TESTICULAR APOPTOTIC ACTIVITY IN TWO BIO-SENTINEL FISH SPECIES INHABITING AN AQUATIC ECOSYSTEM IN AN AREA WHERE CONTINUAL DDT SPRAYING OCCURS: UTILITY OF IMMUNOHISTOCHEMICAL ASSAYS

by

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DISSERTATION SUMMARY

Testicular apoptotic activity in two bio-sentinel fish species inhabiting an aquatic ecosystem in an area where continual DDT spraying occurs: utility of immunohistochemical assays

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Endocrine disrupting chemicals (EDCs) such as DDT have the ability to disrupt hormonally controlled processes, such as spermatogenesis, which is the maturation of germ cells into spermatozoa. During normal spermatogenesis, germ cell apoptosis can occur, but the degree of apoptosis within the testis could possibly be affected by exposure to EDCs.

In 2004, a pilot study on the reproductive health of two freshwater fish species, *Oreochromis mossambicus* and *Clarias gariepinus*, from three impoundments in the Luvuvhu River, found concerning levels of DDT and its metabolites in both species from the Nandoni Dam, and in *O. mossambicus* from the Xikundu Weir. This was not surprising as a large part of the Luvuvhu River catchment is located within an area where ongoing DDT-spraying occurs for vector control purposes. Hence, in 2006, a larger WRC-funded project began to further investigate the findings from the pilot study. A subsidiary study, spanning two seasons, was initiated to investigate testicular apoptosis in fish from the polluted systems, the Nandoni Dam (ND) and the Xikundu Weir (XW), as well as a reference site, the Albasini Dam (AD), utilizing caspase-3 and TUNEL immunoexpression as apoptotic markers. In addition, three fixatives, Bouin's Fluid (BF), Neutrally Buffered Formalin (NBF) and Paraformaldehyde (PFA), were used to determine which would be the optimal fixative for both histological and immunohistochemical assessments.

Sampling occurred during season 1, the low-flow season (October 2007), during DDT spraying of the surrounding area, and season 2, the high–flow season (February 2008),

two months after the DDT-spraying was completed. The testes of *O. mossambicus* (n = 19 season 1, n = 25 season 2) and *C. gariepinus* (n = 19 season 1, n = 20 season 2) were fixed in the above-mentioned fixatives, embedded in paraffin wax, prepared for immunohistochemistry, and exposed to caspase-3 antibodies and TUNEL antibodies individually.

The results indicated that the residues of *p,p´*-DDT - DDD and - DDE were found in the fat samples of both *O. mossambicus* and *C. gariepinus*, in AD, ND and XW. Testicular apoptotic assessment using the caspase-3 assay clearly labeled spermatocytes in the process of cellular death in both seasons, in all three fixatives. When comparing the two assays, a significant difference is found between the caspase-3 and TUNEL positive cells. The results further show that, when comparing the three sampling sites, the highest amount of positive cells are found at the XW. The decrease observed in season two, in both the caspase-3 and TUNEL assay may possibly be linked to the stage of spermatogenesis, coinciding with hormonal changes associated with the different sampling seasons (i.e. breeding and non-breeding seasons). The levels of DDT found in the fat tissue, could not be correlated to an up-regulation in apoptotic cells. The results indicated that the choice of fixative, could affect the identification of the amount of positive cells.

The utility of the caspase-3 and TUNEL assays, in conjunction with all three fixatives, proves a successful tool in assessing and quantifying modulated testicular apoptosis, creating greater research potential in the assessment of the effects of aquatic pollution.

Keywords: DDT, Spermatogenesis, Apoptosis, Caspase-3 immunohistochemistry, TUNEL, *Oreochromis mossambicus*, *Clarias gariepinus*

SAMEVATTING VAN VERHANDELING

Testikulêre apoptotiese aktiwiteit in twee bio-indikator visspesies van ‘n akwatiese ekosisteem in ‘n area waar DDT tans gespuit word: gebruik van immunohistochemiese toetsmetodes

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Endokrien-steurende chemiese stowwe (EDCs), waarvan DDT ‘n voorbeeld is, het die vermoë om liggaamlike prosesse, soos spermatogenese, wat deur hormone beheer word, te versteur. Spermatogenese is die proses waartydens geslagselle ontwikkel tot volwasse sperme. Apoptose van geslagselle, kan gedurende die proses van normale spermatogenese voorkom, maar die hoeveelheid selle wat apoptose ondergaan, kan beïnvloed word deur die blootstelling aan EDCs.

‘n Loodsstudie is in 2004 gedoen op die twee varswater visspesies, *Oreochromis mossambicus* en *Clarias gariepinus,* van drie versamelingspunte in die Luvuvhu rivier. Die doel van hierdie studie was om die gesondheid van die visse te ondersoek. Hoë vlakke van DDT en DDT metaboliete is in vetmonsters van albei visspesies van die Nandoni dam gevind, maar slegs in vetmonsters van *O. mossambicus* van die Xikundu studam. Die Luvuvhu rivier vloei deur die Vhembe distrik, waar DDT steeds gebruik word vir malaria vektorbeheer. Gevolglik, is ‘n WNK-ondersteunde studie in 2006 gedoen om die bevindinge van die loodsstudie verder te ondersoek. As deel van hierdie projek, is besluit om testikulêre apoptose te ondersoek in vis van die drie versamelingspunte in die Luvuvhu rivier, die Albasini dam (AD), geleë buite die DDT sprei-gebied en die Nandoni dam (ND) en die Xikundu studam (XW), geleë in die DDT sprei-gebied. Om die apoptotiese selle te identifiseer, is die twee immunohistochemiese toetsmetodes, die kaspase-3 en die TUNEL toetsmetode toegepas. Om die optimale fikseermiddel vir beide die histologiese en immunohistochemiese ondersoeke te bevestig, is drie verskillende fikseermiddels, Bouins vloeistof (BF), 10% neutraalgebufferde formalien (NBF), asook 4% paraformaldehied (PFA) gebruik. Vis is tydens twee seisoene

