SEASONAL VARIATION IN HAEMATOLOGICAL PARAMETERS AND OXIDATIVE STRESS BIO-MARKERS FOR SELECTED FISH SPECIES COLLECTED FROM THE FLAG BOSHIELO DAM, OLIFANTS RIVER SYSTEM, LIMPOPO PROVINCE, SOUTH AFRICA

by

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Declaration

I declare that the dissertation hereby submitted to the University of Limpopo, for the degree of Master of Science in Physiology has not previously been submitted by me for a degree at this or any other University; that it is my work in design and execution, and that all material contained herein has been duly acknowledged.

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Mogashoa, ME (Miss)   Date

DEDICATION

I dedicate this dissertation to my family and friends. I would like to express special gratitude to my loving parents, Freeman and Rebecca Mogashoa, who continuously encouraged me throughout my studies. My sister, Regina and brother, David for being understanding, supportive and always on my side. Furthermore, I also dedicate this dissertation to my grandparents (Magdeline Mogashoa and Regina Gafane) and my aunts (Betty, Merriam, Elizabeth and Vivian) for their wonderful support. I would also like to thank my dear friends, Monene Nyama and Adolph Ramogale, for their frequent words of encouragement and being there throughout my study.

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ABSTRACT

Water is an essential and yet scarce resource, which has a vital role for human use and also serve as a habitat for numerous organisms in aquatic environments. Despite its scarcity there have been many reports indicating that it is continually polluted by domestic, agricultural, mining and other anthropogenic activities; subsequently affecting the health of organisms residing in such water bodies. Fish have been selected as the bio-monitoring species due to its direct interaction with the environment; thereby making it an appropriate model to monitor and evaluate the health status of the environment. The feral population of the alien species, *Hypopthalmichtys molitrix* (Valenciennes, 1844) commonly known as the silver carp in Flag Boshielo Dam has been considered a healthy population. However, this perception changed considerably after reports of lethargic, dying fish were first noted in 2011. Currently the sporadic deaths amongst mature specimens (>0.7m) persist; and the reason(s) for their demise remains unclear. Therefore, the aim was to employ a seasonal study design to investigate the health status of selected fish species such as *H. molitrix* in Flag Boshielo Dam by evaluating haematological parameters, oxidative stress biomarkers and bio-accumulation levels of particular transition metals.

Seasonal surveys were carried out from February 2012 to January 2013 at Flag Boshielo Dam, Olifants River System, Limpopo Province. The locality surrounding the dam is known to be in an agriculture and mining catchment. *Hypopthalmichtys molitrix* and *Labeo rosae* (Steindachner, 1894) commonly known as the rednose labeo were collected with the use of scoop nets, conventional angling gear and gill nets.

Following collection, morphometric measurements were taken and blood was collected. The blood samples required for further analysis at the Medical Science Department, University of Limpopo were kept on ice (4˚C). After the collection of all blood samples the specific fish was sacrificed and muscle samples were collected for bio-accumulation analysis and gills and liver samples were collected for the measurement of oxidative stress biomarkers. These tissue samples were rapidly frozen and kept frozen (-85˚C) until further analysis.

Haematological parameters from the study reflected a variation amongst comparison of the inter- and intra-species. It was observed that mature *H. molitrix* suffered from anaemia. The response of glutathione-*S*-transferase (GST) and thiobarbituric acid reactive substances (TBARS) was relatively constant throughout all

ABSTRACT

seasons when the young (<0.5m) and mature *H. molitrix* (0.6 – 0.90m) specimens were compared. However, the catalase (CAT) response of mature *H. molitrix* was dramatically impaired. This would increase their vulnerability to oxidative stress. Bio-accumulation levels of the eleven selected transition elements exhibited various trends. Metals such as Molybdenum (Mo), Vanadium (V), (Chromium) Cr, Cobalt (Co), Zinc (Zn), Cadmium (Cd) and Mercury (Hg) exhibited seasonal bio-accumulation levels that were in support of the various feeding behaviours of the fish species in this study. On the other hand, metals such as Manganese (Mn), Iron (Fe), Copper (Cu), Zinc (Zn), Cadmium (Cd) and Mercury (Hg) also illustrated the potential to be contributing factors in the death of the mature specimens.

In conclusion, the findings from this study illustrate the complex nature of metabolic disturbances resulting in the death of mature *H. molitrix* specimens. It is clear that no single aspect investigated in this study could be solely implicated as the major cause of death. This multifactorial presentation necessitates further haematological assessment focussing on blood cell morphology and pathology, as well as investigations into other oxidative stress biomarkers in liver and gill tissue. In addition, identifying the most appropriate tissue type for future bio-accumulation measurements of transition metals in this feral population is necessitated. It is further suggested that neuro-muscular assessments, focussing on neurotransmitters such as γ-aminobutyric acid (GABA) and acetylcholine (Ach), form part of the investigation into the lethargic behaviour of the mature fish.

TABLE OF CONTENTS

CHAPTER 1

LITERATURE REVIEW

TABLE OF CONTENTS

TABLE OF CONTENTS

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

CAT	Catalase
CDNB	1-Chloro-2,4-Dinitrobenzene
DNA	Deoxyribonucleic Acid
DWAF	Department of Water Affairs and Forestry
EDTA	Ethylenediaminetetraacetic Acid
GST	Glutathione- -transferase
HAI	Health Assessment Index
HCT	Haematocrit
Hb	Haemoglobin Concentration
MCH	Mean Corpuscular Haemoglobin
MCHC	Mean Corpuscular Haemoglobin Concentration
MCV	Mean Cell Volume
MDA	Malondialdehyde
RBC	Red Blood Cell
RDW	Red Blood Cell Distribution Width
ROS	Reactive Oxygen Species
SD	Standard Deviation
SPSS	Statistical Package for the Social Sciences
TBARS	Thiobarbituric Acid Reactive Substances
UNEP	United Nations Environment Programme
WBC	White Blood Cell
CO_2	Carbon Dioxide
fL	Femtoliters
μm^3	Cubic Micrometer
H_2O	Water
HCO_3^-	Bicarbonate
GPx	Glutathione Peroxidase
H_2O_2	Hydrogen Peroxidase
O_2	Oxygen
$NADP^+$	Nicotinamide Adenine Dinucleotide Phosphate
ROH	Alcohol

LIST OF ABBREVIATIONS

NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
GSH	Glutathione
GSSG	Glutathione Disulfide
GR	Glutathione Reductase
NO	Nitric Oxide
$ONOO^-$	Peroxynitrite
ROOH	Carboxylic acid
g/dL	Gram/decilitre
pg	Picogram
PBS	Phosphate Buffer Saline
SDS	Sodium Dodecyl Sulphate
V	Vanadium
Cr	Chromium
Mn	Manganese
Fe	Iron
Co	Cobalt
Ni	Nickel
Cu	Copper
Zn	Zinc
Mo	Molybdenum
Cd	Cadmium
Hg	Mercury
AAS	Atomic Absorption Spectroscopy
mm^3	Cubic Millimetre
ROS	Reactive Oxygen Species
SOD (Cu/ZnSOD)	Superoxide Dismutase
ATP	Adenosine Triphosphate
GABA	γ-Aminobutyric acid
Ach	Acetylcholine

FLAG BOSHIELO DAM

GENERAL INTRODUCTION
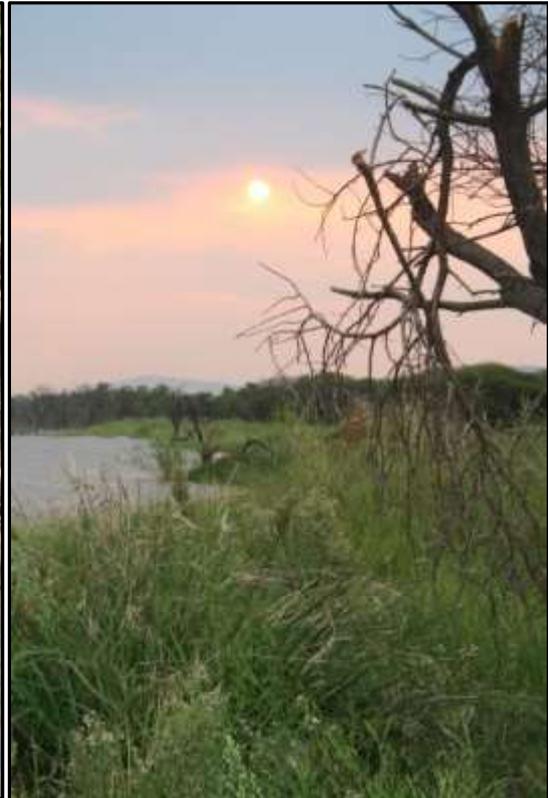
Water as a natural resource supports life and is essential for the survival of all living organisms. Abuse and exploitation result in pollution and ultimately the depletion of this invaluable resource (Water for People, 2003). Anthropogenic activities such as settlements, mining, industrialisation, agriculture and deforestation can significantly contribute to pollution of this important resource. Subsequently this can result in physico-chemical and biological changes in an aquatic system; which may in turn threaten the sustainability of water quality (Adams *et al.,* 1993; Palma *et al.*, 2015).

Anthropogenic activities contributing to industrial and domestic effluents often result in aquatic environments presenting with complex xenobiotic mixtures (Koutsogiannaki *et al.,* 2014). The ensuing alteration of the natural environment results in various environmental challenges, which amongst others include the presences and accumulation of transition metals such as manganese, copper, cobalt, mercury and vanadium. These metals are of great interest due to their diverse effect on aquatic organisms (Kubrak *et al.,* 2013). However, the deleterious effect of either chronic or acute exposure to any transition metal depends largely on inter- and intra-species differences, the type of tissue (Gabriel *et al.,* 2013), feeding behaviour and age (De Laender *et al.*, 2010). In addition to this, the use of aquatic organisms, including, but not limited to fish, as a bio-monitoring tool is an authenticated approach and serves as an early warning of unfavourable changes and the possible harmful effects following exposure to xenobiotics (Van der Oost *et al.*, 2003; Filimon *et al.*, 2013). Comprehensive haematological profiles focussing on deviations in blood composition as indicative of exposure to pollutants, as well as the use of oxidative stress biomarkers such as catalase, superoxide dismutase and glutathione-*S*-transferase (Eissa *et al.*, 2014) are some of the most frequently used bio-monitoring assessments. In support of this, the appeal of bio-monitoring as an assessment tool for specifically metal pollution in aquatic ecosystems is well described in reviews such as that by Luoma (1983) and Zhou *et al.* (2008). These scientific assessments, predominantly using organisms residing in different aquatic environments to investigate the health status of such environments, have the capacity to detect pollution levels before visible signs of pollution appear. Subsequently it can play a pivotal role in pro-active preventative strategies aimed at either avoiding or minimising the impact of pollution.

1.1 GENERAL LITERATURE REVIEW

1.1.1 Introduction

Water is a limited resource, and water bodies such as rivers and dams play a vital role in sustaining life (Scodanibbio and Mañez, 2005; Kamp *et al.*, 2007). In general of all the water resources present on earth, only a small percentage can be accessed as well as used as freshwater. In addition to this urbanisation and industrialisation have increased the demand for this resource (Smith *et al.*, 1999); thereby illustrating the negative impact of the current growth in the economy and human population dynamics.

The establishment and subsequent increase in the production of goods in various industrial sectors and urbanisation, have not only resulted in an increased demand for water, but also increased rates of water pollution. Subsequently, anthropogenic activities increase the aquatic pollutant load, thereby compromising the overall quality and health value of these resources (Mohommad *et al.*, 2013; Verma *et al.*, 2013). These pollutants tend to settle down and accumulate in aquatic environments, thus negatively impacting on the health status of organisms residing in it (Reece and Richardson, 1999; Banaee, 2013). As a result the monitoring of the health status of such organisms has the potential to assist in the early detection of a compromised aquatic environment.

1.2 AQUEOUS ENVIRONMENTS

Water quality is described as the control of the physical, chemical and biological components (properties) of water in order to be fit for usage (Department of Water Affairs and Forestry, 1996). Globally, water pollution is a major concern (United Nations Environment Programme, 2000). Similarly, it has been reported that concerns regarding the water quality of South African water systems have progressively increased (Wepener *et al.*, 2000). Sources of pollution of aquatic systems can be either point or non-point pollutants; or a combination.

1.2.1   Point and non-point pollutants

Point source pollutants are usually discharged deliberately into water bodies; fortunately most of them can be identified and as a result are relatively easy to control. In contrast, non-point source pollution cannot be traced back to its source and is difficult to control and monitor (Van Zyl and Heath, 2007). Run-off increases the occurrence of non-point source pollution, especially in regions where agricultural activities are taking place, often resulting in pesticide contamination of surface water (Amin and Hashem, 2012). The increase in the amount and types of anthropogenic substances present in an aquatic ecosystem poses a negative impact on a multitude of organisms (Reece and Richardson, 1999); hence the urgency to monitor these systems in an effort to protect natural resources.

1.2.2   Fish

In the process of monitoring aquatic environments numerous organisms can and have been used; including but not limited to fish, macro-invertebrates and even plants. However, of these, fish is more often regarded to be the most reliable organism which can be used to monitor water bodies (Velkova-Jordanoska *et al.*, 2008). This preference seems to relate to their high sensitivity to contaminants deposited into the environment, which normally results in damage to physiological systems and biochemical processes long before any other adverse environmental effects appear (Saravanan *et al.*, 2011; Wu *et al.*, 2015). Therefore, the use of these aquatic vertebrates as bio-monitoring tools can play a significant role in the early detection of environmental threats and the implementation of strategies to minimise the environmental impacts of such exposures (Zhou *et al.*, 2008).

1.2.2.1   *Hypopthalmichtys molitrix*

1.2.2.1.1   Introduction

Hypopthalmichtys molitrix (Valenciennes, 1844) commonly known as the silver carp (Figure 1.1) is a non-native South African species that originated in China and Eastern Siberia. It has now been introduced worldwide in at least 88 countries, including South Africa (Kolar *et al.*, 2007). It is predominantly introduced for aquaculture purposes. However, it is often used to control algal blooms (FAO, 2005).

In its adult stage, it is a predominantly phytoplanktivorous, filter-feeding freshwater species (Xie *et al.,* 2004). However, silver carp in the larval stage, after hatching tend to feed mainly on small zooplankton, such as protozoans and rotifers, with a particle size ranging between 8 – 100µm (Kolar *et al.*, 2007; Liu *et al.*, 2007).

In South Africa this alien species is mainly distributed in the Olifants River System, Limpopo Province; where they are frequently encountered in slow-moving or standing water (Skelton, 2001). Silver carp reaches sexual maturity at the age of 3 – 4 years, with males maturing 1 yr earlier than females (Kolar *et al.*, 2007). The lengths attained for 3 yrs old *H. molitrix* ranged from 54 – 56 cm (total length), with an average weight of 1.4 – 1.6 kg; whilst 4 yrs old fish ranged from 65 – 66cm (total length), and an average weight of 2.9 – 3.1kg (Dumitru *et al.*, 2010).

Even though spawning among silver carps has been reported to take place from mid-May to mid-June in Arkansas (Kolar *et al.*, 2007), their reproductive biology in the Olifants River System, Limpopo Province, South Africa remains largely unknown. Their growth rate is dependent on environmental factors; and the availability of food, as well as population densities can affect growth rate (Hagiwara and Mitsch, 1994).

1.2.2.1.2   Taxonomic description

Kingdom   :   Animalia
Phylum   :   Chordata
Class   :   Actinopterygii

Order	:	Cypriniformes
Family	:	Cyprinidae
Genus	:	*Hypophthalmichthys* Bleeker, 1860
Species	:	*Hypophthalmichthys molitrix* (Valenciennes, 1844)

Figure 1.1: *Hypophthalmictys molitrix* collected from Flag Boshielo Dam, Olifants River System, Limpopo Province. (Courtesy of Dr L.J.C. Erasmus)

1.2.2.2   *Labeo rosae*

1.2.2.2.1   Introduction

Labeo rosae Steindachner, 1894 also known as the rednose labeo (Figure 1.2) is endemic to South Africa and widely distributed in the Limpopo, Incomati and Phongolo River Systems in the lowveld reaches (Skelton, 2001). These teleost fish (teleost = ray-finned fishes) prefer habitats with large perennial rivers with sandy stretches and they mostly feed on algae, detritus and small vertebrates (Reid, 1985; Skelton, 2001). Breeding takes place in summer and sexual maturity is reached when they have a total length of approximately 150 mm.

Figure 1.2: *Labeo rosae* collected from Flag Boshielo Dam, Olifants River System, Limpopo Province. (Courtesy of Dr L.J.C. Erasmus)

1.2.2.2.2   Taxonomic description

Kingdom   :   Animalia
Phylum   :   Chordata
Class   :   Actinopterygii
Order   :   Cypriniformes
Family   :   Cyprinidae
Genus   :   *Labeo* Cuvier, 1816
Species   :   *Labeo rosae* Steindachner, 1894

1.2.3   Biological monitoring

The implementation of biological monitoring, to evaluate and determine adverse environmental changes (Besse *et al*., 2012), is generally based on the use of living organisms. These organisms are normally referred to as "indicator organisms" and when such species are studied the following aspects should be considered: (i) a wide distribution and low mobility, (ii) their ecological characteristics must be known, (iii) they must exhibit high sensitivity to pollutants and, (iv) be suitable for laboratory experiments (Füreder and Reynolds, 2003). Some of the more recognised biochemical

analysis performed, whilst investigating the impact of known or suspected environmental exposure, includes a comprehensive haematological evaluation, the use of various oxidative stress bio-markers and establishing the bio-accumulation levels of numerous biologically active elements such as the transition metals (Lebrun *et al.*, 2015).

1.3 HAEMATOLOGY

The close association of fish with their aquatic environment results in them being exposed to any particulate matter dissolved in the water; many of these substances are capable of adversely affecting physiological mechanisms. It is known that many physiological and environmental factors have the potential to affect the haematological profile and composition; in humans and fish alike (Pyszel *et al.*, 2005; Hamid *et al.*, 2013). In support of this, haematological assessments frequently forms the basis of physiological and toxicological studies (Hamid *et al.*, 2013), and, in the detection of pathological changes resulting from exposure to pollutants (Satheeshkumar *et al.*, 2012). However, haematological alterations are not only subjected to pollutants.

Haematological parameters can be affected by factors such as transportation and physical handling of species, seasonal and genetic variations, gender and the distribution of the organism (Örün and Erdemil, 2002; Gbore *et al.*, 2006). Prior to considering a more comprehensive morphological assessment of the various blood components, the basic haematological evaluation would involve establishing total red blood cell (RBC) count, total white blood cell (WBC) count, haematocrit (Hct or PVC), haemoglobin concentration (Hb) and erythrocyte indices such as mean cell volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) (Campbell, 2004). Additional morphological assessments are recommended when findings from the haemogram are inconclusive or the aetiology unknown.

1.3.1   White blood cell count

The immune system plays an important role in the ability of an organism to successfully manage the detrimental health consequences of diseases, injuries, undesirable exposures and other stressful encounters. White blood cells are key components in specific (adaptive) and non-specific (innate) immune responses (Prasad and Charles, 2010). This is supported by the fact that an increased WBC is indicative of damage due to infection of body tissues, severe physical stress and even leukaemia (Singh *et al.*, 2008). Furthermore, decreased WBCs will increase an organism's vulnerability to stress and infection (Sunomonu and Oyelola, 2008). Therefore, it is a common practice in fish health studies to use WBC to validate immune competence.

