes of Southern Africa*. 2nd edn. Struik Publishers, Cape Town, pp. 395.

Smith C.E. (1979) The prevention of liver lipoid degeneration (ceroidosis) and microcytic anaemia in rainbow trout *Salmo gairdneri* Richardson fed rancid diets: a preliminary report. *Journal of Fish Diseases***2**, 429-437.

Spataru P., Viveen W.J.A.R. & Gophen M. (1987) Food composition of *Clarias gariepinus* (= *C. lazera*) (Cypriniformes, Clariidae) in Lake Kinneret (Israel). *Hydrobiologica***144**, 77-82.

Steffens W.(1997) Effects of variation in essential fatty acids in fish feeds on nutritive value of freshwater fish for humans. *Aquaculture***151**, 97-119.

Stewart L.J. (1993) Nutrition of koi, carp and goldfish.In: *Fish Medicine* (ed. by M.K. Stoskopf), pp. 461-470. W.B. Saunders Company. Harcourt Brace Javanovich, Inc., Philadelphia.

Stoskopf M. K.(1993) Fish histology. In: *Fish Medicine* (ed. by M.K. Stoskopf), pp. 31-47. W.B. Saunders Company. Harcourt Brace Javanovich, Inc., Philadelphia.

Tappel A.L. (1973) Lipid peroxidation damage to cell components.*Federation Proceedings***32 (8)**, 1870-1874.

van den Broek A.H.M., Thoday K.L.(1994) Skin. In: *Feline Medicine and Therapeutics*. 2nd edn (ed. by E.A. Chandler, C.J. Gaskell and R.M. Gaskell), pp. 44-45.British Small Animal Veterinary Association.Blackwell Science Ltd. Oxford, London, Edinburgh; Malden, USA; Carlton, Victoria, Australia.

van Vleet J.A. &Valentine B.A.(2007) Muscle and Tendon. In: *Jubb, Kennedy and Palmer's Pathology of Domestic Animals*. 5th edn (ed. by M.G. Maxie), pp. 185-280.Elsevier Saunders, London, New York, Oxford, Philadelphia, St. Louis, Sydney, Toronto.

van Vuuren J.H.J., Wepener V., Smit N.J. & Vlok W. (2012) Biomarkers of pollution in tigerfish, *Hydrocynus vittatus*. South African National Parks 10th Annual Savannah Science Network Meeting. 5-9 March 2012. Skukuza.

Vélez-Hernández E.M., Constantino-Casas F., García-Márquez L.J. & Osorio-Sarabia D. (1998) Gill lesions in common carp, *Cyprinus carpio* L., in Mexico due to the metacercariae of *Centrocestus formosanus*. *Journal of Fish Diseases***21**, 229-232.

Wada S., Hatai K. & Kubota S.(1991)A histopathological study of cultured striped jack with yellow fat disease. *Fish Pathology***26(2)**, 61-67.

Wagenaar G.M., Smith W.C. & Smit N.J. (2012a) The health status of tigerfish, *Hydrocynus vittatus*, in two rivers in the Kruger National Park using histology as a bio-monitoring tool. South African National Parks 10th Annual Savannah Science Network Meeting. 5-9 March 2012. Skukuza.

Wagenaar G.M., Smith W.C. & Smit N.J. (2012b) Histology as a bio-monitoring tool to assess the health status of selected fish species in the Levuvhu and Olifants rivers in the Kruger National Park. South African National Parks 10th Annual Savannah Science Network Meeting. 5-9 March 2012. Skukuza.

Wallach J.D. & Hoessle C. (1968) Steatitis in captive crocodilians.*Journal of the American Veterinary Medical Association***153**, 845-847.

Watanabe T., Takeuchi T., Wada M. & Uehara R. (1981) The relationship between dietary lipid levels and α-tocopherol requirement of rainbow trout. *Bulletin of the Japanese Society of Scientific Fisheries***47**, 1463-1471.

Weyl O.L.F. & Booth A.J. (2008) Validation of annulus formation in otoliths of a temperate population of adult African sharptooth catfish *Clarias gariepinus* using fluorochrome marking of wild fish.*Journal of Fish Biology***73**, 1033-1038.

White S.D. (2000) Skin as a sensor of internal medical disorders. In: *Textbook of Veterinary Internal Medicine. Diseases of the dog and cat*, 5th edn (ed. by S.J. Ettinger and E.C. Feldman), pp. 26-29.WB Saunders Company. Philadelphia, London, New York, St. Louis, Sydney, Toronto.

Willoughby N.G. & Tweddle D. (1978) The ecology of the catfish *Clarias gariepinus* and *Clarias ngamensis* in the Shire valley, Malawi. *Journal of Zoology***186**, 507-534.

Winston G.W. (1991) Oxidants and antioxidants in aquatic animals.Mini-review.*Comparative Biochemistry and Physiology***100C**, 173-176.

Winston G.W. & Di Giulio R.T.(1991) Prooxidant and antioxidant mechanisms in aquatic organisms.*Aquatic Toxicology***19**, 137-161.

Wolf J.C. & Wolfe M.J. (2005) A brief overview of nonneoplastic hepatic toxicity in fish. *Toxicologic Pathology***33**, 75-85.

Wong E., Mikaelian I., Desnoyers M. & Fitzgerald G. (1999) Pansteatitis in a free-ranging red-tailed hawk (*Buteo jamaicensis*). *Journal of Zoo and Wildlife Medicine***30**, 584-586.

Woodborne S., Huchzermeyer K.D.A., Govender D., Pienaar D.J., Hall G., Myburgh J.G., Deacon A.R., Venter J. & Lücker N. (2012) Ecosystem change and the Olifants River

crocodile mass mortality events. *Ecosphere***3(10)**,87. http://dx.doi.org/10.1890/ES12-00170.1