versamel, gedurende die DDT sprei-seisoen (seisoen 1, Oktober 2007, tydens laagvloei toestande), asook twee maande daarna (seisoen 2, Februarie 2008, tydens hoogvloei toestande). Die middelste gedeelte van die testis van elke vis (*O. mossambicus* n =19 seisoen 1, n = 25 seisoen 2; *C. gariepinus* n = 19 seisoen 1, n = 20 seisoen 2), is gefikseer en vervolgens voorberei vir immunohistochemie analise. Elke snit is onderskeidelik blootgestel aan die kaspase-3 en die TUNEL teenliggaampies.

Die resultate dui aan dat vetmonsters van beide *O. mossambicus* en *C. gariepinus* visse, van al drie versamelingspunte, oorblywende vlakke van *p,p'*-DDT - DDD en DDE gehad het. Deur die toepassing van die twee toetsmetodes, is die verskillende tipe geslagselle wat apoptose ondergaan het, vergelyk. Apoptose van die spermatosiete is hoofsaaklik waargeneem in visse van al drie die versamelingspunte tydens beide seisoene. Deur die resultate van die twee histochemiese metodes te vergelyk, tussen seisoen 1 en 2, is daar 'n beduidende verskil gevind tussen die hoeveelheid geslagselle wat kaspase-3 en TUNEL positief getoets het. Die resultate het verder getoon, dat die totale hoeveelheid positiewe selle wat by elke versamelingspunt geïdentifiseer is, hoër was in vis van die XW. Die verskille gevind tydens die twee seisoene, kan moontlik toegeskryf word aan die stadium van spermatogenese wat ooreenstem met hormoon veranderinge, wat tydens seisoen 1 (broei seisoen) en seisoen 2 (na die broei seisoen) plaasvind. Die DDT vlakke in die vetmonsters kon nie geassosieer word met 'n toename in die hoeveelheid selle wat positief getoets het vir apoptose nie. Daar is ook gevind dat die keuse van 'n fikseermiddel, die identifikasie van die hoeveelheid positiewe selle kan beïnvloed.

Om testikulêre apoptose te kwantifiseer deur die gebruik van beide die kaspase-3 en die TUNEL toetsmetodes, asook die gebruik van die drie fikseermiddels, was suksesvol. Die bogenoemde metodes kan dus bydra om die effek wat akwatiese besoedeling op vis het, verder te ondersoek.

Kernwoorde: DDT, Spermatogenese, Apoptose, Kaspase-3 immunohistochemie, TUNEL, *Oreochromis mossambicus*, *Clarias gariepinus*

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LIST OF ABBREVIATIONS

1°	Primary
1HF	Season 1 High Flow
2°	Secondary
2LF	Season 2 Low Flow
Ab	Antibody
ABC	Avidin-Biotin Complex
AD	Albasini Dam
ABP	Androgen Binding Protein
Ag	Antigen
APAF	Apoptosis Activating Factor
BF	Bouin's Fluid
bp	Base pairs
DAB	3, 3´-diaminobenzidine
Caspases	**C**ysteine **Asp**artyl-specific Prote**ases**
DDD	1,1-dihloro-2,2-bis(*p*-chlorophenyl)ethane
DDE	1,1-dihloro-2,2-bis(*p*-chlorophenyl)ethylene
DDT	1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane
DFF	Defragmentation factor
DNA	Deoxyribose Nucleic Acid
DWAF	Department of Water Affairs and Forestry
EC	Electrical Conductivity
EDCs	Endocrine Disrupting Chemicals
ER	Estrogen Receptor
ERK	Extracellular Kinase
Est	Early spermatids
EtOH	Ethanol
FSH	Follicle Stimulating Hormone
GC	Gas Chromatography
GnRH	Gonadothropin Releasing Hormone
GSI	Gonadosomatic Index
H&E	Haematoxylin and Eosin
HPG Axis	Hypothalamics-Pituitary-Gonadal Axis
IAP	Inhibitor of Apoptosis
IgG	Immunoglobulin G
IHC	Immunohistochemistry
JNK	c-Jun amino-terminal Kinase
LH	Luteinizing Hormone
LOD	Level of Detection
MAPKs	Mitogen Activated Protein Kinases
MI	Multiple Imputation
min	Minutes
MOMP	Mitochondrial Outer Membrane Permibiliazation
MS	Mass Spectrometry
mRNA	messenger Ribose Nucleic Acid
NBF	10% Neutrally Buffered Formalin
ND	Nandoni Dam
OC	Organochlorine

LIST OF ABBREVIATIONS

P450	Cholesterol
P450*scc*	Cholesterol side chain cleavage
PAHs	Polycyclic Aromatic Hydrocarbons
PARP	Poly-(ADP-ribose) polymerase
PBS	Phosphate Buffered Saline
PCBs	Polychlorinated Biphenyls
PCD	Programmed Cell Death
PFA	Paraformaldehyde
ppm	parts per million
PS	Phosphatidylserine
Psc	Primary spermatocytes
Psg	Primary spermatogonia
RT	Room Temperature
SD	Standard Deviation
SPE	Solid Phase Extraction
SHBG	Steroid Hormone Binding Globulin
Ssc	Secondary spermatocytes
Ssg	Secondary spermatogonia
StAR protein	Steroidogenic acute Regulatory protein
Sz	Spermatozoa
TDS	Total Dissolved Salts
TdT	Terminal deoxynecleotidyl transferase
TIAR	T-cell restricted Intracellular Antigen Related protein
TNF	Tumor Necrosis Factor
TNF-RI	Tumor Necrosis Factor Receptor 1
TRAIL DR4/ DR5	TNF-related apoptosis-inducing ligand death receptor 4 and 5
TUNEL	Terminal deoxynecleotidyl transferase-mediated dUTP Nick end-Labeling
UPR	Unfolded Protein Response
µg/l	Micrograms per litre
µg/kg	Micrograms per kilogram
XW	Xikundu Weir