1.3.2   Red blood cell count

Red blood cells (erythrocytes) are well-known for the life-supporting role they play in the gaseous exchange of vertebrates; more specifically their primary role in the transport of oxygen (O_2) to tissues, but also the removal of carbon-dioxide (CO_2), a metabolic waste product. Even though differences exist (when comparing human erythrocytes to fish erythrocytes) with regard to their structure and nucleated or non-nucleated nature (Vazquez and Guerrero, 2007), it is generally agreed that haemoglobin in these cells are responsible for the binding and transport of O_2 to tissues. The mechanism employed in the removal of CO_2 is not related to haemoglobin *per se*, but rather involves carbonic anhydrase to facilitate the production of bicarbonate (HCO_3^-) from CO_2 and H_2O; a process that amongst others plays an essential role in the control of blood pH. These critical physiological roles emphasise the necessity to tightly control red blood cell numbers within a specific range; in an effort to sustain proper metabolic function(s).

In teleost fish, similar to other vertebrates, homeostatic mechanisms keep RBC relatively stable in an attempt to maintain the count within its optimal physiological range (Adeyemo, 2007). Exposing an organism to environmental pollutants and irritants has an impact on the RBC (Adeyemo *et al.,* 2008); and it has been found that

a decrease in RBC correlates positively with the level of stress experienced by fish (O'Neal and Weirich, 2001). In addition, it should be remembered that RBC also affect the oxygen carrying capacity of blood; therefore, the presence of either anaemia or polycythaemia will affect the delivery of oxygen to the tissues (Rashidi *et al.*, 2012; Diyaware *et al.*, 2013). Anaemia is a condition characterised by a deficiency in RBC and/or haemoglobin; whereas, polycythaemia indicates an increased concentration of haemoglobin caused by either a reduction in plasma volume or an increase in RBC. Fortunately, anaemia and polycythaemia can be determined via the measurement of the haematocrit and other related erythrocyte indices.

1.3.3   Haematocrit and haemoglobin

Haematocrit, often referred to as the packed cell volume (PCV), is an essential blood parameter used to determine the presence of anaemia or polycythaemia in fish (Archer and Jeffcott, 1977; Lowe, 2004), and other vertebrates. Anaemia in teleost fish has been observed when haematocrit values are <20% (Tonya *et al.*, 2008). However, this value should be approached with caution during the diagnosis of anaemia as some species, such as the sandbar shark (*Carcharhinus plumbeus*) and the Port Jackson shark (*Heterodontus portusjacksoni*) have a normal PCV≤20% (Campbell, 1988; Arnold, 2005). In contrast, the diagnosis of polycythaemia, in general, is confirmed when the PCV≥45%; a condition that can easily result from aspects such as sexual maturity, exposure to hypoxia, stressed-induced catecholamine release and erythrocyte swelling (Blaxhall, 1972; Fange, 1992; McDonald and Milligan, 1992). In addition, the relationship between haematocrit and haemoglobin concentration is also relevant; the oxygen carrying capacity relates better to the concentration of haemoglobin (Rashidi *et al.*, 2012; Diyaware *et al.*, 2013) than the haematocrit itself. It has been reported that fish possess a higher number of multiple haemoglobins when compared to other animals (Giardina *et al.*, 2004); a physiological advantage demonstrated in its ability to adapt with relative ease to aquatic environments where the availability of environmental oxygen can differ tremendously (Landini *et al.*, 2002).

1.3.4   Red blood cell indices

The basic haematological profile is often employed as a diagnostic tool for evaluating the health status of organisms (Ramesh *et al.*, 2013). Amongst these, the various erythrocyte indices have been found to be of particular interest in the assessment and diagnosis of anaemia in organisms exposed to stressful conditions (Coles, 1986). Clauss *et al.* (2008) applied the following basic principles and descriptive terminology in addressing anaemia in fish:

- Three primary types of anaemia
  - Haemorrhagic – blood loss
  - Haemolytic – RBC destruction
  - Hypoplastic – poor erythropoiesis
- Descriptive terminology
  - Reference to cell size – microcytic, normocytic or macrocytic
  - Indication of haemoglobin concentration – hypochromic, or normochromic
  - Indicative of cell loss – haemolytic or haemorrhagic
  - Reference to haemopoietic status – regenerative or non-regenerative

This approach (Clauss *et al.*, 2008) highlights the relevance, extensiveness and focus areas of the following erythrocyte indices used to investigate the presence and aetiology of anaemia in fish populations.

1.3.4.1   Mean cell volume

The mean cell volume (MCV), also called the mean corpuscular volume, refers to the measurement of the average red blood cells and it enables the diagnosis (Sandhaus and Meyer, 2002) and classification of the different categories of anaemia into microcytic, normocytic or macrocytic (Kim *et al.*, 2014).

1.3.4.2   Mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration

The mean corpuscular haemoglobin (MCH) is deduced from the red cell count and the MCV; and mean corpuscular haemoglobin concentration (MCHC) is calculated from the measured Hb and the deducted PCV (Dacie and Lewis, 1984; Sarma, 1990). These measurements (MCH and MCHC) are used to evaluate anaemic conditions (Sachar and Raina, 2014); and they have clinical relevance in the identification of conditions such as macrocytosis, aplastic anaemia, iron deficiency and sideroblastic anaemia (Eastham and Slade, 1992).

1.3.4.3   Red blood cell distribution width

Red blood cell distribution width (RDW, expressed as a percentage) is the coefficient of variation, a product of the standard deviation of erythrocyte cell size divided by the MCV. Thus it expresses the heterogeneity observed within the size distribution curve of the red blood cell population of a specific sample (Harrington *et al.*, 2008); hence its preference as a semi-quantitative measure of erythrocyte anisocytosis (Rezende *et al.*, 2014). In real terms no condition exists that frequently yields a RDW less than normal, thus by default the clinical practice is to consider it as either normal or elevated (Schrier and Landaw, 2012). At present RDW is applied as an ancillary index in the differential diagnosis of specifically microcytic anaemia (Rezende *et al.*, 2014); where increased RDW levels, revealing a greater level of heterogeneity, is an indication of iron deficiency anaemia (microcytic), Vitamin B_{12} deficiency and also the impairment of erythropoiesis (Magri and Fava, 2014). This is in contrast to thalassemia syndromes, resulting from the production of fewer erythrocytes and smaller quantities of haemoglobin, where normal RDW values are indicative of a more homogenous erythrocyte size (Rezende *et al.*, 2014).

1.4   OXIDATIVE STRESS

1.4.1   Introduction

Biomarkers are indicators of biological changes which occur in fish, and other organisms, as a result of exposure to various environmental stressors. Furthermore, these metabolic parameters are considered to be practical measures that can effectively be used as bio-monitoring tools in environmental risk assessment (Van der Oost *et al.*, 2003). This is of particular interest as it reveals how environmental stressors affect biological systems at a sub-cellular level (Adams *et al.*, 2001). It is therefore not surprising that the use of biomarkers have increased tremendously to detect, amongst others, metabolic responses to stressful environmental conditions. Subsequently many investigative efforts focussed on the ability of organisms to efficiently manage the metabolic impact of oxidative stress.

Oxidative stress results from a homeostatic imbalance which occurs when the amount of reactive oxygen species (ROS) produced during aerobic processes, exceeds the rate at which they are eliminated by antioxidant mechanisms (Ellah, 2011; Ameur *et al.*, 2012). Inadequate physiological responses to elevated ROS levels can contribute significantly to protein oxidation, DNA damage as well as the onset of lipid peroxidation in tissues; where the end result will be oxidative damage (Vinodhini and Narayanan, 2009). Thus, the vulnerability of any tissue type to oxidative stress depends on its ability to activate appropriate antioxidant responses.

Catalase, GST and lipid peroxidation are amongst the most commonly used biomarkers for cellular stress, resulting from environmental contaminants (Amado *et al.*, 2006; Vinagre *et al.*, 2014). Their enzymatic activities and responses are subjected to environmental factors such as temperature, seasonal variation and salinity (Madeira *et al.*, 2013). In addition to this the metabolic processes in different body tissues is tightly regulated by their respective homeostatic demands; as a result the function and location of such tissues can significantly influence the impact of oxidative stress on them (Vinagre *et al.*, 2014). This concept is well illustrated in the susceptibility of the

brain to oxidative stress; a vulnerability that results from its incredibly high demand for oxygen, the abundant presence of lipid peroxidation

targets such as unsaturated fatty acids and compared to other tissues its lower antioxidant enzyme activities (Ho *et al.*, 1997; Dringen, 2000). However, it is also known that organisms have the physiological capability to adjust metabolic pathways in an effort to cope with environmental stress, especially in cases of chronic exposure. Ibrahim and Harabawy (2014) reported that fish, as a result of the elevated production of ROS, developed a mechanism that enables them to counter the detrimental impact of pollutants via the release of xenobiotic metabolising enzymes. In addition to this it was found that CAT serves as the first line of defence against oxidative stress (Ibrahim and Harabawy, 2014), whilst GST acts as a cytotoxic detoxifying enzyme capable of limiting lipid peroxidation and as a result it suppresses the occurrence of apoptosis (Banaee, 2013). These findings support the use of such biomarkers in the present study.

The vulnerability of any biological system to oxidative stress depends on the demand for oxygen, the availability of suitable substrates such as unsaturated fatty acids that would act as lipid peroxidation targets, as well as the presence of antioxidant enzymes such as catalase (CAT) and glutathione-*S*-transferase (GST) (Figure 1.3) to regulate metabolic processes (Ho *et al.*, 1997; Dringen, 2000). Enzymes are biochemical macromolecules involved in metabolic processes and activities; and are responsible for the control of various physiological functions within living organisms (Hunter, 1995). Alterations in environmental conditions initiate physiological responses that will assist the organism in coping with the metabolic impact of such stressful circumstances (Somero, 1992); if these coping mechanisms are inadequate it can result in oxidative stress and ultimately tissue damage.
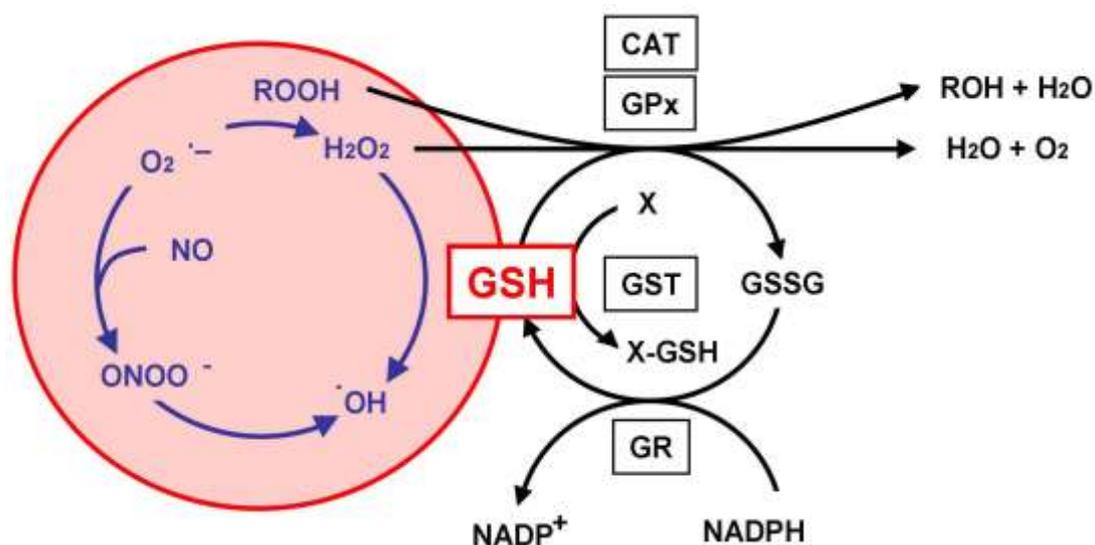
Figure 1.3. An illustration of enzymatic and non-enzymatic processes involved in the oxidative stress response (Adapted from Aoyama and Nakaki, 2012).

Exposure to pollutants induces a stressed state, where reactive oxygen species (ROS) can accumulate and cause damage. Subsequently oxidative stress occurs since the organism's defence mechanisms are incapable of handling the amount of ROS in circulation (Simonato *et al.*, 2011). Reactive oxygen species are important in numerous physiological processes (Oliveira *et al.*, 2010); however, at high concentrations they have a tendency to result in oxidative damage. This has the potential to modify DNA, lipids and proteins, ultimately resulting in mitochondrial bio-energetic failure followed by apoptosis and/or necrosis (Chuang, 2010). An increase in oxidative stress biomarkers, such as catalase (CAT) and glutathione-*S*-transferase (GST) can act as an early warning of possible environmental pollution (Haux and Forlin, 1988). In addition, lipid peroxidation can be used to indicate cell and tissue damage caused by ROS (Valavanidis *et al.*, 2006) and has been proven a useful and reliable tool in monitoring exposure to pollutants (Park *et al.*, 2006; Srivastava *et al.*, 2006).

1.4.2 Catalase

Catalase (EC 1.11.1.6), a tetrameric haem-containing enzyme, present in almost all aerobic cells, catalyses the decomposition of hydrogen peroxide (H_2O_2) to water (H_2O) and molecular oxygen (O_2) (Prakash *et al.*, 2011). Thus, it protects the cells against the toxic effects of ROS such as hydrogen peroxide (Hermes-Lima and Storey, 1993). It is an enzyme with one of the highest turnover rates; a single CAT molecule has the potential to convert millions of hydrogen peroxide molecules to water and oxygen every second (Goodsell, 2004). The down regulation of this enzyme can render the organism more susceptible to oxidative stress resulting from environmental exposure.

1.4.3 Glutathione-*S*-transferase

Glutathione-*S*-transferase (EC 2.5.1.18) is primarily responsible for detoxifying endogenous and exogenous electrophiles (Zhang *et al.*, 2012). The detoxification mechanism is dependent on the conjugation of reduced glutathione (GSH) with toxic compounds, to increase the hydrophilicity and assist in the excretion of such toxicants (Ketterer *et al.*, 1983). Even though most fish species exhibit GST catalytic activity, it has not been as well described as for their mammalian counterparts (Schlenk *et al.*, 2008). It plays a major role in alleviating oxidative stress (Lee *et al.*, 2008) and is used as biomarker in monitoring environmental pollution (Cunha *et al.*, 2007).

1.4.4 Lipid peroxidation

Lipid peroxidation is an autocatalytic mechanism which results in oxidative stress, which further causes the development of oxidative damage of cellular membranes and even organelles (Cheeseman, 1993; Niki, 2009). Lipid peroxidation in teleost fish is measured as thiobarbituric acid reactive substances (TBARS) (Stepić *et al.*, 2012); which is frequently used as an indicator of oxidative stress (Olakolu *et al.*, 2012) resulting from exposure to environmental pollutants (Almroth *et al.*, 2005).
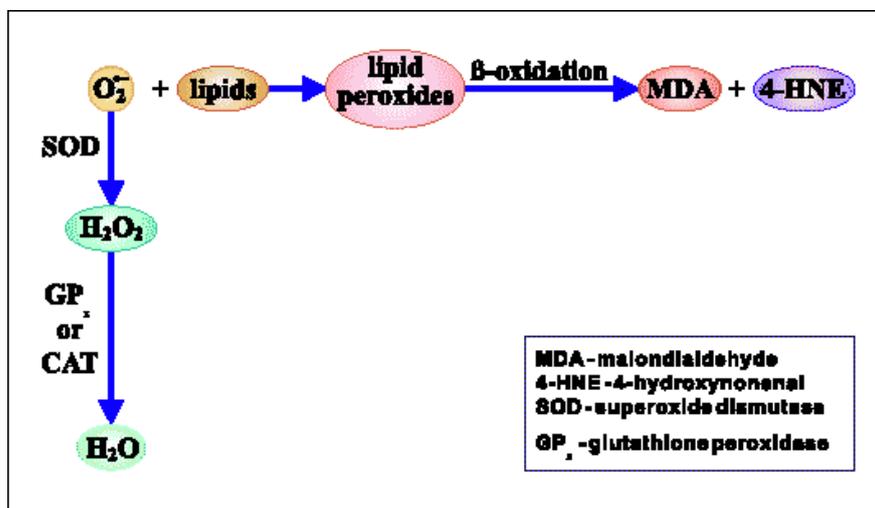
Figure 1.4: The transformation pathways of a superoxide radical anion. The superoxide molecule is either metabolized via the process of lipid peroxidation or participates in neutralization to form H_2O_2 and H_2O (Adapted from Kwiecien *et al.*, 2002).

Lipid peroxidation occurs mainly because of the decomposition of polyunsaturated fatty acid peroxides found in membrane lipids (Azevedo *et al.*, 2013), and it yields an end product known as malondialdehyde (MDA) (Figure 1.4) (Niedworok and Bielaszka, 2007). It is used as a reliable marker for free radical-mediated and oxidative stress (Atip *et al.*, 2010), and also as an important biomarker for monitoring the effects caused by the presence of pollutants such as petroleum products (King *et al.*, 2012), cyclic aromatic hydrocarbons (Otitoloju and Olagoke, 2011), and heavy metals (Osuala, 2012). It is known to react with the free amino group of proteins, phospholipids and nucleic acids; thereby, resulting in structural transformation that can impede optimal functioning of the immune system (Lee *et al.*, 2004). It is therefore not surprising that lipid peroxidation has been implicated in the pathogenesis of various diseases and clinical conditions (Repetto *et al.*, 2012).

1.5   BIO-ACCUMULATION

1.5.1   Introduction

Bio-accumulation refers to a time-dependent increase in the overall concentration of contaminants, which have the ability to induce detrimental health effects, in the tissues of organisms. Some of these contaminants, including but not limited to the various transition metals, are known to be non-biodegradable; therefore, they have an inclination to accumulate in the environment and eventually reach harmful levels (Blanco *et al*., 2014). It has been reported that high concentrations of metals are found in sediments and as a result might present an increased risk of exposure to benthic feeders (Velusamy *et al*., 2014). In addition, the persistence of metals in the aquatic environment is attributed to their non-degradable nature, which promotes bio-magnification (Clark, 1992). However, metal bio-accumulation in fish is a remarkably complex process and encompasses so much more than only feeding behaviour.

The basic principles in fish nutrition, as primarily noted in reports on developments in the aquaculture sector, is aimed at the stimulation of optimal growth and health as well as the effective control of waste production. Even though basic nutritional requirements seem to be relatively similar for captive and feral populations; feral populations are at a distinct disadvantage due to the structure and composition of their environment. A very important nutrient group is the micronutrients which includes the numerous minerals and vitamins. These minerals, as a primary focus of the current study, refer to a group of inorganic elements required by the body for optimal homeostatic performance. The implication of these elements, more specifically those forming part of the transition metal group, in many regulatory processes such as acting as metalloenzymes, the production of blood cells and as part of the oxidative stress response (Gatlin, 2010), identified them as relevant to this study. Subsequently, the bio-accumulation levels of 11 selected transition metals were measured in muscle tissues of the pelagic feeder *H. molitrix* and the benthic feeder *L. rosae*.