CHAPTER 1: INTRODUCTION

1.1. General introduction

Environmental pollution has been the "buzzword" for the past few decades, and at the crux of scientific driven research towards 'saving' the world. Countless researchers have embarked on the task to identify the end-points of pollution, and elucidate its origins and mechanisms. The frequent reports on the decline of male reproductive health, in both humans and wildlife, attributed to the exposure of various compounds, still in use and discontinued, is disquieting (1, 2). These environmental, synthetic, and/or industrial toxic compounds, known as endocrine disrupting chemicals (EDCs), have the potential to act as hormone modulators, thus interfering with the endocrine pathways, and ultimately altering reproductive efficacy (3). The concern from the scientific world is the concern, not only for human health, but also for wildlife, which are exposed to these toxicants in their natural environments (2). The risk of a polluted environment stretches past the present, but rather unto the capacity to reproduce viable offspring in the future, to ensure the sustainability of resources for humankind.

Pesticides are poisonous substances that are ideally targeted at eradicating a particular organism, whilst being harmless to other organisms. Complete selectivity of a pesticide is virtually impossible, thus there exists a potential of causing damage to man or to other non-target organisms. Less acutely poisonous pesticides may however be persistent, and elicit long-term ecological effects and pollution of the environment. A class of pesticides, the insecticides, is divided into two groups, the organophosphorous and the organochlorine compounds, such as DDT (1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane.

DDT and its metabolites are one of numerous endocrine disrupting chemicals (EDCs) that exhibit estrogenic or anti-androgenic effects. These effects may alter the functioning of the endocrine system, and in so doing result in adverse effects on the health of the exposed organism (4). The observed adverse effects of DDT exposure on wildlife

include: reproduction abnormalities in birds, mammals, and the feminization of males (1, 5).

The male reproductive system is vulnerable to the effects of persistent environmental pollutants and/or physical factors. Reproduction is an event that is the culmination of a host of biological processes. Reproduction is controlled by the central nervous system, the endocrine system, as well as external cues. These processes ensure that there is functional maturation of the sexual organs, appropriate sexual behavior, and correct maintenance of hormone levels (3). Alterations in normal hormonal levels may result in alteration in cell maturation and viability of these cells, thus interfering with normal bodily processes. Under normal circumstances the process of apoptosis regulates the optimal cell number and proliferation rates of cells (6). Thus interference with normal bodily processes will up-regulate the process of apoptosis.

Apoptosis is one form of programmed cell death (PCD), in which the cell has the ability to activate an inherent suicide mechanism that ultimately eliminates the cell in an ordered fashion (49). The cellular mechanism of apoptosis is mediated through the action of the caspase family of proteases. Activation of the 'executioner' caspase -3, 7, or 9 results in the initiation of cellular death, however, caspase-3 is considered the main executioner caspase (7). It is clear that caspase activation is a hallmark of almost all apoptotic systems (8). Thus, an immunohistochemical technique to assess caspase-3 expression proves useful to assess the presence of caspase-3 as well as to quantify the extent of apoptosis (9).

Following executionary caspase activation, the cell undergoes apoptotic cascades, resulting in cellular dismantling, which causes DNA fragmentation and fragmentation of cellular components. The fragmented cellular components of the cell are then packaged into vesicles, and are removed from the system through phagocytosis (10). The utility of the TUNEL (terminal deoxynecleotidyl transferase-mediated dUTP nick end-labeling) technique is accepted as the most widely used histochemical method for fragmentation detection (10). The utility of a caspase 3/7 assay in identification of apoptosis which has

been up-regulated by apoptotic ligand activation through the action of DDT has previously been demonstrated (11). In another study, it was reported that exposure to various toxic chemicals may cause strand breaks in the DNA, directly or indirectly (12). Thus, both the caspase-3 and TUNEL assays can be useful in assessing the extent of upregulation of apoptosis induced by DDT and its metabolites.

1.2. Motivation for the current study

In South Africa, malaria has been a growing concern since the late 1930s. The country is plagued by malaria in three of its provinces, namely, the Limpopo Province, Kwa-Zulu Natal, and Mpumalanga. As a method to curb the rising incidence of malaria cases, the use of DDT was elicited for vector control purposes (13). The Limpopo Province has a higher risk of malaria, as compared to the other two provinces (17) (fig 1). DDT has been used for malaria vector control in Limpopo, Mpumalanga, and KwaZulu-Natal, since 1948 (14). DDT was briefly discontinued in KwaZulu-Natal during the 1996/'97 periods, but this decision resulted in insect resistance to the new safer pyrethroid insecticide, thus resulting in DDT application resurging in 2000. However, DDT was never discontinued in the Vhembe district of the Limpopo province (fig 2), where it has been sprayed annually sprayed for the past 60 years (fig 1, 2). (15).

The Luvuvhu River catchment in the Vhembe District Municipality in the Limpopo Province, South Africa (16) is unique in a sense that the river originates outside of the DDT-sprayed area, flows through the DDT-sprayed area and joins the Limpopo River in the Kruger National Park. Thus a potential gradient of higher levels of DDT concentrations could be expected as the river flows towards the Kruger National Park.