Apart from the pollutants which are contributed by anthropogenic activities, various metals have been identified to be of particular concern worldwide (Mohamed *et al*., 2012). They are potentially toxic (Censi *et al*., 2006) and have been reported to

accumulate in sediments and organisms, to result in ecological damage and also elicit adverse effects on human health as humans often consume affected organisms such as fish (Malik *et al.*, 2010; Jooste *et al.*, 2014). The focus on muscle tissue relates to the fact that it is the 'part' of the fish most often consumed; which formed part of a study looking at the human health risks involved in utilising such food sources (Jooste *et al.*, 2014). These metals usually accumulate when organisms, living in a particular environment, are exposed to them via the food they consume and/or directly from the environment (Hellawell, 1986). The rate of accumulation of metals vary significantly (Kotze, 2003), due to diffusion gradients which are created as they are absorbed by the tissue until they reach the internal organs (Retief *et al.*, 2006). The importance of metals, especially many of those classified as transition metals, has been implicated for various physiological processes. It is equally true that such physiologically active elements should be regulated within an optimal physiological range; and that any deviation from this set point can result in homeostatic imbalances.

1.5.2 Molybdenum

Molybdenum (Mo) is a transition metal that can be found in living organisms where it is considered an essential micronutrient (Goyer, 1986; Eisler, 1989). It is classified as an inorganic element which comprises approximately 0.00015% of the earth's crust (Chappell, 1975). This metal is frequently used in industry especially in the manufacturing of steel alloys and weapons (Eisler, 1989), and even as a catalyst in petroleum refining (Galadima and Ibrahim, 2010). Anthropogenic activities can significantly contribute towards its environmental deposition.

These residues can be found in elevated amounts near Mo mines; as a result its presence might affect animals and plants alike. From the work done by Davies *et al.* (2005) it seems highly unlikely that signs and symptoms of toxicity would appear as exposure levels have to be extremely high for this to happen (LC_{50} values ranging from 70 – 2000mg/L). Nonetheless, this contradicts earlier reports stating that 28 day Mo exposure, in rainbow trout, had an LC_{50} value of 0.73 – 0.79mg/L (Birge, 1978; Birge *et al.*, 1980). In agreement with the latter two studies, Fletcher and Warburton (1997),

accepts 73µg/L as a water quality guideline employed in the protection of aquatic organisms. In support of this exposure disparity, and the fact that at this moment comprehensive information regarding the physiological and toxicological impact of Mo in fish is lacking, contributed to Saiki *et al.* (1993) and Reid (2002) proposing that this metal is non-toxic to fish.

1.5.3   Vanadium

Vanadium (V), a transition element that is widely distributed in the earth's crust has an average abundance of 0.14mg/kg (Amorim *et al.*, 2007); however, the World Health Organisation (1988) estimates its range to be between 3 – 310mg/kg in soil. Irrespective of the fact that it is considered to be a non-volatile metal it has been reported to be toxic when found in sediments and aquatic bodies (Gummow, 2011). Even though V is not considered to be an essential element, it is involved in affecting the physiological functioning of organs such as the liver and kidney (Mahmoud *et al.*, 2011). The detrimental health impact of V has been reported in conjunction with Cd, where it was found to induce hepatotoxicity (Valko *et al.*, 2005). Furthermore, its metabolic potential is reflected in its ability to accumulate in organisms, to inhibit enzymes such as Na^+/K^+-ATPase and proteinkinases (Mukherjee *et al.*, 2004; Zaki *et al.*, 2007), and to regulate lipid peroxidation via its impact on antioxidant enzymes such as CAT and glutathione peroxidase (Gummow, 2011).

1.5.4   Chromium

Chromium (Cr), regarded as one of the heavy metals, is found in the environment in two forms, Cr (III) the beneficial micronutrient and Cr (VI) the harmful contaminant (Hubicki and Kolodyńska, 2012). It has been identified as a very scarce metal with very low concentrations in the aquatic environment (Nussey *et al.*, 2000). However, anthropogenic activities such as industrial effluents, industrial emissions and also combustion processes (Bielicka *et al.*, 2005) increase the environmental concentration of this metal to such an extent that it reaches toxic levels, which can ultimately be

detrimental to the health of organisms. In addition, the toxicity of Cr not only depends on its own chemical speciation, it is also influenced by the animal species, body size, feeding patterns and even environmental conditions such as the pH of water, salinity, water hardness and also temperature (Holdway, 1988).

1.5.5   Manganese

Manganese (Mn) is an abundant and naturally occurring element which is easily released into water bodies through runoff by agricultural activities (Apori *et al.*, 2012; Farina *et al.*, 2013). This element does not occur in a natural or pure state; therefore, it exists as salts, chelates or even in oxidative states (Farina *et al.*, 2013); the various forms are used as gasoline additives, paint manufacturing and production of ceramics (Su *et al.*, 2012). Anthropogenic activities, such as those mentioned, are known to contribute towards environmental pollution; thus often contributing to excessive environmental levels.

Manganese is known as an essential trace element, which is required for normal cellular functioning, especially in the brain. In addition, it is involved in carbohydrate and lipid metabolism, and it is also known as a co-factor for certain enzymes (Watanabe *et al.*, 1997; Strydom *et al.*, 2006). Although it has been reported to exhibit relatively low toxicity to aquatic organisms (Seymore *et al.*, 1995); it is well known for its neurotoxicity. The latter is most probably attributed to the capacity of Mn to induce oxidative stress and disrupt neurotransmission (Erikson *et al.*, 2004). The damage to neuronal cells, glial cells and neurons, might be a consequence of the preferential accumulation of Mn in mitochondria; which can induce disturbances to the process of oxidative phosphorylation (Malthankar *et al.*, 2004).

1.5.6   Iron

Iron (Fe), an abundant transition element (Weber *et al.*, 2006), can exist in one of three oxidative states, *viz.* Fe(II), Fe(III) and Fe(IV). Even though many of its physiological functions are documented elsewhere, of relevance in the current context is its

structural inclusion in haemogloblin (Anim *et al*., 2011), as well as the regulation of enzyme activity (Wachtershauser, 2007) such as that of CAT (Watanabe *et al*., 1997).

1.5.7   Copper

Copper (Cu) is classified as an essential trace nutrient, which is required in small amounts in all forms of living organisms including fish (Bury *et al*., 2003); it also functions as a co-factor for numerous biological processes (Valko *et al*., 2005). It has been reported to play an important role in stimulating erythropoiesis (Samanta *et al*., 2011) and haemoglobin production (Shingadia, 2012), and the synthesis of enzymes *viz*. cytochrome oxidase, CAT and superoxide dismutase (Anim *et al*., 2011). Its deficiency in humans and other vertebrates, including fish, results in anaemia while in excess it is associated with toxicity and liver damage (Kanumakala *et al*., 2002).

1.5.8   Nickel

Nickel (Ni) is classified as an essential nutrient which has numerous cellular functions including activation and inhibition of enzymes such as hydrogenases, transaminases and α-amylase (Alexandrovn *et al*., 2006; Das, 2009). It is required in small amounts; its deficiency is associated with poor absorption of ferric acid, alteration of the metabolism of calcium and anaemia; the latter most probably due to a decreased Hb and Hct (Samal and Mishra, 2011). In addition to this, Ni can also enter the Fenton reaction to partake in the oxidative stress response.

1.5.9   Cobalt

Cobalt (Co), another naturally occurring element is widely distributed and can be found in soil, animals, plants and even aquatic bodies (Gál *et al*., 2008). It has two common oxidative states (Co^{2+} and Co^{3+}), which contributes to its industrial application in cutting tools, surface coatings, ceramics and pigmentation to name a few (Gál *et al*., 2008). Not only is Co toxic and carcinogenic, it is also known to enter the Fenton reaction inducing oxidative stress through the production of hydroxyl radicals (Leonard *et al*.,

1998). The ability of Co(II) to generate various oxidising species is well known (Parejo *et al.*, 2000). In contrast to these harmful effects of Co, this transition metal plays an important role as an essential element in the formation of Vitamin B_{12} (Tripathi and Srivastava, 2007). This vitamin, also known as cobalamin, is a key factor in the synthesis of haemoglobin (Das, 2009); where a deficiency is associated with anaemia while an excess results in polycythaemia (Anim *et al.*, 2011). Unlike the study by Murtala *et al.* (2012), reporting that the bottom feeding fish in their study exhibited higher accumulation levels compared to the other species; the current study found that *L. rosae* had far lower accumulation levels throughout all seasons ($P = 0.000$). Cobalt therefore, seems more accessible to *H. molitrix* than to *L. rosae*. In addition, the muscle tissue levels of Co reached its lowest concentration during the winter survey; most probably due to the impact of lower water temperatures.

1.5.10   Zinc

Arguably the most apt and provocative description, regarding the physiological relevance of zinc (Zn), was that of Plum *et al.* (2010), who depicted it as an "essential toxin". The magnitude of information on the physiological versatility of Zn, indicate that there could be no doubt that either deficient levels or excessive exposure to it will have detrimental health impacts irrespective of the organism involved (Cuevas and Koyanagi, 2005; Ackland and Michalczyk, 2006; Bhowmik *et al.*, 2010; Yoshikawa *et al.*, 2013; Khan and Awan, 2014; Khan *et al.,* 2014). Of particular interest in this discussion, based on the haematological findings, oxidative stress biomarkers and behavioral observations in the current study, is the role of Zn in physiological mechanisms implicated in the development of signs and symptoms recorded here.

1.5.11   Cadmium

Cadmium (Cd) is a highly toxic metal, even at low concentrations, and is capable of interfering with the optimal functioning of various metabolic pathways (Mohanty *et al.*, 2013). Heavy metals, such as Cd, become toxic when the body cannot metabolise

them and they accumulate in soft tissue. Subsequently, the liver, placenta, kidneys, lungs and brain are highly susceptible to Cd toxicity (De Lurdes Dinis and Fiuza, 2011). Of particular interest in the current study was to determine if Cd exposure, as reflected in its seasonal bio-accumulation levels in muscle tissue of *H. molitrix* and *L. rosae*, could be implicated in the following (i) the development of anaemia in mature *H. molitrix* as reported in Chapter 3, (ii) disturbing the oxidative stress response, thus contributing to excessive ROS production, and (iii) contribute to the observed behavioural and morphological abnormalities, i.e. excessive mucous production, abnormal swimming behaviour and quiescence. The latter is relevant in the current context as Mohanty *et al.* (2013), studying *Labeo rohita* after exposure to sub-lethal Cd exposure, found that exposure to this metal can indeed induce various behavioural and morphological changes.

1.5.12 Mercury

The ubiquitous nature of environmental mercury (Hg), as well as the fact that all of its forms exhibit toxic effects (Zalups, 2000), makes it practically impossible for organisms to avoid exposure to some form of it (Valko *et al.*, 2005). The more prominent toxicological characteristics of Hg relate to impacts on the nervous system (Farina *et al.*, 2013), renal and gastro-intestinal systems (Rice *et al.*, 2014), and haematology (Maheswaran *et al.*, 2008). Prior to considering some of these toxicological features of Hg as relevant in explaining the deaths of mature fish, it was important to establish whether a disparity exist in (i) bio-accumulation levels of fish species with different feeding behaviours and (ii) if seasonal and/or pooled seasonal Hg levels in young and mature *H. molitrix* support the theory that the latter should exhibit elevated levels in order to implicate Hg as a causative factor.

1.6   AIM AND OBJECTIVES

1.6.1   Aim of the study

To use haematological parameters, oxidative stress biomarkers and bio-accumulation levels of selected transition metals in an effort to analyse the impact of environmental contaminants on the health status, of selected fish species such as *H. molitrix* and *L. rosae*.

1.6.2   Objectives of the study

The objectives of the study are to:

a)   employ various haematological parameters, *viz*. Hct, Hb, RBC, WBC and erythrocyte indices to verify the presence of diseased states such as anaemia and polycythaemia;

b)   examine the relationship between seasonal bio-accumulated metal levels and oxidative stress responses employing the measurement of CAT, GST and TBARS levels from liver and gill tissue;

c)   determine the contribution of seasonal variation on muscle bio-accumulation levels of various metals, reflecting on the relevance of feeding behaviour in the accumulation of the selected transition metals;

d)   examine the interaction between oxidative stress bio-markers and selected metals in an effort to identify possible homeostatic mechanisms that have been compromised.

1.7   COMPOSITION OF THE DISSERTATION

The composition of this dissertation entails six chapters. Chapter 1 is the literature review and the aim and objectives for this study. The focus of Chapter 2 is a comprehensive methods and materials discussion that includes the description of the sampling site, as well as the fish species. Chapters 3 to 5 are the result chapters; where Chapter 3 deals with all relevant haematological aspects related to *H. molitrix* and Chapter 4 with the biomarkers for oxidative stress in *H. molitrix.* Chapter 5

investigates the bio-accumulation of selected transition metals in both *H. molitrix* and *L. rosae*. Chapter 6 is the concluding chapter followed by the reference list.

1.8   SCOPE OF THE STUDY

At the onset the focus of the study was purely to conduct a comparative study of homeostatic responses to environmental pollutants between selected freshwater fish species. However, the focus had to be adjusted as it was noted that sporadically some of the bigger specimens of *H. molitrix* were floating lethargically at the water surface; subsequently dying shortly thereafter. This observation created an urgency to understand why these fish were dying in an effort to determine if the reason(s) was species-specific with little risk to other aquatic organisms or if the evidence support the possibility that other organisms might be affected in due time.

1.9   LIMITATIONS

This study, similar to most research endeavours, identified a number of aspects that were either not available during this study or specifically addressed in it, thus limiting the extent to which the data could be interpreted. The collection of blood smears to investigate morphological abnormalities of red and white blood cells could have added value. The biomarkers used in this study were considered adequate for the purpose of this investigation; however, a more comprehensive approach including the measurement of superoxide dismutase and GSH levels might have been helpful. Except for the opportunity to use bio-accumulation values for trends, the lack of baseline values for transition metal bio-accumulation levels in *H. molitrix* made it very difficult to relate this to specific metabolic impacts.

Figure 1.5. Field work on the bank of Flag Boshielo Dam (Courtesy of Dr L.J.C. Erasmus).

Methods and Materials

2.1   STUDY SITE

The Olifants River originates from the east of Johannesburg and flows northwards before curving eastwards towards the Kruger National Park (KNP) through to Massingir Dam where it joins the Limpopo River in Mozambique (Heath *et al.*, 2010). The Olifants River System is divided into three sub-catchments; upper Olifants River sub-catchment, middle Olifants River sub-catchment and the lower Olifants River sub-catchment. The upper sub-catchment area originates in Gauteng near Breyton and flows through the Highveld grasslands. Loskop Dam, located approximately 60 km from Flag Boshielo Dam, is located in this sub-catchment area and is the major impoundment in Mpumalanga (De Lange *et al.*, 2003).
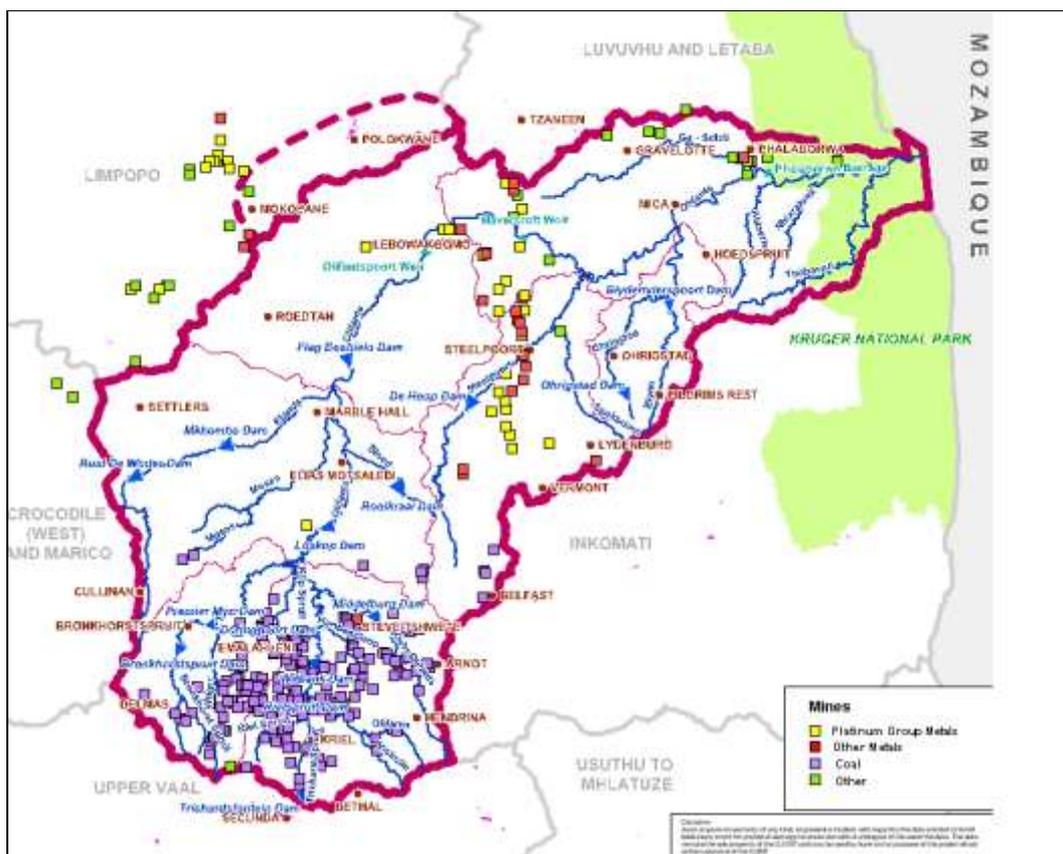
Figure 2.1: Olifants River catchment area (Adapted from dwaf.gov.za).

The middle sub-catchment is divided into the upper middle and lower middle Olifants River. It consists of the portion of the Olifants River Basin between Loskop Dam and the junction of the Steelpoort and Olifants rivers and stretches for approximately 300 km along the Olifants River from below Loskop Dam (De Lange *et al.*, 2003). The lower Olifants sub-catchment stretches from the Drakensberg escarpment through the KNP to Massingir Dam in Mozambique.