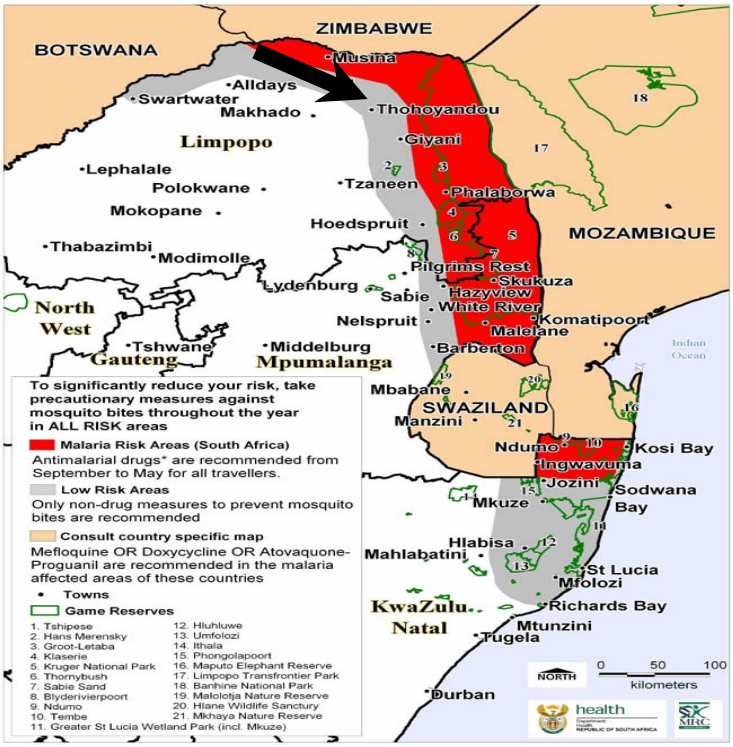


Fig. 1: Map of the malaria affected regions of South Africa (17). Copyright permission obtained from the MRC and Department of Health, South Africa.

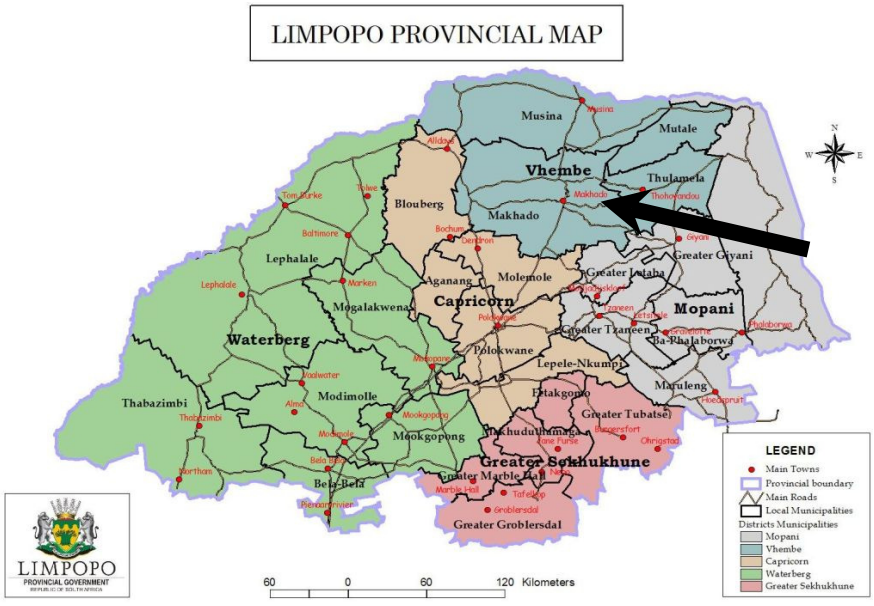


Fig. 2: The Limpopo Provincial Map, indicating the Vhembe district, where DDT is currently being sprayed (18). Copyright permission obtained from the Limpopo government.

In 2004, a Water Research Commission funded study entitled "A pilot investigation of environmental endocrine disruptive chemicals in a currently DDT-sprayed area" was conducted at the Albasini and Nandoni Dams and the Xikundu Weir, in the Luvuvhu River. The aim of the investigation was to analyze water, sediment and fish tissues for DDT residues as well as to assess the estrogenicity of collected water samples (19).

The results showed levels of DDT in the fat from *O. mossambicus* and *C. gariepinus* specimens from the Nandoni Dam, but only in *O. mossambicus* from the Xikundu Weir, as no *C. gariepinus* was found. In addition, the histological assessment of the testes showed that 56% (17 of n=30) of *O. mossambicus* showed an incidence of testicular oocytes. No DDT was found in the fish from the reference site, the Albasini Dam, as no fat was available for chemical target analysis (19).

The presence of DDT in the fat of the fish is not unfounded as DDT is lipophillic, and is fat-soluble (20). The cause of concern is that steroid hormones are produced from the breakdown of fat in the body. During steroid hormone synthesis, the breakdown of fat may also result in the breakdown of DDT, thus allowing DDT and its metabolites to elicit an action (21).

It is known that the pesticide, DDT, is a hormone-mimicking agent that can alter the hormonal profiles in the body by binding to various receptors. Technical grade DDT consists of 65-80% of the active component *p,p´*-DDT, 15-21% *o,p´*-DDT, which is nearly inactive, 4% of *p,p´*-DDD, and up to 1,5% 1-(p-chlorophenyl)-2,2,2-trichloroethanol . It is however the persistent metabolite of *p,p´*-DDT, *p,p´*-DDE, that has been reported to act as an androgen receptor antagonist and testosterone inhibitor (5). There have been reports that technical grade DDT possesses estrogenic properties, resulting mainly from *o,p´*-DDT (45).