The Olifants River catchment (Figure 2.1) is characterised by intensive mining activities focusing mainly on coal, platinum, copper and phosphate from the Witbank, Middelburg, Burgersfort and Phalaborwa mines. It is therefore not surprising that it was described as the most polluted river system in South Africa (Van Vuuren *et al.*, 1999; Heath *et al.*, 2010; Ashton and Dabrowski, 2011; Jooste *et al.*, 2015).
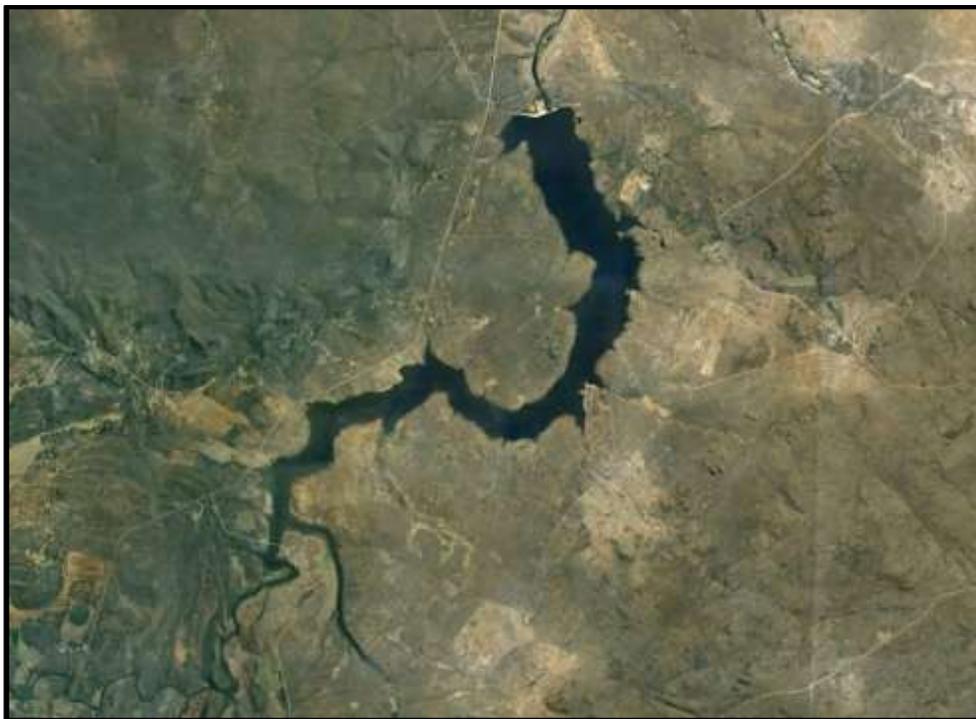
Figure 2.2: Aerial view of Flag Boshielo Dam, Limpopo Province, South Africa (Adapted from https://www.google.com).

Methods and Materials

2.1.1   Flag Boshielo Dam

Flag Boshielo Dam (formerly known as Arabie Dam) (Figure 2.2) was built in the 1980s, to provide water for irrigation, domestic, industrial use and also for recreational purpose (McCartney *et al.*, 2004). Flag Boshielo Dam (24°49'05" S; 29°24'59" E), Limpopo Province, South Africa is located in the middle catchment of the Olifants River System (Clark, 1997).

Upstream from Flag Boshielo Dam is Loskop Dam, which is mainly polluted by acid mine drainage decanting from abundant mines in the Witbank and Middelburg area, as well as pesticides and herbicides from agricultural activities in the area (Driescher, 2008). Water flows from Loskop Dam to Flag Boshielo Dam and therefore may affect the water quality of the latter dam. The dam wall of Flag Boshielo Dam was raised by 5 m in 2005, dramatically increasing the water capacity of the dam (Ashton, 2010).

2.1.2   Localities where fish were caught

This study formed part of a more comprehensive study and as a result the number of researchers involved in each of the monthly surveys ranged between 8 – 12. These numbers resulted in the gradual move from a camp/laboratory set-up on the bank of the dam (Figure 2.3A) to the better suited accommodation facilities at the Tamboti Ridge Lodge (Figure 2.3 B).

Figure 2.3: Field laboratory setting: (A) on the bank of Flag Boshielo Dam and (B) at Tamboti Ridge Lodge, Schuinsdraai Nature Reserve (Courtesy of Dr L.J.C. Erasmus).

2.2 BIO-MONITORING SPECIES

Fish species used in this study included *H. molitrix* and *L. rosae*. These specimens were collected monthly for the period February 2012 to January 2013.

2.3 COLLECTION OF FISH

The collection of fish for this study involved the combination of various techniques. The mature, lethargic *H. molitrix* specimens were collected with a scoop net from the water surface whilst out on the boat. They were immediately placed in dam water in a big container, taken to the research vehicle and transported to the field laboratory. At the laboratory they were kept in bigger containers filled with dam water under constant aeration; using a commercial air pump to provide the circulating air.

The collection of the smaller *H. molitrix* specimens were more challenging as they were neither floating lethargically at the water surface nor could they be collected via the use of gill nets. Therefore, conventional angling gear presenting floating baits approximately 10 – 20cm of the bottom was used to catch them. Prior to their transport to the field laboratory, the collected specimens were housed in angling approved holding nets that allowed free circulation of dam water.

Labeo rosae, a relatively abundant angling species were easily collected using a combination of conventional angling gear and a composite gill net. On the bank they were kept in nets that allowed the free movement of dam water.

It should be noted that this comprehensive study included amongst others parasitology and as a requirement different species were not housed together, but separately in nets and tanks specifically allocated to each species. This was done to prevent parasite movement between fish species.

2.4 THE HEALTH ASSESSMENT INDEX

The basis of the health assessment index (HAI) (Addenda A and B) involves the post mortem evaluation (i.e. necropsy-based) of external and internal variables related to various organ systems, and capturing data using a pre-set sheet to record values

indicating normality as well as the presence of anomalies (Madanire-Moyo *et al.*, 2012). In addition a colour-chart has been incorporated into the HAI for colour assessment when evaluating the liver, spleen and bile (Watson, 2001; Watson *et al.*, 2012) (Figure 2.4). The application of this method of assessment is cost-effective and it can be used in both laboratory and field setting (Goede and Barton, 1990). The current study formed part of a more comprehensive project and as a result only HAI data relevant to the focus of this study was used.
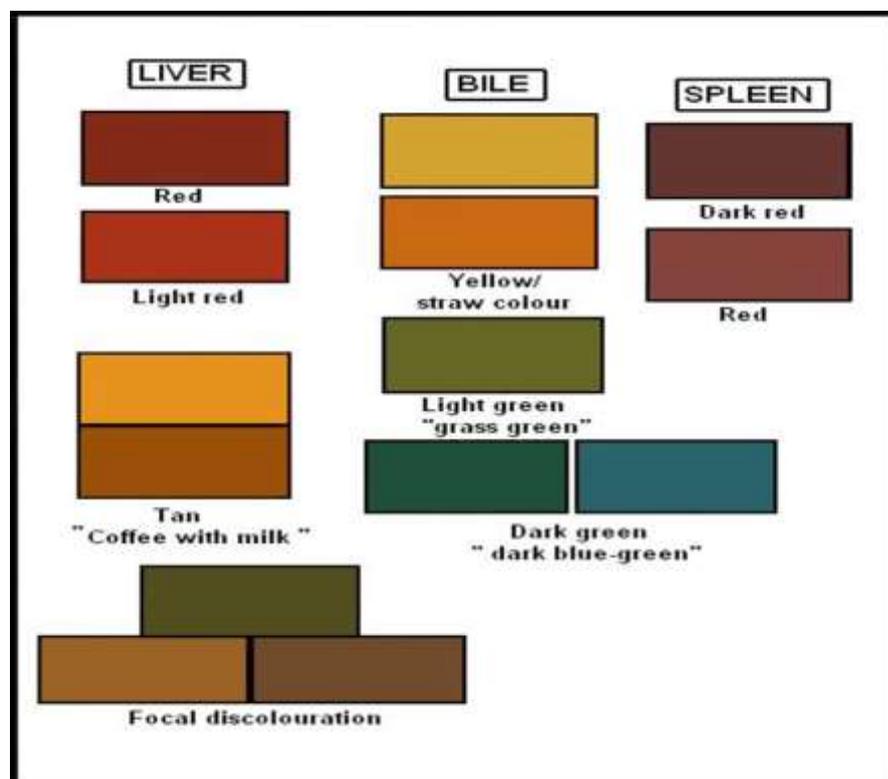
Figure 2.4: Colour chart used to compare the colour of liver, bile and spleen (Adapted from Watson, 2001).

2.5 HAEMATOLOGY

The application of haematology in various studies is essential as it has been identified as an important tool for monitoring and evaluating physiological and pathological

changes which takes place in fish; where the pathological changes denotes alterations caused by disease(s) (Satheeshkumar *et al*., 2012). It has been reported that these parameters are sensitive and effective in determining the health status of fish (Radu *et al*., 2009). Various haematological parameters are available to evaluate the functional status of the oxygen carrying capacity; the most prominent being RBC, WBC, Hct and Hb (Ramesh *et al*., 2013). In addition to this the erythrocyte indices, such as MCV, MCH, MCHC and RDW, can be used for the diagnoses of anaemia (Coles, 1986).

2.5.1   Blood collection and analysis

Whole blood samples were collected from live specimens using the caudal peduncle puncture method (Aqualex, 2004). The measurement of Hct values was done using a disposable, sterile, plastic syringe with a 22 gauge needle to transfer blood into a capillary tube. These tubes were then sealed with commercial critoseal clay and centrifuged for 8 min at 12 000 x g (MSE Haemo Centaur centrifuge, Opto-Labor, Sanyo). All values were recorded using a MSE Micro-haematocrit reader and expressed as percentages.

Whole blood samples for the measurement of other relevant blood parameters, were collected using Vacutainer products, where the Vacutainer tubes contained Ca-EDTA (Eyhylenediaminetetraacetic acid), which prevented coagulation of the whole blood samples. These blood samples were stored in a fridge (4°C) for a period not exceeding 3 days, before further analysis at the Medical Science Department of the University of Limpopo. Using an automated haematology analyser (Coulter AcT 5 Diff, Beckman Coulter, Miami, FL) the following measurements were done red blood cell count (RBC = n x 10^6 cells), white blood cell count (WBC = n x 10^3 cells/µL), haemoglobin (Hb = g/dL), mean cell volume (MCV = fL), mean cell haemoglobin (MCH = pg), mean cell haemoglobin concentration (MCHC = g/dL) and red blood cell distribution width (RDW = %).

The normal range of MCV has been recorded to be between 70 and 80fl, and the MCV is calculated as follows:

$$MCV \quad = \quad \frac{\text{Volume of packed cells/1000ml of blood}}{\text{Red blood cell count in millions/ml}} \quad \text{fl or } \mu m^3$$

The MCH and MCHC can be calculated applying the formulas suggested by Sarma (1990):

$$MCH \quad = \quad \frac{\text{Haemoglobin in g/1000ml of blood}}{\text{RBC count in millions/ml}} \quad \text{Pg/cell}$$

$$MCHC \quad = \quad \frac{\text{Haemoglobin in g/100ml X 100}}{\text{Volume of packed cells /100ml of blood}} \quad \% \text{ or g/dl}$$

2.5.2 Sacrificing of fish

This procedure was performed after the blood collection, but prior to the collection of other tissue samples. The spinal cord was severed, in a single action, just behind the operculum. In the absence of an operational animal ethics committee at the University of Limpopo, this procedure was submitted to and approved by the Animal Ethics Committee of the University of Pretoria (T001-12).

2.6 BIOCHEMICAL ANALYSIS OF OXIDATIVE STRESS BIOMARKERS

Biochemical tests were conducted using liver and gill samples collected from *H. molitrix*. Upon dissection, at the field laboratory, the samples were placed in cryotubes (Figure 2.5) and kept in a freezer (-20°C). On our return to the laboratory (UL) these samples were transferred to a bio-freezer (-85°C) until required for further analysis.

The biochemical analysis of gill and liver tissue samples collected from healthy younger fish and larger lethargic fish (Figures 2.6 A&B), were conducted at the Genetics Department, Stellenbosch University (SU). All tissue samples were pre-weighed to the nearest 0.1 gram and triple-washed with 1 X Phosphate Buffer saline

(PBS, pH 7.4). It was subsequently centrifuged at 10 000 X g (2 minutes, at 4°C) to remove cell debris, nuclei and mucus. These samples were then used to conduct assays for the measurement of CAT, GST and TBARS.

Figure 2.5: Tubes and specimen jars used during collection of various tissue samples. (Courtesy of Dr L.J.C. Erasmus)

2.6.1   Catalase

Catalase activity was determined using the method described by Aebi (1984). The final volume of the assay was 240µl in the 96 wells; all reagents excluding the samples were equilibrated at room temperature before the commencement of the assays. The reaction mixture contained diluted hydrogen peroxide, potassium hydroxide and catalase potassium periodate. The assays were done in triplicate and the 96-well microplates (Nunc™, Denmark) were read using a GloMax-Multi microplate reader (Promega) at an absorbance of 540nm.
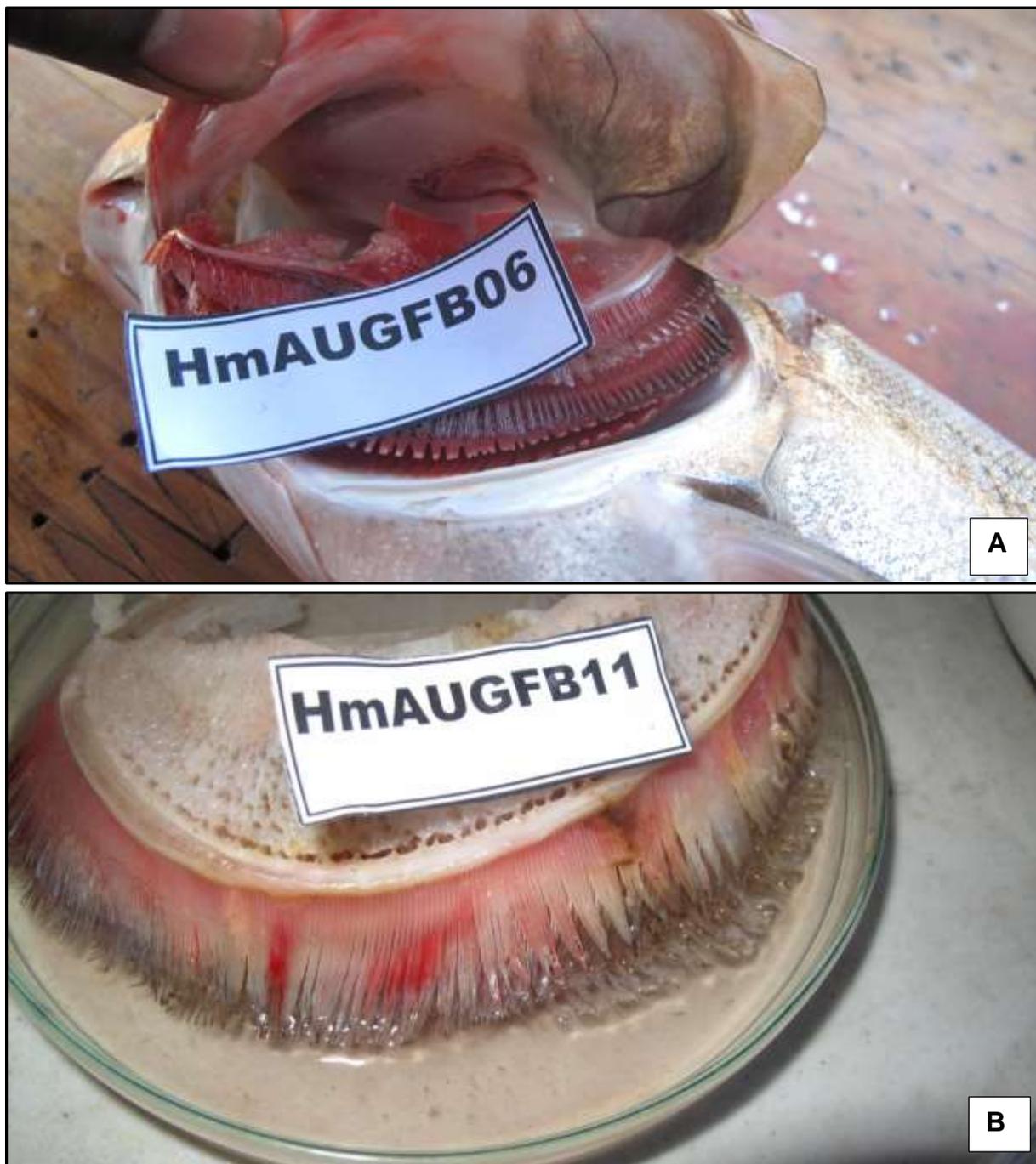
Figure 2.6: Comparison of healthy gill morphology observed in a younger fish (A) (♀, 0.628 kg, TL = 0.4 m) in contrast to a sample collected from a larger, lethargic fish (B) (♀, 4.503 kg, TL = 0.90 m). (Courtesy of Dr L.J.C. Erasmus)

2.6.2 Glutathione-*S*-transferase

The activity of this biomarker was determined by the method described by Habig *et al*. (1974). The final volume of the assay was 200µl in the wells and the assay temperature was 25°C. The reaction mixture containing assay buffer, GSH and 1-chloro-2,4-dinitrobenzene (CDNB) (Cayman, USA) was added to initiate the reaction. The assays were done in triplicate for each sample, but in duplicate for the standards in 96-well microplates (Nunc™, Denmark) using a GloMax®-Multi microplate multimode reader (Promega Instruments, France) at the absorbance of 340nm every minute until five minutes was reached. As a reference, one unit of the GST was described as the quality of enzyme required to catalyse the formation of 1.0nmol of CDNB with reduced GSH/minute at 25°C. Glutathione-*S*-transferase activity was expressed as nmol/min/ml; and the following equation was used to calculate GST activity:

GST activity = $\dfrac{\Delta A340/min}{0.00503\mu M^{-1}}$ X $\dfrac{0.2ml}{0.02ml}$ X sample dilution l

= nmol/min/m

Where 0.00503 µM^{-1} represents the extinction coefficient for the CDNB conjugate at 340 nm.

2.6.3 Lipid peroxidation

The TBARS test for the presence of MDA is the most widely used method to measure and quantify lipid peroxidation; especially in liver and gill samples (Di Guilio *et al.*, 1989). Therefore, as a measure of lipid peroxidation the OxiSelect® Thiobarbituric Acid Reactive Substances (TBARS) Assay kit was employed to determine the levels of the reactive compound MDA (Ohkawa *et al.*, 1979). All liver and gill samples were homogenised in 1 X PBS (containing 1 X butylated hydroxytoluene) and sonicated (Omni-Ruptor 400, OMNI International Inc.); where after the homogenates were

centrifuged (10 000 X g, 10 minutes, 4°C), supernatants collected and placed on ice for protein concentration using the NanoDrop® Spectrophotometer, where after the supernatants were assayed to determine TBARS levels.

All samples were assayed in triplicate, unless indicated otherwise, adding 100 µl of the MDA standard and the samples to separate micro-centrifuge tubes. In addition 100µl of sodium dodecyl sulphate (SDS) lysis solution (0.2% w/v) was added to the samples and the standards, it was mixed thoroughly and incubated at room temperature for 5 min. Samples and standards were further diluted when 250µl of TBA reagent was added to the tubes; this reaction was allowed 45 – 60 min (95°C) to proceed before the samples were centrifuged (3000 rpm, 15 min) and the supernatant was removed for further analysis. Spectrophotometric measurements were performed using 200 µl of the MDA standard and samples transferred to a 96-well micro titre plate (Nunc™, Denmark), where the absorbance was measured at 532 nm (Glomax®-Multi Microplate Reader, Promega, France).

2.7 BIO-ACCUMULATION

Various tissue types can be used to determine the accumulation of metals; however, in this study muscle tissue were used. The muscle tissue was removed from the section located laterally to and in close proximity of the dorsal fin. These tissue samples were stored in cryotubes and frozen until further analysis.

The measurement of transition metals in muscle samples were done at the CSIR CAS Stellenbosch Analytical Laboratory. Flame atomic absorption spectroscopy (AAS), a fast and easy technique with extremely high sensitivity, was used to determine the levels (µg/kg) of Vanadium (V), Chromium (Cr), Manganese (Mn), Iron (Fe), Cobalt (Co), Nickel (Ni), Copper (Cu), Zinc (Zn), Molybdenum (Mo), Cadmium (Cd), and Mercury (Hg).

2.8 STATISTICAL ANALYSIS

SPSS version 22 (SPSS, Chicago, USA) was used for statistical analysis. Descriptive statistics were done and tabulated results are expressed as means ± SD. Where

necessary inference were conducted employing relevant statistical methods. Group means were compared using the student's *t*-test; where $P \leq 0.05$ was considered significant. The limited number (n) of subjects / samples in some data sets forced the inclusion of outliers that affected not only the range of these values, but also the SD.

Results and discussion of the seasonal haematological assessment of
Hypopthalmichtys molitrix and *Labeo rosae*

3.1   RESULTS

The seasonal variation of all relevant haematological parameters is summarised in Table 3.1. Furthermore, Table 3.1 compared the selected fish species in an effort to record differences between (i) *L. rosae* and *H. molitrix* and (ii) young and mature *H. molitrix* specimens. Missing data sets relates to younger *H. molitrix* specimens, as it was more often challenging to obtain adequate blood samples from the smaller fish. Similarly, the young *L. rosae* hampered the collection of adequate blood samples for comprehensive analysis. Reference haematological values could not be located for this feral population of *H. molitrix*. However, the discovery of the Pieterse Ph.D. Thesis (Pieterse, 1982) at the University of Limpopo resulted in the creation of Table 3.2 and added tremendous value to the most recent findings.

3.2   DISCUSSION

3.2.1   White blood cell

In the current study no data was available for the summer and autumn surveys of the younger *H. molitrix* specimens, thus creating a challenging scenario for the interpretation of the data. The mature specimens exhibited a relatively stable WBC counts throughout the year, with a decrease from winter to spring although not statistical significant. Similarly the younger specimens exhibited a decrease during this period; however, it was not nearly as pronounced as that of the mature fish (21% vs 56%).

In comparison with other studies, some insight was gained into the extremely variable nature of the WBC counts. Whilst it is fair to argue in favour of the relationship that exists between immune challenges versus WBC counts, studies on *Cyprinus carpio* (Kondera *et al.*, 2012), *Cichlasoma dimerus* (Vazquez and Nostro, 2014) and *Betta splendens* (Motlagh *et al.*, 2012), clearly illustrated that baseline values, for what can be considered to be healthy fish, varied considerably

Results and discussion of the seasonal haematological assessment of *Hypopthalmichtys molitrix* and *Labeo rosae*

Table 3.1: A comparison of the seasonal variation in haematological parameters recorded for mature and young *Hypopthalmichtys molitrix* and individual *Labeo rosae* collected from Flag Boshielo Dam.

	Fish	SEASONS											
		Summer			Autumn			Winter			Spring		
		Mature	Young	*L. rosae*	Mature	Young	*L. rosae*	Mature	Young	*L. rosae*	Mature	Young	*L. rosae*
Haematological parameters	RBC (n x 10^6 µL^{-1})	0.27±0.15 (n=9)	___	___	0.55±0.13 (n=7)	___	___	0.48±0.30 (n=7)	1.46±0.22 (n=13)	___	0.49±0.19 (n=15)	1.42±0.58 (n=10)	___
	WBC (n x 10^3 µL^{-1})	29.00±22.5 (n=9)	___	___	35.65±6.7 (n=4)	___	___	33.99±26.32 (n=7)	57.11±20.87 (n=13)	___	15.06±17.84 (n=16)	44.86±21.94 (n=10)	___
	Hb (g/dL)	2.63±1.07 (n=8)	___	___	3.09±0.68 (n=7)	___	___	2.88±1.60 (n=6)	7.05±1.12 (n=13)	___	2.22±1.74 (n=10)	4.93±1.95 (n=6)	___
	Hct (%)	12.2±2.38 (n=5)	35.9±5.2 (n=21)	38.6±2.5 (n=27)	13.67±5.9 (n=12)	38.40±6.8 (n=5)	44.2±1.0 (n=27)	12.88±4.02 (n=8)	25.87±4.71 (n=15)	27.9±3.4 (n=18)	9.97±4.23 (n=13)	28.85±6.37 (n=12)	23.1±4.4 (n=17)
	MCV (fL)	149±5 (n=7)	___	___	143±3 (n=5)	___	___	143±4 (n=7)	138±7 (n=13)	___	157±14 (n=7)	151±10 (n=7)	___
	MCH (pg)	89±4 (n=5)	___	___	56±0.1 (n=6)	___	___	46±23 (n=6)	49±3 (n=13)	___	53±4 (n=2)	45±0.21 (n=2)	___
	MCHC (g/dL)	59±1 (n=5)	___	___	39±1 (n=5)	___	___	32±16 (n=6)	35±2 (n=13)	___	37±0.21 (n=2)	30±0.92 (n=2)	___
	RDW (%)	5.37±0.73 (n=7)	___	___	5.43±0.81 (n=7)	___	___	5.97±0.67 (n=7)	7.32±2.04 (n=13)	___	10.17±8.61 (n=7)	12.57±8.25 (n=7)	___

Key: RBC = red blood cell count, WBC = white blood cell count, Hb = haemoglobin, Hct = haematocrit, MCV = mean cell volume, MCH = mean cell haemoglobin, MCHC = mean cell haemoglobin concentration and RDW = red cell distribution width, n = number of specimens. " ---" indicates that no relevant data was collected .

Results and discussion of the seasonal haematological assessment of *Hypopthalmichtys molitrix* and *Labeo rosae*

Table 3.2: Comparison of the annual haematological values from the current study (feral population of *Hypophthalmichtys molitrix*) with that of a population reared in captivity at the Fisheries Research Station at Marble Hall (Pieterse, 1982).

HAEMATOLOGICAL PARAMETER			
	Mature fish	**Young fish**	**Pieterse (1982)**
RBC ($X10^6$/mm^3)	0.45±0.21 (n=38)	1.44±0.41 (n=23)	1.36±0.23 (n=20)
WBC ($X10^3$/mm^3)	24.51±21.29 (n=36)	51.78±21.75 (n=23)	59.83±21.29 (n=20)
Hb (g/dL)	2.65±1.35 (n=31)	6.38±1.71 (n=19)	8.75±1.51 (n=20)
Hct (%)	12.04±4.76 (n=38)	31.7±7.22 (n=53)	22.90±5.17 (n=20)
MCV (fL)	148.27±9.33 (n=26)	142.30±10.14 (n=20)	167.99±16.15 (n=20)
MCH (pg)	60.77±20.87 (n=19)	48.16±2.65 (n=15)	65.07±9.49 (n=20)
MCHC (g/dL)	41.74±13.98 (n=18)	34.41±2.50 (n=15)	38.92±3.11 (n=20)

Key: RBC = red blood cell count, WBC = white blood cell count, Hb = haemoglobin, Hct = haematocrit, MCV = mean corpuscular volume, MCH = mean corpuscular haemoglobin, MCHC = mean corpuscular haemoglobin concentration, n = number of specimens.

Various factors such as age and seasonal fluctuations can also contribute to haematological changes. Therefore, it makes sense to propose that a fish species such as *H. molitrix*, collected from the same aquatic environment and with approximately the same age profile should be used to establish a baseline haemogram. Vazquez and Guerrero (2007) suggested that such reference values

should be established under standardised conditions; thereby, being able to provide substantial diagnostic information. To establish such a set of standardised reference values for the Flag Boshielo Dam feral population, is a very challenging endeavour as the capture, effective transport and housing of *H. molitrix* is currently problematical.

With regard to seasonal variations and its impact on the haemogram; WBC counts can be altered, maybe not as a primary response to temperature fluctuations, but in response to other related environmental challenges such as parasitic load and pH changes. This being said, the fact that these blood cells play an important role in the immune response of an organism, leads to the argument (even if partially obscured by incomplete data sets) that both the mature and younger specimens experienced reduced immune challenges in the transition from winter to spring. The reason(s) for the disparity in the age-response is as yet not clear, and requires further investigation. It is difficult to establish whether the Pieterse study (Pieterse, 1982), was of an annual or seasonal design; subsequently, the only plausible comparison is with annually pooled values from the current study. This comparison revealed that the mature specimens exhibited annual WBC counts far less than that reported for both groups of younger fish. The WBC counts for the younger fish seems to indicate that it would be fair to support a baseline value of >50X10^3 cells/mm^3.

3.2.2   Red blood cell count

The more mature fish were lethargic, but still alive, and was easily collected from the water surface using a scoop net. This was in contrast to the younger fish that had to be collected using conventional angling equipment. Closer investigation of the gills (Figure 2.8B) clearly illustrated that blood flow and oxygenation in the mature fish was not optimal, in many cases the gills of the mature fish also had large necrotic areas. This can effectively alter the ability of such fish to utilise and transport oxygen, possibly explaining the sluggish behaviour observed. In the absence of more focused laboratory tests, it was theorised that anaemia was a possibility. However, at this stage the type of anaemia and why only the more mature fish were affected is not clear.

Results and discussion of the seasonal haematological assessment of *Hypopthalmichtys molitrix* and *Labeo rosae*

Further laboratory findings illustrated that the RBC were significantly reduced in mature fish as compared to younger specimens. To some extent this finding partially supported the theory regarding anaemia. Nevertheless, further investigation into the Hb, Hct and other related erythrocyte indices was required to clarify this scenario. When comparing the young and more mature *H. molitrix* specimens to the baseline values for species such as *C. carpio* (1.17±0.21 µL^{-1}) (Kondera *et al.*, 2012), *B. splendens* (1.84±0.13 µL^{-1}) (Motlagh *et al.*, 2012), and *C. dimerus* (3.23±0.97 µL^{-1}) (Vazquez and Nostro, 2014), it was noted that even though only mature specimens had anaemia, the younger specimens had RBC that were far lower than those of the mentioned species. It was therefore theorised that this lower RBC count observed in the younger *H. molitrix* feral population from the Olifants River System could possibly be species-specific.

The work done by Pieterse (1982) on healthy 2-year old *H. molitrix*, held at the Marble Hall Fisheries Research Station, is the only source that could be located containing baseline values for this species in South Africa. The current feral population in the Olifants River System originated from these captive fish after their accidental release into this system in the early 1980's. When comparing current findings to that of the earlier study, it is clearly observed that the younger specimens exhibited RBC counts values closely resembling that of the earlier study. However, based on this and even when considering that RBC numbers can decrease somewhat with an increase in age, the mature specimens definitely seemed anaemic.

3.2.3   Haematocrit

In contrast to the incomplete RBC and WBC counts, the complete seasonal Hct profile was available for both age groups of *H. molitrix* as well as *L. rosae*. It did not only confirm the RBC results for the mature specimens, but it also shed light on the missing RBC data of the younger fish. The Hct results without doubt supported the presence of anaemia amongst the mature fish; it was evident from the fact that the Hct of the younger fish was always significantly higher than that of the mature specimens (P =

0.005). Similarly, seasonal Hct values of *L. rosae* and mature *H. molitrix* were also significantly different ($P = 0.01$); *L. rosae* had consistently higher values. In addition, the seasonal Hct values for young *H. molitrix* and *L. rosae* did not differ significantly ($P = 0.66$). The Hct values (%) of the younger fish compared very well with those reported for *H. molitrix* (22.9±5.17) (Pieterse, 1982), *C. carpio* (27±2.6) (Kondera *et al.,* 2012), *B. splendens* (33.65±2.34) (Motlagh *et al.*, 2012), and *C. dimerus* (32.98±2.98) (Vazquez and Nostro, 2014). However, despite the fact that some fish species such as the sandbar shark and Port Jackson shark have a normal Hct of less than 20% (Campbell, 1988; Arnold, 2005), this principle doesn't seem to apply to *H. molitrix*. Current findings unequivocally support the view that the Hct values of the mature fish are far too low to sustain life and maintain metabolic demands. In addition to this it is important to also consider the haemoglobin concentration; as even a normal Hct in the absence of optimal levels of haemoglobin, has the potential to result in reduced oxygen transport and supply.

3.2.4 Haemoglobin

Haemoglobin, as the primary transporter of oxygen, plays an important role in the distribution of oxygen to tissues. If for some reason either the ability of Hb to carry oxygen or its concentration in erythrocytes is impaired the metabolic rate and subsequently homeostatic control will be affected. Haemoglobin results confirmed that its concentration (g/dL) in mature fish was far less than that reported for the younger fish. It was therefore not surprising that the older fish behaved sluggishly; the bigger fish should exhibit higher metabolic demands which were obviously not met in the current scenario.

It is of interest to note that the Hb values of the younger fish compared very well with those of *C. dimerus* (6.82±1.04) (Vazquez and Guerrero, 2007), and *B. splendens* (8.08±0.69) (Motlagh *et al.,* 2012). Current findings, for both young and mature specimens, in combination with that of Pieterse (1982), indicated that the mature fish had incredibly low Hb content. Therefore, the circulatory set-up with reduced Hct and

Hb can, even if only partially, explain the phenomenon of lethargy observed amongst mature specimens. In addition to this, it is of great importance to understand the aetiology of this anaemic presentation; therefore the use of erythrocyte indices can assist in establishing this.

3.2.5   Red blood cell indices

3.2.5.1   Mean corpuscular volume

The MCV exhibited a clear seasonal effect as it decreased during autumn and winter, and increased again in the transition from winter to spring. Findings from this study support the notion by Motlagh *et al.* (2012), that erythrocyte indices such as MCV and MCH have a wide range of physiological variation. This was emphasised by the fact that present MCV values (fL) differed from those of *B. splendens* (182.48±5.37) (Motlagh *et al.,* 2012), *C. carpio* (236±38) (Kondera *et al.*, 2012), and *C. dimerus* (111.08±27.11) (Vazquez and Nostro, 2014). However, when compared to the report by Hedayati and Ghaffari (2013) on *H. molitrix*, the current MCV values across all seasons compared very well with their control measures. In addition to this, the fact that their MCV values were slightly less than that of the present study could probably be explained by the fact that their study was on captive-bred juvenile fish (200 g). From the current results some evidence exists in support of macrocytic anaemia as the erythrocytes of mature specimens were bigger in size than those of the younger fish.

3.2.5.2   Mean corpuscular haemoglobin

Erythrocyte indices can be useful for the measurement of physiological disturbances in stressed fish. Thus it not only plays an important role in environmental monitoring, it can also be used as indicators of disease and stress (Shaluei *et al.*, 2013). It is unfortunate that seasonal MCH values for the younger fish were incomplete; thereby hindering sensible interpretation. However, data for the mature fish was complete and

clearly illustrates a seasonal impact; a gradual decrease from summer to winter followed by an increase during spring.

The MCH values (pg) reported for *C. carpio* (56.9±13.6) (Kondera *et al.*, 2012) and *B. splendens* (43.81±2.55) (Motlagh *et al.*, 2012) compared well with that of the present study. At first glance these values for other teleost fish seems to indicate that the current seasonal variation in MCH values should not be a source of concern as it falls within what could be considered as a normal physiological range. However, Kopp *et al.* (2010) and Shaluei *et al.* (2013), investigating *H. molitrix* reported control values that were less than that observed in the studies conducted in the Olifants River System, both the current study and that of Pieterse (1982). Shaluei *et al.* (2013) reported that juvenile *H. molitrix* were found to have a MCH of 34.57±1.92pg; which compared very well to results from Kopp *et al.* (2010) who reported that 2-year old specimens had an MCH of 37±5pg. This discrepancy in values clearly illustrates the importance of creating and documenting environment- and species-specific baseline values; in so doing establishing the much needed diagnostic criteria that is currently lacking for fish from the Olifants River System to facilitate the interpretation of relevant data.

3.2.5.3   Mean corpuscular haemoglobin concentration

This measurement reflects the Hb concentration in a given volume of packed red blood cells; a measure that is most often employed to determine the type and severity of anaemia. It is therefore reasonable to argue that a decreased MCHC would indicate iron-deficiency; iron being a key component of the heme-group in the haemoglobin molecule. This in itself might be the result of insufficient iron in the diet or due to severe blood loss.

Current incomplete results for the young specimens don't support a sensible seasonal comparison between the mature and young specimens. However, using annual values it becomes clear that the MCHC levels in mature specimens were elevated when compared to the younger fish, as well as those recorded by Pieterse

(1982). The values for the mature fish were even higher than those reported by Shaluei *et al.* (2013) (27.91±2.96g/dL). An elevated MCHC can be indicative of Vitamin B_{12} or folic acid deficiency (Firouz *et al.*, 2013), sickle cell disease or even homozygous haemoglobin C disease (Fabry *et al.*, 1982; Bookchin and Balazs, 1986). These findings propose a multifactorial causality for mature *H. molitrix*; firstly nutritional deficiencies related to the antipernacious anaemic factor (Vitamin B_{12}) as well as that of folic acid which prevents anaemia, and secondly a hereditary component with sickle cell diseases and an autosomal recessive disorder as key focus areas.

3.2.5.4   Red blood cell distribution width

The pathological and clinical value of this erythrocyte index relates to the fact that it essentially describes the normal variation in red blood cell size; in essence it is a reflection of the homogeneity or lack thereof in a specific red blood cell population. This is of relevance as it should be considered that erythrocytes are continuously produced; therefore, some cells are new, some are older and some will be fragmented. Subsequently, what might be considered a normal RDW is calculated somewhere between these values; where the conventional reference range for humans is considered from 12 – 15% (Lippi and Plebani, 2014). A low RDW is not considered of pathological relevance. However, the RDW value increases parallel with anisocytosis and as a result when an increased RDW is observed a noticeable anisocytosis is expected. This is in agreement with the fact that an elevated RDW reflects an abnormal red blood cell population which is conventionally used to reflect on conditions such as iron deficiency, deficit in Vitamin B_{12} and/or folic acid, and impaired bone marrow function (Sahli *et al.*, 2013).

A comprehensive literature survey failed to confirm the consistent use of RDW in haematological studies on fish, especially in *H. molitrix*. Subsequently, locating the elusive species-specific RDW-values could not be done. However, existing literature confirmed that these values can vary significantly, depending on aspects such as species diversity and the uniqueness of their aquatic environment. This concept is

supported by studies where control RDW values (%) for species such as *Oreochromis andersonii* were 29.3±8.55 (Kefi *et al.*, 2013), which was far greater than that reported for *Gobius niger* (19.44±1.62) (Fazio *et al.*, 2012) and for *Clarias gariepinus* (13.05±0.35) (Solomon and Ochume, 2013), illustrating the importance of establishing a species-specific reference range for RDW.

Even though reference values for *H. molitrix* in the Olifants River System is lacking; current findings albeit preliminary, reflects positively on the following: (i) both young and mature specimens exhibited an increased RDW for the transition period from winter to spring, most probably due to environmental conditions related to deficient nutritional intake, and (ii) with the exception of spring values for the mature fish, their RDW values remained relatively constant throughout the remainder of the year. Within an environmental context the latter is currently difficult to explain, especially with no young fish data for this period to serve as a comparison. The use of RDW in blood pathology can play an important role in confirming the presence of anaemia; it is therefore disconcerting to note that fish studies; in general, do not appreciate its value as a diagnostic tool.