In so doing these compounds, elicit an inappropriate hormonal response, which may result in the production of inappropriate cell numbers, either too many or too few, or the production of malformed and damaged cells. Thus, studies have investigated the ability

of a host of tools to quantify the effects of this endocrine disrupting activity (22). One such tool is the investigation of the modulation of the normal cellular process of apoptosis.

The process of apoptosis normally removes extraneous and damaged cells from the organism. In the event of incorrect maturation resulting from various factors such as hormonal interference, this process is up-regulated (23). There are various techniques to quantify apoptosis, ranging from biochemical assays to electron microscopy (23).

The use of caspase-3 and TUNEL assays for apoptosis, due to a host of insults has been investigated in various species (24, 25). A study investigated testicular apoptosis in *C. gariepinus* in an urban nature reserve in South Africa. This particular study used both caspase-3 and TUNEL assays, and concluded that both assays have utility in determining the types of cells affected, as well as quantification of apoptotic cells (26).

To further investigate the results from the pilot study, a larger study was initiated entitled, "Environmental exposure and health risk assessment in an area where ongoing DDT spraying occurs". The aim of this ongoing study is to assess the health and reproductivity of fish within the Luvuvhu River. As part of, and related to this study, it was decided to include an investigation on testicular apoptosis in fish from the Luvuvhu River, using immunohistochemical techniques.

1.3. Aim of the study

To assess the testicular apoptotic activity in *O. mossambicus* and *C. gariepinus*, inhabiting the Albasini Dam, outside of the DDT-sprayed area, the Nandoni Dam and the Xikundu Weir within the DDT-sprayed area, in the Luvuvhu River in the Limpopo Province.

Objective 1:

- To compare the degree of testicular apoptosis between *O. mossambicus* and *C. gariepinus* from the three sites.

Objective 2:

- To compare and confirm the efficacy of the TUNEL and cleaved caspase-3 immunostaining of apoptosis.

Objective 3:

- To compare the efficacy of three different fixatives for optimal immunostaining.

Objective 4:

- To assess the concentrations of DDT in the fat tissue of both *O. mossambicus* and *C. gariepinus,* and to correlate these values with the degree of observed testicular apopotosis.

CHAPTER 2: LITERATURE REVIEW

2.1. Testes

The site of reproduction in the male is the testis. The two main functions of the vertebrate testes are spermatogenesis and androgen synthesis (27). In teleost fish, the testes are elongated sac-shaped organs that develop directly from the peritoneal epithelium. However, the seminiferous tubules of teleosts lack the permanent germinal epithelium that is observed in mammals (28).

In the teleost fish, two types of testicular structures can be identified namely, the lobular and the tubular type arrangement (Fig 3). Typical in most teleosts is the lobular type of arrangement, which is comprised of numerous, variably arranged lobules that are separated by a thin fibrous connective tissue layer, with the spermatozoa being freed into a central lumen.

The tubular type of testicular arrangement has no central lumen, instead the cysts move towards a central cavity, in which the spermatozoa are released. The tubular type of arrangement is uncommon and is only restricted to a few species such as the guppy, *Poecilia reticulate* (28, 29).

The lobular type of testicular arrangement is comprised of numerous cysts (Fig 4). The cysts are compartments within the testes that house the germ cells that are undergoing the process of spermatogenesis. Within a particular cyst the germ cells mature in a synchronous pattern, resulting in one cyst containing germ cells at the same stage of development.

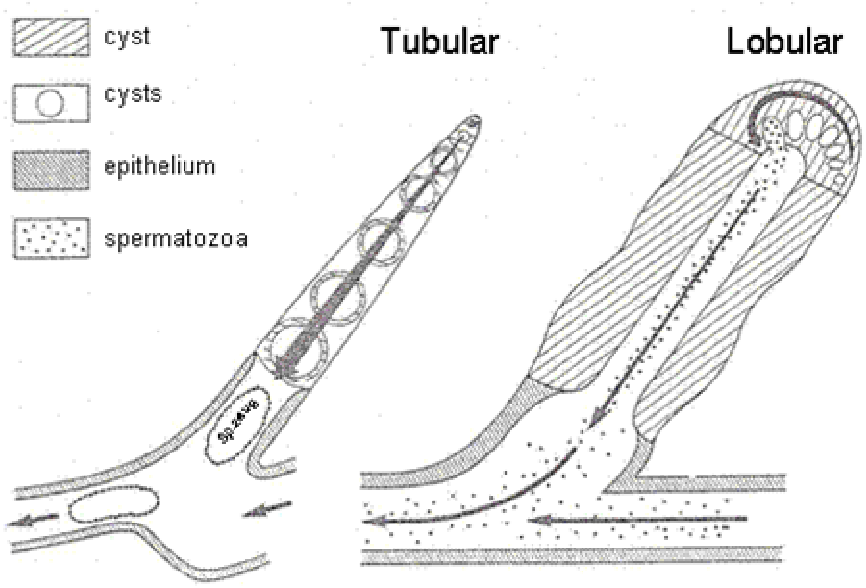


Fig. 3: Two testicular subtypes present in teleost fish (28). Copyright permission obtained from the NRC, Canada.

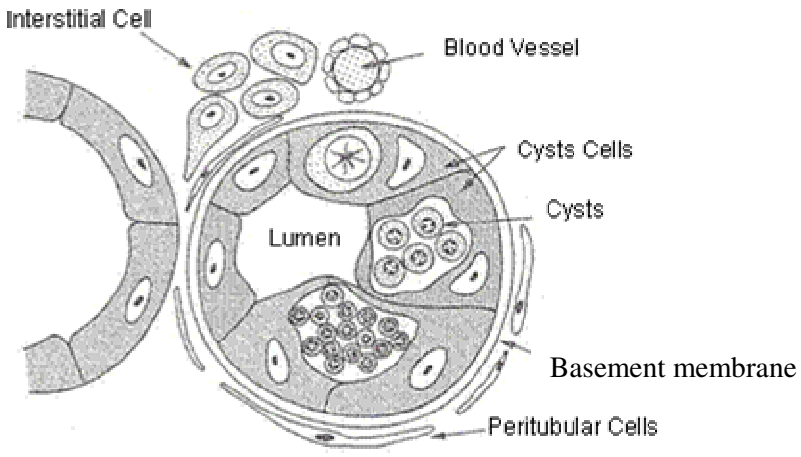


Fig. 4: Cross section of a lobule of a teleost fish, indicating cellular arrangement (28). Copyright permission obtained from the NRC, Canada.

2.2. Spermatogenesis

The seminiferous tubules of the testes are the site for the spermatogenic process (27). Spermatogenesis is defined as a process in which germ cells undergo differentiation and metamorphosis to mature into functional spermatozoa (30). The process of spermatogenesis occurs continually during the reproductive lifetime, where the germ cells

undergo stages of mitotic division, differentiation and ultimately meiosis (31). The stages of the spermatogenic cycle are defined as: primary spermatogonia, secondary spermatogonia, primary spermatocytes, secondary spermatocytes, and spermatids spermatozoa (28).

2.2.1. Primary spermatogonia

Primary spermatogonia are identified by light microscopy as large cells, with a large amount of cytoplasm that are usually found outside of a cyst. Primary spermatogonia, the products of stem cells, undergo mitotic division to produce secondary spermatogonia (31).

2.2.2. Secondary spermatogonia

Secondary spermatogonia are observed as an aggregation of cells within a cyst. Less cytoplasm is observed as compared to primary spermatogonia. The secondary spermatogonia further proliferate and differentiate to form primary spermatocytes (31).

2.2.3. Primary spermatocytes

Primary spermatocytes can be distinguished from secondary spermatogonia in that the cell membrane is uneven and the cell is smaller in size. The primary spermatocytes undergo meiosis to produce secondary spermatocytes (27).

2.2.4. Secondary spermatocytes

The secondary spermatocytes are smaller than the primary spermatocytes. In addition the nuclear size is much smaller than that of primary spermatocytes. The secondary spermatocytes also undergo meiosis to produce spermatid (27).

2.2.5. Spermatids

Spermatids are characterized by dense chromatin in the nuclei, and are smaller than secondary spermatocytes. It is these spermatids that differentiate and ultimately become spermatozoa (31).

2.2.6. Spermatozoa

Spermatozoa are the final stage of spermatogenesis. The spermatozoa are densely packed in the cyst and are released into the lumen (31, 32).

2.3. The Endocrine System

The endocrine system is one of the major integrating systems in humans and animals. This communication system consists of glands, hormones and cellular receptors that control the homeostasis of the body (33). Through utilization of circulating hormones, the endocrine system ensures correct delivery of hormones to their target organs to elicit a specific response. These hormones can exert their action at low concentrations (10^{-9} or 10^{-12} g/ml) in the bloodstream, and thus bind to specific hormone receptors. This receptor bound state results in the initiation of a cascade of events that influence pivotal developmental, growth, regulatory, and homeostatic mechanisms throughout the body (34). A component of the endocrine system, the Hypothalamic-Pituitary-Gonadal (HPG) axis (Fig 5), is the main regulator of spermatogenesis and reproduction in general (35).

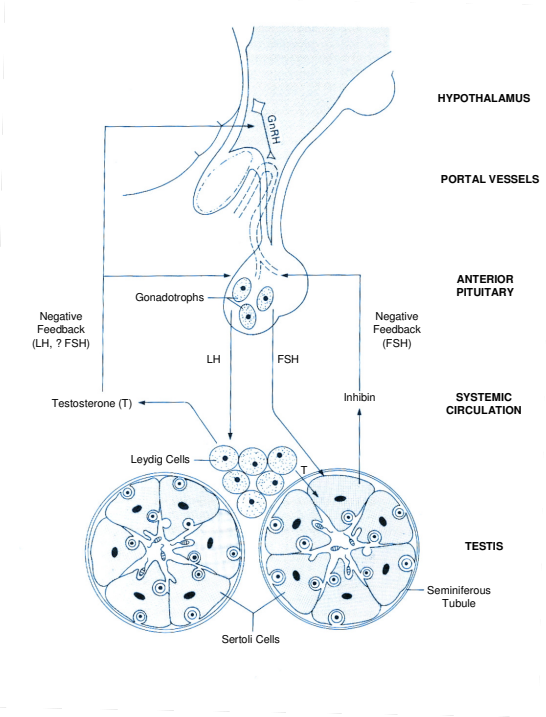


Fig. 5: Diagrammatic representation of the HPG axis (36). Copyright permission obtained from Blackwell-Scientific Publishers.

In mammals, this axis is comprised of three components; gonadotropin-releasing hormone (GnRH) neurons, from the hypothalamus; secretion of gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary; and the somatic cells of the testes, the Leydig, and Sertoli cells (3).