Results and discussion of the seasonal variation in oxidative stress biomarker levels of *Hypopthalmichtys molitrix*

4.1 RESULTS

The levels of three oxidative stress biomarkers; CAT, GST and TBARS, were measured in liver and gill tissue samples. Seasonally determined values, expressed in µmol/min, for these enzymes are summarised in Table 4.1.

The use of the colour chart (Figure 2.6) to identify macroscopic deviations found that the liver of smaller *H. molitrix* specimens displayed a healthy red colour. However, the mature specimens had a number of abnormalities ranging from focal discolouration to general discolouration. In addition, the focal discolouration often presented with pigment granules associated with macrophage aggregates. Comparing the activity of ROS enzymes (i.e. CAT, GST and TBARS) measured in the liver of *H. molitrix* specimens revealed the following. The young *H. molitrix* specimens exhibited higher CAT activity than the mature fish. In contrast, little seasonal and age-dependent differences in GST and TBARS were observed.

4.2 DISCUSSION

The preference of fish as a sentinel organism in ecotoxicological assessments is predominantly based on the role that they play in the trophic web, their ability to accumulate harmful substances and the fact that they easily respond to low levels of mutagens (Van der Oost *et al.*, 2003). Consequently an early warning system for the detection of aquatic environmental disturbances would rely substantially on the ever increasing popularity of fish biomarkers as pollution indices. Within this group of biological indices the various oxidative stress biomarkers such as CAT, glutathione peroxidase, superoxide dismutase and GST are of particular interest in host responses to environmental stressors.

The focus of the current study on CAT, GST and TBARS activity was an effort to identify possible multifactorial role players in the sporadic deaths reported for mature *H. molitrix* specimens in Flag Boshielo Dam. It is unfortunate that an incomplete seasonal data set of the gill enzyme activities hampers a practical interpretation of this data. However, access to a full seasonal data set for liver enzyme activity did succeed

Results and discussion of the seasonal variation in oxidative stress biomarker levels of *Hypopthalmichtys molitrix*

in identifying CAT as a possible role player in the dysfunctional oxidative stress response of mature *H. molitrix* specimens.

Table 4.1: Summary of the seasonal variation in oxidative stress biomarkers recorded for young and more mature *Hypophthalmichtys molitrix* specimens collected from Flag Boshielo Dam.

LIVER					
Season	**CAT** (µmol/min)		**GST** (µmol/min)		**TBARS** (µmol/min)	
	Young	**Mature**	**Young**	**Mature**	**Young**	**Mature**
Summer	0.91±0.02 (n=2)	0.53±0.1 (n=4)	0.31±0.01 (n=2)	0.30±0.01 (n=4)	0.51±0.14 (n=3)	0.37±0.37 (n=4)
Autumn	0.69±0.56 (n=2)	0.39±0.18 (n=4)	0.31±0.01 (n=2)	0.30±0.02 (n=4)	0.32±0.31 (n=2)	0.41±0.18 (n=4)
Winter	1.47±0.25 (n=4)	0.33±0.05 (n=2)	0.30±0.01 (n=4)	0.29±0.01 (n=2)	0.14±0.08 (n=4)	0.38±0.45 (n=2)
Spring	1.31±0.86 (n=3)	0.41±0.15 (n=3)	0.30±0.02 (n=3)	0.30±0.02 (n=3)	0.60±0.22 (n=3)	0.57±0.16 (n=2)
GILL					
Summer	---	0.19±0.02 (n=3)	---	0.34±0.08 (n=3)	---	0.21±0.22 (n=3)
Autumn	---	---	0.71±0.38 (n=2)	0.33±0.02 (n=4)	---	---
Winter	1.04±1.12 (n=2)	---	0.32±0.01 (n=3)	0.32±0.02 (n=2)	0.06±0.01 (n=3)	0.18±0.00 (n=2)
Spring	0.17 (n=1)	0.19±0.03 (n=5)	0.32±0.00 (n=2)	0.32±0.03 (n=2)	0.07±0.00 (n=2)	0.07±0.02 (n=4)

Key: CAT = catalase, GST = glutathione-S-transferase, TBARS = thiobarbituric acid reactive substances, n = number of samples analysed. All data sets expressed as averages.

Unlike CAT that consistently exhibited higher levels for the younger fish than the mature ones, GST remained relatively constant throughout the year in both young and

CHAPTER 4

Results and discussion of the seasonal variation in oxidative stress biomarker levels of *Hypopthalmichtys molitrix*

mature specimens. Similarly, lipid peroxidation values exhibited a diverse seasonal response making it very difficult to implicate it as a contributing factor in the demise of these mature fish.

Even though control (unstressed) CAT values are not available at present for this feral population of *H. molitrix*, a number of possibilities are proposed based on the recorded values. Firstly, in a seemingly seasonal-independent fashion the mature fish always exhibited noticeably lower CAT activity when compared to the younger fish. In essence this supports the probability of an impaired metabolic response towards oxidative stress; however, it might also hint on some, as yet, undetermined external factor to which mature specimens are highly susceptible without any noticeable impact on younger fish. Secondly, if it is theorised that these values observed for both age groups represents a metabolic response to an environmentally stressed state; then the fact that the mature fish exhibited much lower CAT values might implicate the excessive build-up of H_2O_2. In a non-enzymatic process excessive H_2O_2 levels can promote the formation of hydroxyl radicals, which, in turn will contribute to the formation of peroxynitrate, a potent oxidant. Thirdly, a single CAT molecule can catabolize numerous H_2O_2 molecules; is it therefore reasonable to argue that the consistently observed differences in the recorded values necessarily validate that the CAT levels in the mature fish is inadequate to sustain normal antioxidant responses?

Disregarding the latter statement, for a moment at least and arguing in favour of the possibility that impaired CAT activity in mature fish did indeed result in the excessive production and subsequent accessibility of H_2O_2. Even though erythrocytes exhibit CAT activity and can therefore produce its own H_2O_2; it is critical to acknowledge the fact that this molecule readily permeates membranes and are as a result not compartmentalised in the cell where it is produced. Not only can hydrogen peroxide act as a substrate in oxidation reactions to produce complex organic molecules; it can also partake in the Fenton reaction where the inclusion of a metal reductant facilitates the formation of the highly reactive hydroxyl radical. The hydroxyl radical, as a ROS, is the strongest oxidising agent known and reacts with organic molecules at a diffusion-limited rate (Hartung and Greb, 2002). Reflecting on, and in

Results and discussion of the seasonal variation in oxidative stress biomarker levels of *Hypopthalmichtys molitrix*

support of the findings in Chapter 3, a worst case scenario is potentially created when H_2O_2 is not neutralised after entering the erythrocyte. In this locality the oxidation of haemoglobin results in the formation of numerous potentially cytotoxic products such as iron, free haem, haemichromes, and methaemoglobin (Fe^{3+}) (Ahn and Kim, 1998; Vallelian *et al.*, 2008). This impact is further exacerbated with the release of ROS during the auto-oxidation of oxyhaemoglobin to methaemoglobin (Mandal *et al.*, 2007). Furthermore, the presence of free haemoglobin in the extracellular fluid, due to extravasation or haemolysis of erythrocytes is known to enhance oxidation-related toxicity associated with the incidence of inflammation, haemolytic disorders and ischaemia (Vallelian *et al.*, 2008).

It is therefore concluded that the disparity observed between the mature and young fish CAT reactions seems to be of relevance, even if only partially, in the poor prognosis of the mature fish. However, the findings from this chapter in postulating the presence of elevated levels of H_2O_2 necessitate an investigation into the physiological significance of the transition metals as reductants in the Fenton reaction.

Results and discussion of the bio-accumulation measurements in *Hypopthalmichtys molitrix* and *Labeo rosae*

5.1   RESULTS

The bio-accumulation results for this study are summarised in Tables 5.1 and 5.2. Table 5.1 compares the impact of seasonality on the bio-accumulation levels of 11 selected metals in the muscle tissue of two fish species, *H. molitrix* and *L. rosae*. In a more focused attempt to understand the unexplained deaths amongst mature *H. molitrix* species, Table 5.2 draws a comparison between the muscle tissue metal levels for young and mature specimens. Table 5.3 presents the seasonal water levels for 5 of the transition metals; these were the only metals that formed part of the water assessment panel. The inclusion of graphical illustrations, Figures 5.1 through to 5.4, was done to support the discussion and indicate trends. This was only deemed necessary for Mn, Cu, Zn and Cd, and, as such the illustrations were inserted into the discussion of the specific transition metal.

Comparing selected transition metal levels revealed a seasonal and behavioural trend in the accumulation level of metals such as Mo, Fe, Cu and Zn. When comparing the accumulation levels of Mn, measured in the muscle of young and mature *H. molitrix* specimens, it was noticeable that its levels in mature fish were consistently higher than that of the younger specimens. Similar bio-accumulation patterns were detected for Cu and Zn in young *H. molitrix.* Concentrations of dissolved Mn, Fe, Cu, Zn and Hg were never above instrument detection levels; clearly illustrating the challenges faced when trying to establish background concentrations for inorganic pollutants.

Results and discussion of the bio-accumulation measurements in *Hypopthalmichtys molitrix* and *Labeo rosae*

Table 5.1: Comparison of the seasonal fluctuations in the bio-accumulation levels (µg/kg) of selected metals, measured in the muscle tissue of *Hypopthalmichtys molitrix* and *Labeo rosae* specimens collected in Flag Boshielo Dam.

Metals	Seasons							
	Summer		Autumn		Winter		Spring	
	Hm (n=14)	Lr (n=19)	Hm (n=15)	Lr (n=12)	Hm (n=15)	Lr (n=17)	Hm (n=18)	Lr (n=16)
Mo	46±27	71±65	24±24	12±4	25±13	23±10	15±6	42±14
V	161±85	229±181	90±67	87±50	66±23	104±54	73±32	106±33
Cr	213±174	197±141	71±44	94±40	73±21	151±78	77±27	98±23
Mn	403±478	221±102	1267±2441	182±65	526±479	146±120	632±1217	121±33
Fe	15392±14518	8709±7208	14517±11434	4938±3062	6584±3632	5851±4423	5971±2291	5637±2716
Co	52±24	7±4	44±33	4±3	29±21	7±3	47±32	3±1
Ni	163±120	109±75	135±118	73±37	85±139	129±60	54±15	61±19
Cu	458±354	360±249	347±270	259±128	131±64	301±221	181±60	239±59
Zn	3916±1868	3535±1299	5708±4537	3849±908	5739±1510	2063±784	5264±1895	3637±668
Cd	68±50	144±109	75±51	83±75	82±67	82±181	49±35	75±60
Hg	73±36	52±38	63±51	25±14	65±69	37±19	22±19	27±7

All values are expressed as averages ± standard deviation. Hm = *Hypopthalmichtys molitrix* and *Lr = Labeo rosae.*

Accumulated levels determined from wet tissue samples.

Results and discussion of the bio-accumulation measurements in *Hypopthalmichtys molitrix* and *Labeo rosae*

Table 5.2: Seasonal fluctuations in the bio-accumulation levels (μg/kg) of selected metals measured in the muscle tissue of young and mature *Hypophthalmichtys molitrix* collected in Flag Boshielo Dam.

Metals	Seasons							
	Summer		**Autumn**		**Winter**		**Spring**	
	Young (n=9)	Mature (n=5)	Young (n= 4)	Mature (n=11)	Young (n= 8)	Mature (n=7)	Young (n=8)	Mature (n=10)
Mo	61±21	18±4	45±42	17±6	24±12	26±15	14±9	16±4
V	214±51	64±15	105±57	85±72	62±14	72±30	72±32	74±34
Cr	273±187	106±77	109±74	57±17	71±12	75±30	86±32	70±23
Mn	507±571	215±149	637±466	1496±2839	488±305	570±650	232±59	951±1593
Fe	16134±16832	14056±10718	17007±13518	13611±11171	5362±2597	7980±4319	4403±1668	7226±1952
Co	58±26	41±15	43±63	45±17	22±11	38±27	28±8	62±36
Ni	195±136	106±61	164±155	124±109	41±20	134±197	48±15	59±13
Cu	589±385	222±37	598±410	256±134	128±64	133±68	162±35	167±73
Zn	4572±1990	2735±838	9742±7039	4242±2253	5253±1319	6293±1617	5469±2320	5101±1590
Cd	69±61	66±26	110±71	62±38	77±88	86±35	56±47	42±23
Hg	64±18	90±56	98±85	50±29	31±8	105±87	21±9	23±25

Results and discussion of the bio-accumulation measurements in *Hypopthalmichtys molitrix* and *Labeo rosae*

Table 5.3: Detected seasonal water levels (mg/ml) of selected transition metals from Flag Boshielo Dam for the period February 2012 to January 2013.

Variables	Autumn		Winter		Spring		Summer		TWQR
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Manganese (Mn) (mg/R)	<0.01	<0.00	<0.01	<0.00	<0.01	<0.00	<0.01	<0.00	0.18
Iron (Fe) (mg/R)	<0.01	<0.00	<0.01	<0.00	0.01	0.00	0.01	0.00	0.001–0.5##
Copper (Cu) (µg/')	<0.01	<0.00	<0.01	<0.00	<0.01	<0.00	<0.01	<0.00	0.0003
Zinc (Zn) (µg/')	<0.01	<0.00	<0.01	<0.00	<0.01	<0.00	<0.01	<0.00	0.002
Mercury (Hg) g/'	<0.01	<0.00	<0.01	<0.00	<0.01	<0.00	<0.01	<0.00	<0.04

= Kempster and Van Vliet (1980) and Canadian Water Quality Guidelines (CCREM, 1987).

Values highlighted in grey are interpreted according to DWAF (1996).
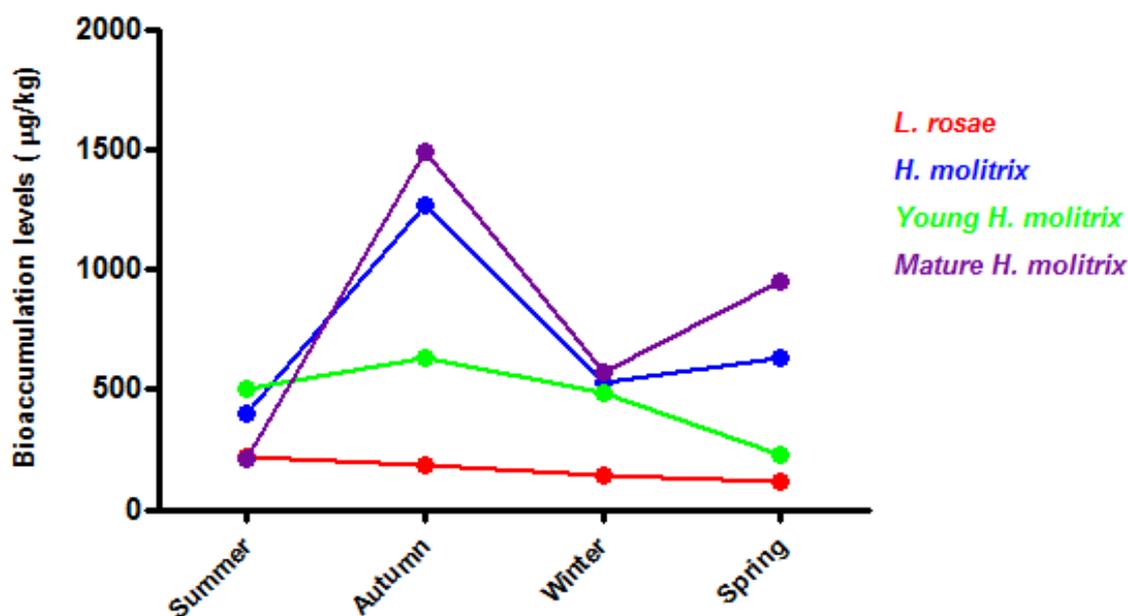
Figure 5.1: Comparison of the seasonal fluctuations in the bio-accumulation levels of Mn (µg/kg) of two fish species at Flag Boshielo Dam.

Results and discussion of the bio-accumulation measurements in *Hypopthalmichtys molitrix* and *Labeo rosae*
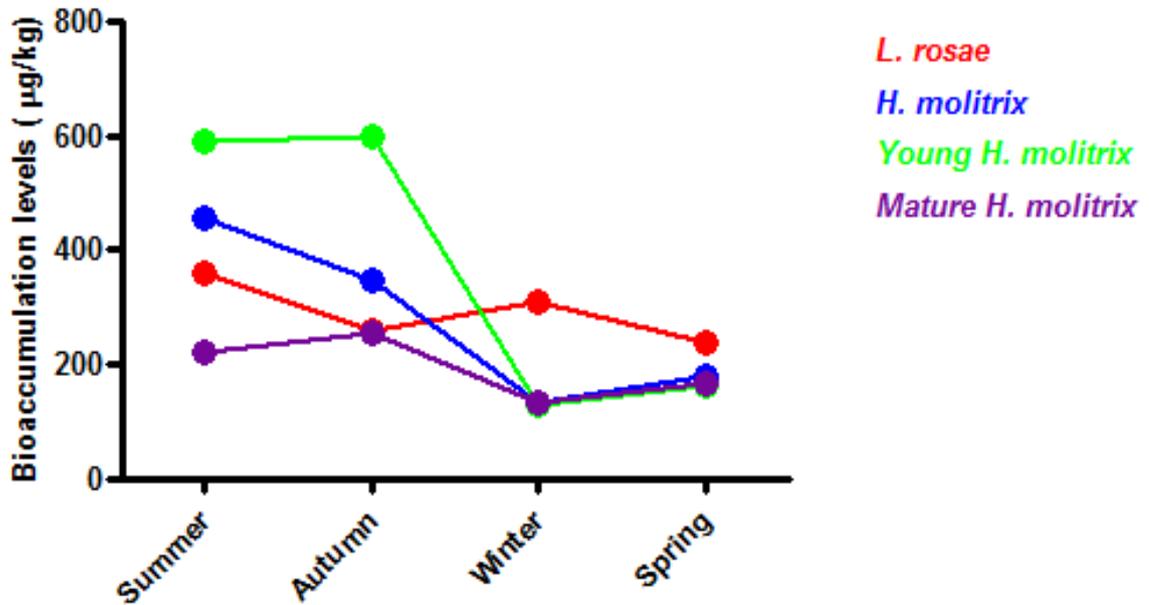
Figure 5.2: Comparison of the seasonal fluctuations in the bio-accumulation levels of Cu (µg/kg) of two fish species at Flag Boshielo Dam.
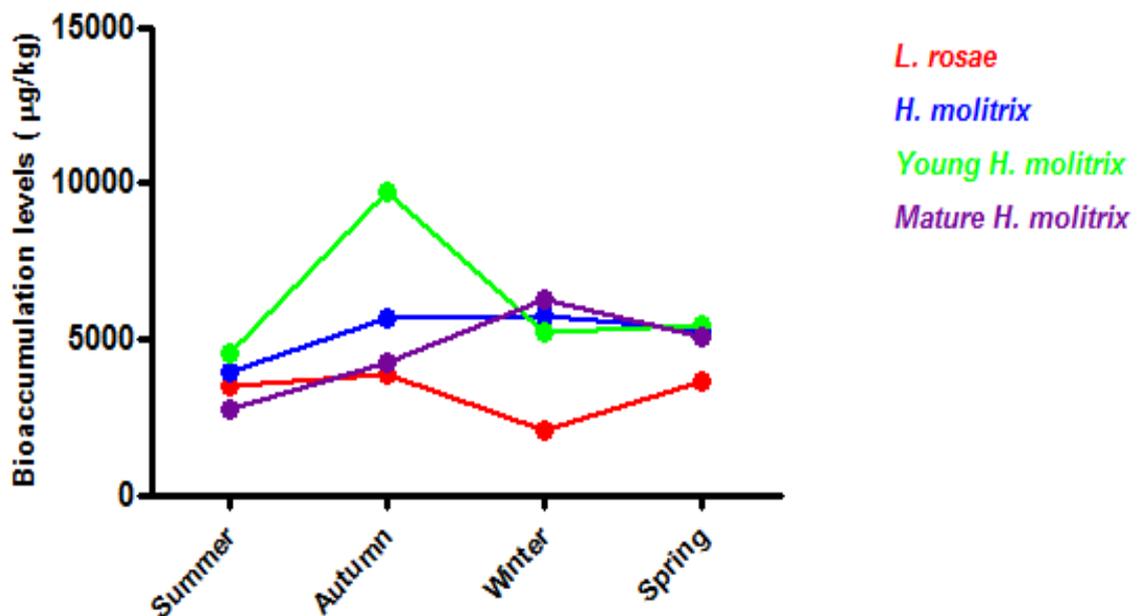
Figure 5.3: Comparison of the seasonal fluctuations in the bio-accumulation levels of Zn (µg/kg) of two fish species at Flag Boshielo Dam.