2.3.1. Somatic cells of the testis

The survival and spermatogenic progression of germ cells is vastly dependent on the somatic cells of the testes (32). The Sertoli cells plays a vital part in the maturation of the germ cells by providing and sustaining an optimal supportive and nutritive microenvironment during germ cell proliferation and maturation. The Sertoli cells are located within the interstitial tissue (Fig 5), which is neighbouring the cysts (32). FSH targets receptors within the Sertoli cells to regulate spermatogenesis by stimulating Sertoli cell factors (31). Within the cysts the Sertoli cells perform multiple specialized functions, which are carried out simultaneously. The Sertoli cells have the ability to function as a phagocyte thus eliminating residual cytoplasmic bodies at spermiation (37). Additionally, adjacent Sertoli cells form intercellular tight junctions, thus forming the blood-testis-barrier. The blood-testis-barrier separates the seminiferous epithelium into two compartments, namely: the basal compartment housing the spermatogonia and spermatocytes, and the adluminal compartment, housing the meiotic spermatocytes and spermatids (38).

In addition to the Sertoli cells, the Leydig cells are the somatic cells with the capacity for steroidogenesis. Leydig cells are typically found in the interstitial tissue between the lobules (Fig 5), and usually occur in a cluster of cells (38). Leydig cells are primarily the androgen synthesis sites of the testes, which secrete testosterone. LH targets the Leydig cells to stimulate testosterone, which activates androgen receptors in the seminiferous epithelium to control spermatogenesis. The testosterone produced by the Leydig cells is concentrated in the seminiferous tubules by an androgen-binding protein (ABP) induced by FSH (33). Spermatogenesis requires both FSH and very high levels of testosterone to promote germ cell maturation. There is evidence that supports a control pathway which co-ordinates Leydig cell functions with germ cells and Sertoli cells (39). This evidence

stems from the observation that the structure of the Leydig cell and its function varies with the germ cell/Sertoli cell development observed in animals with 'zonal testes' such as teleosts.

In teleost fish, the hormonal homologues for the mammalian FSH and LH have been identified. These fish hormones function similarly to their mammalian counterparts, as the vertebrate endocrine system varies slightly among species. In the control of spermatogenesis, the product of the pathway is not a hormone, but a cell that remains in the lobule that cannot exert feedback control to the brain. Thus, the Sertoli cell appears to monitor spermatogenesis, and when needed, releases the hormone inhibin (Fig 5), which specifically inhibits FSH secretion (21).

2.3.2. Estrogen

The steroidal hormone estrogen is involved in the control of reproductory processes, including sexual differentiation, maturation, control of the cell cycle and proliferation (40). Estrogens have generally been associated with the regulation of female functions, however, they are produced in male vertebrates (21), and estrogen receptor (ER) activity is expressed throughout the HPG axis and in the testes of a variety of vertebrate species (31). This observation led to the suggestion that estrogens take part in the regulation of testicular function (3). Natural estrogens bind to ER with a high affinity and specificity, while xenoestrogens bind to the ER with a lower affinity. Nonetheless, xenoestrogens occur at concentrations that are sufficient to elicit a range of biological effects (41).

2.3.3. Aromatization of testosterone

Conversion of androgens to estrogens in the last step of estrogen biosynthesis is mediated by the cytochrome P450 enzyme aromatase (42). Aromatase is regarded as a potential xenoestrogenic target since variation in its expression and thus, its function, can disrupt the estrogen production rate that will lead to changes in estrogen levels, resulting in disruption of estrogen-affected processes (37). Aromatase and ERs localization during various stages of murine germ cell development, suggest that there is a possibility of

direct estrogenic action on the germ cells (31). Chemical disruption of any part of the process of steroidogenesis, either by androgen inhibition or estrogenic stimulation, may consequently compromise the progression of hormonal dependent processes such as spermatogenesis (43).

2.4. Endocrine Disrupting Chemicals (EDCs)

Compounds known as EDCs exhibit the potential to interfere with the normal functioning of the endocrine system by mimicking, inhibiting or enhancing the actions of endogenous hormones (2, 4). EDCs are either natural or synthetic, exogenous compounds that provoke adverse health effects by potentially interfering with hormone synthesis, subsequent hormonal secretion, storage, release, transport, binding, efficacy and gene expression (44). The past few decades have seen numerous industries worldwide utilizing various synthetic compounds for a host of needs. Thus, as a result chemical pollutants including polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), heavy metals and pesticides were synthesized and eventually released in to the environment.

2.4.1. DDT (1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane)

The pesticide DDT was first synthesized in 1874, used as an insecticide since 1939 (13) and is heralded for being the panacea to malaria. DDT, due to its cost-effectiveness, has been the most widely used insecticide for agricultural and epidemiological purposes (1, 13). In South Africa, DDT has been used for malaria vector control in three provinces, KwaZulu-Natal, Limpopo and Mpumalanga, since 1948. However DDT was never discontinued in the Limpopo province, where it has been annually sprayed for the past 60 years (14).

In addition to possessing estrogenic properties, DDT is also lipid soluble (20). This characteristic allows the DDT not to dissolve in water, but rather be deposited in the fat tissues of organisms. Bio-concentration occurs over the period of exposure, when the DDT from the surrounding media is stored in the fat of the organism, at higher levels than

that found in the surrounding environment (20). During this period the potential exists for DDT to elicit its hormonal mimicking properties, either directly or through its breakdown into metabolites.

Steroidogenesis requires the breakdown of fat stores in order for hormonal synthesis to take place (43). Thus, during fat breakdown, DDT metabolites are released into the blood stream. The use of fat from a specimen can be subjected to target chemical analysis, in order to determine the composition of chemicals contained within the sample (46). This gives an indication of the composition and levels of DDT, its metabolites, DDD and DDE, and other EDCs present.