Results and discussion of the bio-accumulation measurements in *Hypopthalmichtys molitrix* and *Labeo rosae*
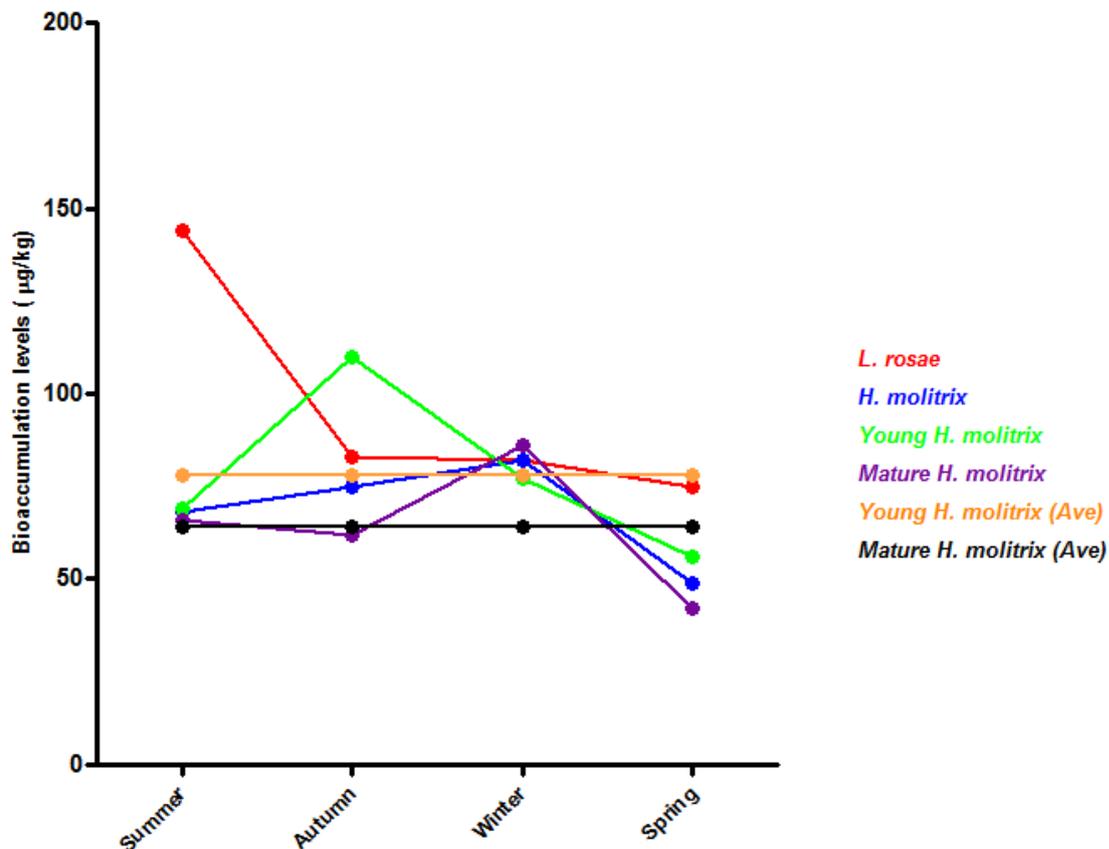
Figure 5.4: Comparison of the seasonal fluctuations in the bioaccumulation levels of Cd (µg/kg) of two fish species at Flag Boshielo Dam.

5.2   DISCUSSION

5.2.1   Molybdenum

The bio-accumulation level of Mo seems to be relevant as an indicator of current exposure, as it is widely recognised that there is no accumulation potential as tissue levels return to normal once exposure stops (Scientific Committee on Food, 2000). Molybdenum is part of a number of metalloenzymes, such as xanthine oxidase which is well known that it can generate ROS (Sharma *et al.*, 2004). However, when considering the possible physiological impacts of Mo that might clarify, even if only partially, why the mature *H. molitrix* perished; the lack of discernable bio-accumulation

seasonal trends when comparing them to the younger specimens and *L. rosae*, would fail to sensibly support such a physiological response.

Current findings do support behavioural and seasonal traits as observed between the two investigated species, as well as the sub-groups within the *H. molitrix* population. It is important to note that even though this part of the discussion does not address the issue of unexpected deaths, it does establish the integrity of the bio-accumulation data. The Limpopo Province, is a predominantly summer rainfall area. It is therefore, reasonable to argue that Mo will gradually precipitate with the progression from summer through to spring as decreased water flow [speed] will facilitate sedimentation of Mo. Subsequently, comparing the accumulation levels of Mo for a benthic feeder (*L. rosae*) with an open water filter feeder (*H. molitrix*) should support an increase in *L. rosae* and a decrease in *H. molitrix* bio-accumulation levels for this period. Not only was this assumption correct, it was also statistically significant ($P = 0.02$).

In addition to this, when comparing young and mature *H. molitrix* specimens, the only anticipated difference relates to the improved ability of the younger specimens to filter out smaller particles than the mature fish. With this in mind it was postulated that Mo levels in the younger fish should present at higher levels during summer and autumn, and thereafter (winter to spring) follow a decreasing pattern for both age groups as accessibility becomes less due to alluviation. Once more, the recorded data confirmed that the seasonal Mo muscle levels complimented this assumption; and that the intra-species comparison was statistically significant ($P = 0.001$).

5.2.2   Vanadium

Current seasonal findings were erratic and didn't exhibit a distribution pattern that would implicate V in the deaths observed amongst mature *H. molitrix* specimens. This is relevant as dying, mature specimens were collected throughout the year. Personal observations during this period could not confirm that some seasons produced more dying fish than others. Despite the fact that bio-accumulation levels failed to identify V as a causative factor in mature fish mortality; these values did exhibit a similar trend

to that reported for Mo, where its seasonal levels reflected positively on the feeding behaviour of the species investigated.

5.2.3   Chromium

The environmental content of Cr have been reported to vary greatly and range from trace quantities to 250mg/kg in the soil (Bielicka *et al.*, 2005); whereas aquatic distribution levels were found to range from 1.0 – 10µg/l in freshwater lakes and rivers (Merian, 1991). Recently, Praveena *et al.* (2013), reported that fish species such as *Labeo rohita*, developed anaemia when exposed to sub-lethal dosages (1/10th of LC_{50} 96 hrs, 10ppm) of Cr. It was previously found (Chapter 3) that the mature *H. molitrix* specimens in this study are anaemic. Therefore, in light of these findings and that reported by Praveena *et al.* (2013) implicating Cr in the development of anaemia, it is proposed that Cr is involved in the anaemic presentation of mature *H. molitrix*. Validation of this theory was dependent on providing evidence that mature specimens had consistently higher seasonal bio-accumulation levels of Cr than the younger fishes and *L. rosae.* However, consistently higher Cr levels in mature fish was not found; regardless of the fact that Cr levels in young and mature *H. molitrix* differed significantly (P=0.01). Despite this, reflecting on reports such as that by Abdel-Baki *et al.* (2011), raised the question whether measuring bio-accumulation levels for Cr from muscle tissue is indeed the most appropriate and relevant indicator of its potential to interfere with physiological systems. It is therefore suggested that future investigations on Cr and probably a number of the other transition metals, include assessing its levels in other tissues, e.g. liver, and use these levels to explore their value in predicting physiological disturbances.

5.3.4   Manganese

Exposure to Mn, at sub-lethal levels, was found to elicit a stress response in fish, as confirmed by the increased production of cortisol after exposure (Gupta *et al.*, 2012).

Observing current Mn data it can be argued, albeit cautiously, that neither *L. rosae* nor young *H. molitrix* experienced high enough exposure levels to induce detrimental Mn effects. In addition, it should be contemplated that the dose-response of a species will be affected by various factors, including but not limited to age and gender. However, the findings for the mature specimens indicate that these fish presented consistently with levels far exceeding that of *L. rosae* and the younger fish (Figure 5.1). Subsequently, and in agreement with the report by Sharma and Langer (2014) who recorded decreased Hct, Hb and RBCs after Mn exposure, it is accepted that the Mn bio-accumulation data can, even if only partially, explain the presence of anaemia in mature specimens. In addition to this, it is anticipated that: (i) the ability of Mn to induce oxidative stress, as well as the resulting increase in ROS can contribute to tissue damage, especially when CAT activity is compromised and (ii) the Mn-induced impaired neurological and oxidative phosphorylation activity present plausible explanations for the non-responsive lethargic behaviour.

5.3.5   Iron

The pathogenesis of anaemia and impaired CAT activity identified Fe as a possible role player in the demise of mature silver carp specimens. Iron-deficiency can significantly contribute to the development of anaemia. In general the capacity of Fe to cause oxidative stress, relates to its role as a metal reductant in the Fenton reaction; subsequently producing ROS. Even though, not statistically significant ($P = 0.85$), the current data do support a relatively consistent decline in accumulation levels, throughout the sampling period (summer to spring), for all species in this study. Regrettably, no distinct age-dependent accumulation pattern was found to explicitly implicate Fe as a causative factor.

5.3.6   Copper

In the present study the Cu values for *L. rosae* were consistently higher than that of the mature *H. molitrix* specimens, throughout all seasons (Figure 5.2). Higher values

were also measured in young *H. molitrix* fish during winter and spring surveys. The seasonal bio-accumulation levels, when comparing *L. rosae* with *H. molitrix* (young and mature pooled) did not differ significantly (P = 0.53). However, a significant difference was found when comparing the young and mature *H. molitrix* specimens (P = 0.02). Unlike the dramatic seasonal fluctuations observed in the Cu levels for the young *H. molitrix*, those reported for *L. rosae* and mature *H. molitrix* remained relatively stable. Calculating the average Cu values indicated that levels in mature specimens (195μg/kg) were less than that of *L. rosae* (289μg/kg) and even more so when compared with the young *H. molitrix* (369μg/kg). Subsequently, it is postulated that these lower annual Cu levels in mature fish could support impaired production of haemoglobin as well as decreased erythropoietic activity. Haematological support (Table 3.1) for this argument highlighted that mature fish presented with lower Hb, Hct and RBC levels than their younger counterparts.

In addition, it should also be remembered that Cu together with Zn plays an important role in the ROS-scavenging activity of SOD (Cu/ZnSOD complex), thereby increasing the capacity to eliminate superoxide anions (Zhang *et al*., 2013) produced via aerobic respiration. This mechanism plays an important role in eliminating excessive production and accumulation of ROS. The discussion on Zn bio-accumulation (section 5.3.9) will confirm that similar to Cu, Zn levels were found to be consistently higher in the younger *H. molitrix* than in the mature fish. Despite omitting the measurement of SOD activity from the scope of this study, the fact that both Cu and Zn were present at such high levels in healthy young *H. molitrix* demands urgent attention in prospective studies to investigate the likelihood that the Cu/Zn/SOD interaction is significantly impaired in the mature *H. molitrix* specimens.

5.3.7   Nickel

Current seasonal and complete sampling period averages for the bio-accumulation levels of Ni, in *H. molitrix* (young and mature groups) and *L. rosae*, were inconclusive. The bio-accumulation levels of Ni for inter-species (*L. rosae / H. molitrix*) and intra-species (young fish / old fish) comparisons were not significant (P = 0.53 and P = 0.89

respectively). No distinct patterns were detected to implicate its involvement in disturbing any of the various physiological mechanisms identified thus far as possible role players in the death of the mature fish.

5.3.8   Cobalt

The lack of species-specific bio-accumulation reference values makes it very difficult to establish what can be considered normal. On average the mature *H. molitrix* fish exhibited higher Co levels than younger fish ($P = 0.18$). That being said, considering that young fish was not anaemic then it would be reasonable to propose that, in accordance to the statement by Anim *et al.* (2011), mature fish should have presented with polycythaemia and not with anaemia as is currently noted. It is therefore highly unlikely that Co is directly involved in the aetiology of anaemia and the subsequent death of the fish. Indirectly, based on the fact that mature fish on average had higher Co levels than younger fish and that no reference values are known for this species, another set of feasible suggestions are the following. Firstly, the ability of Co^{2+} to oxidise sulfhydryl groups, GSH, and pyridine nucleotides (Battaglia *et al.*, 2009), thereby inducing oxidative stress, cannot be ignored and is a key concept for future research on this feral population. Secondly, existing literature highlights the impact of Co on mitochondria, especially inducing aspecific damage to the inner membrane with specific focus on impaired cytochrome *c* action (Battaglia *et al.*, 2009). Cytochrome *c* plays an important role in mitochondrial respiration, and if this process is impaired, ATP production will decrease. Mature fish were lethargic, and a future investigation into the integrity of mitochondrial respiration can add tremendous value to understanding their energy requirements.

5.3.9   Zinc

Current findings support the impact of feeding behavior on Zn accumulation; *H. molitrix* presenting with much higher levels than *L. rosae* (Figure 5.3). Therefore, on average Zn seems to be more accessible to pelagic feeders. In addition to this, irrespective of

CHAPTER 5

RESULTS **and discussion of the bio-accumulation measurements in** *Hypopthalmichtys molitrix* **and** *Labeo rosae*

feeding behavior both species in this study exhibited a steady increase in bio-accumulation of Zn from summer through to autumn (*L. rosae* and young *H. molitrix*) or all the way through to winter (mature *H. molitrix*). Flag Boshielo Dam falls in a summer rainfall area; therefore, the observed increased bio-accumulation is most probably due to increased Zn-availability following run-off. However, average Zn values illustrated the following (i) that at species level *H. molitrix* had noticeably higher levels (5157µg/kg) compared to that of *L. rosae* (3535µg/kg), and (ii) that young *H. molitrix* in turn had higher levels (6259µg/kg) than the mature specimens (4593µg/kg). These average values, for *H. molitrix*, will form the basis for establishing to what extent Zn can be implicated in the deaths observed amongst mature fish.

The haematological findings in Chapter 3 confirmed the presence of anaemia amongst mature *H. molitrix*. This was evident from the substantially lower RBC, WBC, Hb and Hct values of the mature fish, compared to those recorded for the younger group. Kori-Siakpere and Ubogu (2008) indicated that chronic sub-lethal Zn exposure exhibited a dose-dependent decrease in blood parameters such as Hb, Hct and RBC; typified in the presentation of anaemia. The fact that younger fish displayed a consistently higher Zn level, had a relatively normal blood profile [when compared to those reported by Pieterse (1982)], and no anaemia, confounds sensible interpretation at this level.

On the other hand, appropriate oxidative stress responses can minimise oxidative damage. As was previously mentioned (section 5.3.6) the interaction between Zn/Cu/SOD plays an important role in an organism's anti-oxidant response. It is well-known that oxidative stress develops when Zn levels are deficient, subsequently causing tissue damage (Oteiza *et al.*, 2000; Bhowmik *et al.*, 2010). Zinc and Cu exhibited similar seasonal distribution patterns in the younger *H. molitrix*, which illustrates that unlike mature fish they were inherently better equipped to activate optimal anti-oxidant responses via enhanced Cu/Zn-SOD activity (not tested in this study). Thus more effectively cycling the O_2^- produced by the mitochondria during ATP production, to generate H_2O_2. In addition to this, the excessive levels of H_2O_2 can be controlled via enzymatic or non-enzymatic pathways. The former involving CAT, which

was found to exhibit remarkably better activity in the younger fish (Chapter 4, Table 4.1), to facilitate the following reaction: $H_2O_2 \rightarrow H_2O + O_2$. Similarly, yet another anti-oxidant function of the redox-inert metal Zn relates to the reduction of hydroxyl radical ($\cdot$OH) formation from H_2O_2 via antagonism of redox-active transition metals (Bray *et al.*, 1990).

The fact that mature fish were quiescent, hinted on a possible neurodegenerative impact. A recent review by Szewczyk (2013) on Zn homeostasis and neurodegenerative disorders focussed, amongst others, on the impact of aging. It was indicated that various progressive pathological features such as neuronal loss, altered cell metabolism and oxidative stress are associated with aging (Mocchegiani *et al.*, 2005). From existing literature no evidence could be located to indicate that Zn disrupts muscle activity at the neuromuscular junction per se; however, its interaction with γ-Aminobutyric acid (GABA) seems to be of particular interest. This focus is relevant because GABA is the primary inhibitory neurotransmitter in the central nervous system and plays a fundamental role in neural excitability. It is known that Zn attenuates GABA receptor-mediated responses (Sharonova *et al.*, 2000), thereby regulating muscle tone and as a result contribute to decreased spasticity (Boucetta *et al.*, 2014). Even though there seems to be some support from this for the lethargic behaviour of the mature *H. molitrix*, it is proposed that future research efforts include the seasonal measurement of both GABA and acetylcholine; the latter due to its impact on muscle contraction.

5.3.10   Cadmium

Investigating the impact of Cd bio-accumulation followed a dual approach. Firstly, to do a comparison of *H. molitrix* (young and mature fish combined) with *L. rosae* to establish if a discernible seasonal distribution pattern based on feeding behaviour was present (Figure 5.4). This would indicate whether benthic feeders are indeed, as expected, more vulnerable to Cd toxicity than pelagic fish. Secondly, to compare young and mature *H. molitrix* specimens in an effort to find evidence supporting the

involvement of Cd in the deaths of the mature specimens; or at least implicate it in the development of the observed behavioural and morphological changes reported in this study. The comparison of *H. molitrix* with *L. rosae* (Figure 5.4) clearly illustrated that the latter exhibited consistently higher Cd levels throughout all seasons. This distribution pattern highlights the fact that Cd was more readily available to *L. rosae*. However, the fact that no deaths were reported or personally noted for *L. rosae* during the entire study indicate to the probability that recorded Cd levels were not high enough to produce symptoms of toxicity or be lethal. Further assessment, comparing the seasonal Cd levels in the two *H. molitrix* subgroups, did not find consistently elevated Cd levels in the mature specimens. The younger specimens, on average (pooled seasonal averages: 78 µg/kg vs 64 µg/kg respectively), had accumulation values higher than that of the mature fish. In essence current findings cannot explicitly attribute the observed deaths and behavioural and morphological changes to Cd levels *per se*. Nonetheless, the fact that many physiological mechanisms exhibit an age-dependent loss in functionality proposes that the physiological stress induced by the Cd levels in mature fish were beyond their homeostatic response-capacity. This concept warrants further investigation, especially regarding the integrity of the renal system which is known to be the most important target organ for Cd toxicity.

5.3.11 Mercury

Seasonal findings indicate that feeding behaviour reflects positively on the availability of Hg, and subsequently its bio-accumulation in benthic and pelagic fish in this study. A benthic feeder such as *L. rosae* consistently presented with lower accumulation levels than the pelagic feeder *H. molitrix*. This suggests that most of the available Hg were dissolved in the water itself and not trapped in the detritus. When comparing the young and mature *H. molitrix* specimens, an unexpected seasonal bio-accumulation pattern was revealed. With the exception of spring, all other seasonal measurements, between these two age groups, exhibited an inversely proportional relationship. Based on this, and with the assumption that the bio-accumulation levels was indeed high enough to indicate excessive exposure; there should have been an increased mortality

rate during the summer and winter surveys. However, current recorded numbers for dying fish collected during these surveys do not support this; as a matter of fact it indicates the opposite with higher numbers collected during autumn and spring. That being said, it would be irresponsible to use the recorded numbers as an accurate indication of the number of fish that died during the period of this study because (i) all monthly surveys lasted 3 – 5 days and the number of dying fish collected might not be considered representative of the total number that perished in a specific month, and (ii) the lay-out and mere size of the Flag Boshielo Dam would make it relatively easy to miss dying fish while on the dam. Thus in a further investigative effort it was opted to use pooled seasonal values to determine if that might be a better predictor of detrimental health outcomes.

The pooled Hg values for the mature and young fish were 67 µg/kg and 53 µg/kg respectively. In addition to this, and contemplating similar environmental exposure levels, these bio-accumulation values indicate that younger fish tolerated exposure to Hg better than their older counterparts. Within this context it would be reasonable to consider the following arguments regarding the suspected deleterious health impacts. Firstly, the presence of anaemia in mature fish is in accordance with previous studies emphasising that Hg exposure does so via inducing a decrease in both RBC and Hb concentration (Patil and Jabde, 1998; Maheswaran *et al.*, 2008). The decreased Hb is most probably the result of Hg effectively competing with Fe (Pyszel *et al.*, 2005). Secondly, exposure to Hg is known to induce oxidative stress (Vassallo *et al.*, 2011). This predisposition to oxidative stress due to Hg exposure is most probably attributed to the high affinity of Hg for thiol-containing compounds such as glutathione (Houston, 2007). Subsequently, decreased intracellular GSH will trigger the production of ROS; a result that can also be achieved through increased lipid peroxidation. The similarity in GST and TBARS results for young and mature fish in this study effectively eliminates this as a possible mechanism. However, the impact of Hg on the free radical quenching enzyme CAT is of interest (Benov *et al.*, 1990); as this enzyme was found to exhibit poor activity in the mature fish. This seems to be a more likely mechanism for Hg-induced oxidative stress in this study.

Finally, currently available literature was used to establish whether Hg exposure could have played a role in the development of lethargic behaviour observed in mature fish. Here, the focus shifted to confirmation of the capacity of Hg to disrupt neurological activity. Even though this aspect was not directly investigated in the current study, the sluggish and non-response behaviour of the mature fish resulted in speculation that, amongst other possible physiological impacts, some degree of neurological impairment could have been involved. From the literature it was found that Hg can indeed have a detrimental effect on neurological activities; thereby, contributing to motor neuron deterioration (Praline *et al.*, 2007), abnormal muscle tone (Guzzi and La Porta, 2008) and fatigue (Wu *et al.*, 1985). From this it is evident that Hg could have been a role player in the current scenario. Future investigations focussing on the effect of Hg on neurotransmitters such as acetylcholine and γ-Aminobutyric acid could contribute significantly to our understanding of how *H. molitrix* responds to environmental stressors.

Conclusion

6.1 INTRODUCTION

Aquatic ecosystems, both freshwater and marine, serve as habitats for numerous organisms that are dependent on each other and on the environment they live in. Furthermore, the quality of water as an essential and very scarce resource is compromised with the advent of rapid urbanisation and industrialisation. These developments have significantly contributed to increased disposal of pollutants into the environment. Subsequently, adverse environmental conditions such as those created by pollution will have a detrimental effect on the health status of organisms residing in such aquatic ecosystems. The primary focus of this study was to establish a multifactorial physiological pattern capable of explaining the unexpected deaths reported amongst mature specimens of the alien species *H. molitrix* in Flag Boshielo Dam. In support of this, the impact of seasonality on haematological parameters, oxidative stress responses and bio-accumulation levels of transition metals in selected fish species such as *H. molitrix* and *L. rosae* was investigated*.*

6.2 HAEMATOLOGY

The use of haematological parameters as bio-monitoring tools is an established practice. These assessments have the ability to indicate deficient cell formation, increased or decreased cell production for both erythrocytes and leucocytes, reflect on the O_2-carrying capacity of blood and much more. Findings from this study unequivocally established that mature *H. molitrix* specimens were anaemic; a condition capable of contributing to the reported quiescence, as well as the pale appearance of the gills. However, the aetiology remains uncertain and requires further investigation.

6.3 OXIDATIVE STRESS

By default all aerobic organisms, including but not limited to fishes, are vulnerable to oxidative stress. Homeostatic interferences resulting from either physiological or environmental stressors have the potential to induce oxidative stress. Subsequently

the metabolic capacity of a stressed organism will determine if this event is adequately managed or not. In the event of sub-optimal anti-oxidant responses, oxidative stress is likely to progress in severity and cause tissue damage and ultimately apoptosis. Therefore, the use of oxidative stress biomarkers can play a significant role in assessing the impact of deleterious environmental conditions on the physiological integrity of an organism. In this study the oxidative stress biomarkers CAT, GST and TBARS were used to evaluate anti-oxidant responses. Results, comparing young and mature *H. molitrix* fish, confirmed that GST were unaltered throughout all seasons and were therefore unlikely to be involved in the demise of mature specimens. The seasonal variations in lipid peroxidation were erratic and no definitive pattern was detected to implicate it as a causative factor. However, compelling proof was found that CAT might form part of the multifactorial mechanism responsible for the death of these fish. This is based on the fact that CAT activity was consistently and notably less in mature *H. molitrix* than that of the younger fish; a scenario that supports the presence of excessive H_2O_2 levels.

6.4   BIO-ACCUMULATION

The presence of transition metals in water bodies have been reported to result in an inclination to accumulate (Blanco *et al*., 2014) and may therefore reach harmful levels which may result in tissue and organ damage. Findings from this study, in the absence of supportive water and/or sediment data, indicated that bio-accumulation of transition metals such as Mo, V, Cr, Co, Zn, Cd and Hg, was positively associated with pelagic and benthic feeding patterns. Furthermore, seasonal and in some cases pooled seasonal bio-accumulation levels established that it was reasonable to conclude that metals such as Mn, Fe, Cu, Zn, Cd and Hg were likely involved in disease pathogenesis. Probable mechanisms include participation in the Fenton reaction as a metal reductant or depletion of anti-oxidants such as GSH.

Conclusion

6.5 RECOMMENDATIONS FOR FUTURE RESEARCH ACTIVITIES

Evidence in support of possible mechanisms involved in the impaired health status and therefore the death of mature *H. molitrix* was generated by this study. However, the holistic complexity of life and death was reiterated as it was clear that no single entity or concept could downright explain why the mature *H. molitrix* specimens were dying. This uncertainty regarding the aetiology creates concern, especially within the framework of protecting the biodiversity of this aquatic ecosystem.

Is this a species-specific response indicating an age-dependent vulnerability; thus only affecting older *H. molitrix*? If so, why do we have sporadic deaths and not hundreds or even thousands of mature fish perishing at any given time? Is the life expectancy of *H. molitrix* in the Flag Boshielo Dam really in the region of 10-years (data not shown, but part of the bigger study)? This is relevant as this species was introduced into this system approximately 30 years ago; and it is only in the last 3 – 4 years that these mature specimens started to die in noticeable numbers. At this stage isotope studies (part of the bigger study) can form an important part in establishing whether this filter feeder is progressively forced to adjust its feeding behaviour due to a lack of natural food.

The haematological assessment of *H. molitrix* confirmed the urgent need for further haemocytological investigations into erythrocyte morphology, to detect cell abnormalities that might indicate the specific type of anaemia. Dramatic discrepancies in the existing literature regarding control (reference) haematological values for *H. molitrix* made it very difficult to identify deviations fortunately the availability of the Pieterse (1982) study and the inclusion of younger healthy specimens created an acceptable reference point. However, that being said, research endeavours focussing on creating an age and gender specific haematological reference guide for *H. molitrix* in the Olifants River System, Limpopo Province, will add enormous value to improving our understanding of the interaction of this fish species with its environment.

This study clearly indicated that the mature fish exhibited an impaired anti-oxidant response. The scope of this study was somewhat limited with regard to the number of biomarkers used. A more comprehensive investigation into the oxidative

Conclusion

stress cycle is suggested; with the focus on enzymatic and non-enzymatic components in this cycle. Of particular interest is SOD; the younger fish had higher Zn and Cu bio-accumulation levels during summer and autumn and this hints on the possibility that CuZn-SOD might play a role in their anti-oxidant response. In addition, the role of liver macrophage aggregates in fish pathology is well established (Agius and Roberts, 2003). The fact that focal and general liver discolouration was predominantly recorded amongst mature *H. molitrix* specimens requires further histopathological assessment of liver tissue.

Bio-accumulation efforts focussing specifically on the accumulation potential of transition metals should consider the following: (i) analysing metal levels in various tissue types to identify those most appropriate for bio-monitoring specific metals; (ii) use the information generated in (i) in combination with environmental exposure levels and other physiological parameters (haematology, neurological, etc.) to create a gender- and age-based prediction model. It is envisaged that such a model can play a significant role in establishing acceptable (sub-lethal) exposure levels to metals in the Flag Boshielo Dam.

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HEALTH **A**SSESSMENT **V**ARIABLES

Fish health assessment variables, with assigned characters illustrating the norms and deviation from the norm in the necropsy-based system.

Variables	Variable condition	Original field designation	Substituted value for the HAI
External variables			
Length	Total length	mm	-
Weight	Weight	g	-
Eyes	Normal	N	0
	Exophthalmia	E1/E2	30
	Haemorrhagic	H1/H2	30
	Blind	B1/B2	30
	Missing	M1/M2	30
	Other	OT	30
Fins	No active erosion or previous erosion healed over	0	0
	Mild active erosion with no bleeding		
	Severe active erosion with haemorrhage /	1	10
	secondary infection	2	20
Skin	Normal, no aberrations	0	0
	Mild skin aberrations	1	10
	Moderate skin aberrations	2	20
	Severe skin aberrations	3	30
Opercula	Normal / no shortening	0	0
	Mild / slight shortening	1	10
	Severe shortening	2	20
Gills	Normal	N	0
	Frayed	F	30
	Clubbed	C	30
	Marginate	M	30
	Pale	P	30
	Other	OT	30
Pseudobranch	Normal	N	0
	Swollen	S	30
	Lithic	L	30
	Swollen and lithic	P	30
	Inflamed	I	30
	Other	OT	30

Variables	Variable condition	Original field designation	Substituted value for the HAI
Thymus[a]	No haemorrhage Mild haemorrhage Moderate haemorrhage Severe haemorrhage	0 1 2 3	0 10 20 30
Internal variables (necropsy)			
Mesenteric fat	(Internal body fat expressed with regard to amount present) None Little, where less than 50% of each cecum is covered 50% of each cecum is covered More than 50% of each cecum is covered Cecae are completely covered by large amounts of fat	 0 1 2 3 4	 - - - - -
Spleen	Black Red Granular Nodular Enlarge Other	B R G NO E OT	0 0 0 30 30 30
Hindgut	Normal, no inflammation or reddening Slight inflammation or reddening Moderate inflammation or reddening Severe inflammation or reddening	0 1 2 3	0 10 20 30
Kidney	Normal Swollen Mottled Granular Urolithic Other	N S M G U OT	0 30 30 30 30 30
Liver	Red Light red "Fatty" liver, "coffee with cream" colour Nodules in liver Focal discolouration General discolouration Other	A B C D E F OT	0 30 30 30 30 30 30

Variables	Variable condition		Original field designation	Substituted value for the HAI
Bile	Yellow or straw colour, bladder empty or partially full		0	-
	Yellow or straw colour, bladder full, distended		1	-
	Light green to "grass" green		2	-
	Dark green to dark blue-green		3	-
Blood (Haematocrit)	Normal range		30–45%	0
	Above normal range		>45%	10
	Below normal range		19–29%	20
	Below normal range		<18%	30
Blood (plasma protein)	Normal range		30–69mg/dL	0
	Above normal range		>70mg/dL	10
	Below normal range		<30mg/dL	30
Parasites	No observed parasites		0	0
	Few observed parasites		1	10
Endoparasites	No observed endoparasites		0	0
	Observed endoparasites	< 100	0	10
		101–1000	1	20
		> 1000	3	30
Ectoparasites	No observed ectoparasites		0	0
	Observed ectoparasites	1–10	1	10
		11–20	2	20
		> 20	3	30

Health **A**ssessment **I**ndex

HEALTH ASSESSMENT INDEX				
Date:	Locality name:		Survey no:	
Fish species:			No:	

Fish no	ST cm:	TL cm:	Mass grams:	Sex M/F	Eyes	Skin	Fins	Opercules	Gills	Pseu. branch	Mes. fat	Liver	Spleen	Hind gut	Kidney	Bile	Hct (%)
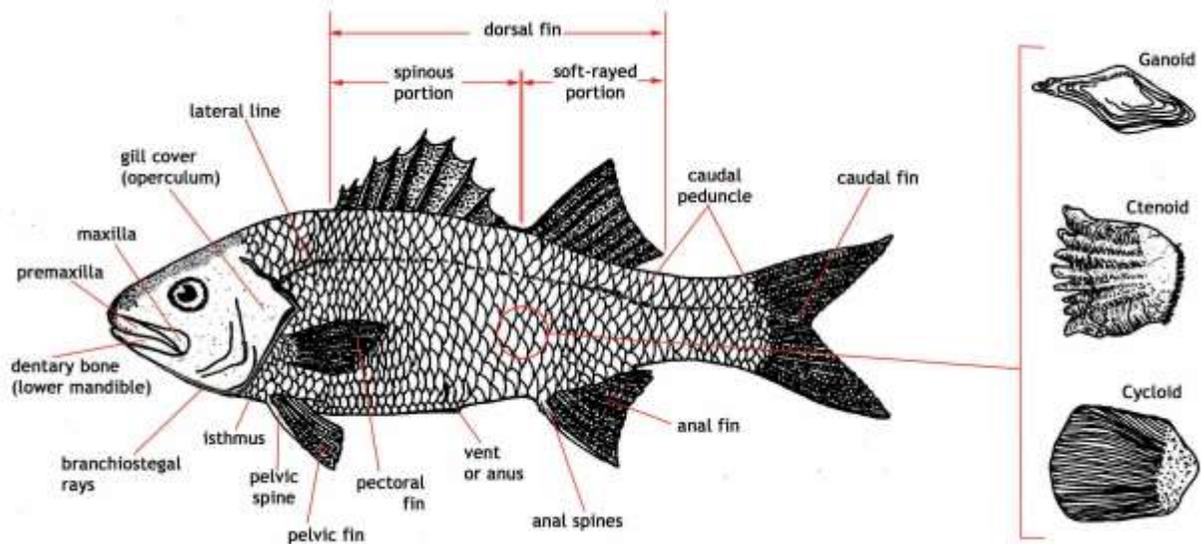
Total ectoparasites =

Total endoparasites =

ECTOPARASITES											
Group	Parasite	Mouth	Head	Body	Dorsal fin	Caudal fin	Anal fin	Pectoral fin	Pelvic fin	Total	Sp.#
Copepoda											
Branchiura											
Cysts (endo)											

SKIN SMEAR / NASAL PIT			GILL SMEAR			
Parasite	Infestation	Specimen #	Parasite	Infestation	Specimen #	Total (external) ectoparasites =
						Total (external) endoparasites =

Health **A**ssessment **I**ndex
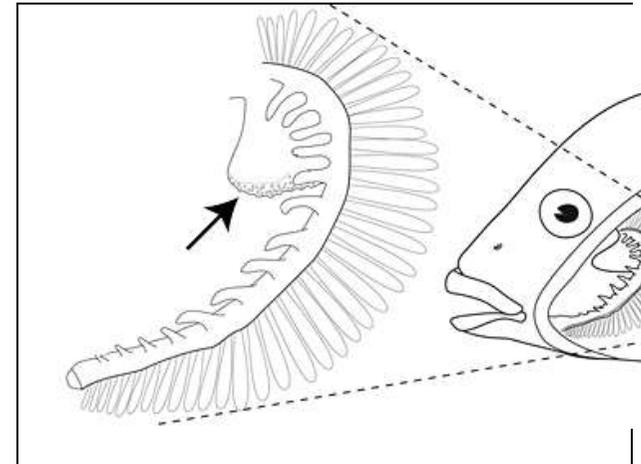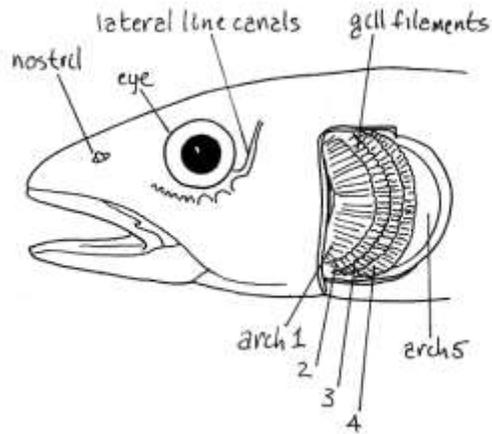
PARASITES ON THE GILLS								
Parasites		Gill no 1	Gill no 2	Gill no 3	Gill no 4	Total / gill	TOTAL	Sp. #
Monogenea	Left							
	Right							
Copepoda	Left							
	Right							
Cysts	Left							
	Right							
Other	Left							
	Right							

Total (gills) ectoparasites =
Total (gills) endoparasites =

ENDOPARASITES						
Group	Parasite	Site / organ	No (infestations)	TOTAL	Ref / sp no	Remarks
Trematoda						
Cestoda						
Nematoda						
Pentastomida						
Total (internal) endoparasites =						