Although DDT is considered to be an EDC, it also belongs to a group of chemicals termed xenoestrogens. Xenoestrogens are defined as chemicals with the potential to mimic the action of the natural hormone estrogen. Even though the structure of the xenoestrogens differs from that of estradiol, the mimicking action still has the potential to disrupt the endocrine system (47). Natural estrogens bind to ER with a high affinity and specificity, while xenoestrogens bind to the ER with a lower affinity. Nonetheless, xenoestrogens occur at concentrations that are sufficient to elicit a range of biological effects (41). This is as a result of natural hormones binding to ABP, which are then transported in the blood bound to steroid hormone binding globulin (SHBG), with a high affinity, to albumin, with a lesser affinity, and unbound in the free circulating form (21). However, xenoestrogens do not bind to SHBG or ABP, but rather circulate in the unbound form, thus enabling a greater hormonal response.

The interaction of these xenoestrogens with androgen receptors may result in the antagonization of endogenous androgens, whilst interaction with estrogen receptors may result in mediation of cellular actions of hormones (22). This mimicking potential enables the compound to interfere with the synthesis of estrogen, its secretion, and the action of the hormone (3). This alteration in hormonal profiles within the organism, leads to disruption of normal hormonal dependent processes, such as spermatogenesis, resulting in the viability of the germ cells, being compromised (43). The viability of germ cells and any other cells of an organism are controlled by the process of apoptosis (6).

2.5. Apoptosis

2.5.1. Background

In order maintain the optimal cell number and cell proliferative rates throughout the body, a mechanism must be in place to eliminate damaged or extraneous cells, to ensure optimal cell viability (6, 7, 48). The mechanisms behind cellular death have intrigued scientists of various disciplines for more than a century. In 1972, a group of researchers studied the ultrastructural morphological changes in dying cells. Based on their findings, they introduced the term apoptosis. The term is a Greek word derived from *apo* meaning "apart" and *ptosis* meaning "fallen" to describe the shedding of leaves from trees (48).

Apoptosis is one form of programmed cell death (PCD), in which the cell has the ability to activate an inherent suicide mechanism that ultimately eliminates the cell in an ordered fashion (49). Apoptosis is observed in humans in the developing fetus, when interdigital webs are removed during the development of the limbs (48). Apoptosis differs from oncosis, autophagy, and mitotic catastrophe (Table 1), in that apoptosis is an active process, in which caspase-3 is activated, which results in the cell nucleus remaining intact, allowing the cell to expend energy and maintain control of its demise (23).

Oncosis is characterized by cellular swelling, which eventually leads to cellular and nuclear breakdown, accompanied by inflammation and leakage of cytosol into the surroundings of the cell, resulting in necrosis (23, 50). This is a passive, pathological process, thus, the cell does not have the ability to control its death, as all the organelles are ruptured (51).

Table 1**:** Characteristics of various types of cell death

	Apoptosis	**Oncosis**	**Autophagy**	**Mitotic Catastrophe**
Stimulus	Intracellular, Cytotoxic agents	Extracellular, ROS	Starvation, cellular remodelling	Aberrant mitosis, DNA damage
Nucleus	Condensation, pyknosis	Intact	Blebbing, segregation possible pyknosis	Intact
Cell Membrane	Intact, blebbing	Swelling, rupture	Blebbing possible	Intact
Cytoplasm	Intact, condensed	Rupture, spillage	Autophagic vacuoles	Intact
Caspase Activity	Dependent	Independent	Independent	Independent
Mitochondria	Intact	Ruptured	Dilation possible	Dilation possible
Result	Apoptotic bodies	Inflammation	Autophagic vesicles	Giant multinucleate cells

Autophagy is an evolutionary conservation process regulated by environmental cues. This form of PCD is characterised by the bulk degradation or recycling of the dysfunctional or damaged cellular components. The cytosolic materials are encapsulated in autophagic vesicles, which fuse with lysosomes or other vacuoles resulting in degradation of contents (52). Mitotic Catastrophe is a conserved stress response mechanism, which is initiated by dysfunctional progression of mitosis. The cells are eradicated during or close to metaphase (53).

2.5.2. Mechanism of Apoptotic cell death

2.5.2.1. Initiation Phase

Cells may undergo apoptosis through the activation of two major signalling pathways, namely the extrinsic pathway and the intrinsic pathway. The extrinsic pathway, referred to as the death receptor pathway, is activated by ligand-bound death receptors, belonging to the Tumor Necrosis Factor (TNF) superfamily of receptors (54):

- TNF receptor 1 (TNFR1)
- TNF-related apoptosis-inducing ligand death receptor 4 and 5 (TRAIL-DR4/DR5)
- Fas

The intrinsic pathway is activated by intrinsic signals such as chemical induced DNA damage, oxidative stress, and growth factor deprivation. The resultant effect is the activation of apoptosis through the involvement of the mitochondria or the endoplasmic reticulum (55). The mitochondria are the most important cells in the process of intrinsic activated apoptosis. Even though the mitochondria do not directly trigger apoptosis, it is the mitochondrial outer membrane permeabilization (MOMP) that is the key event (49). This MOMP results in the release of toxic proteins into the cytoplasm and constitutes a "point of no return" in PCD. The members of the Bcl-2 family, a regulatory family of proteins housed in the mitochondria, control the MOMP. Upon apoptotic signal initiation, the pro Bcl-2 proteins such as Bax are activated and cause MOMP. After initiation of MOMP, cell death precipitates through the release of a host of apoptotic molecules, or the loss survival functions of the mitochondrial (48, 49).

The endoplasmic reticulum is the second organelle participating in the intrinsic process of apoptosis. The endoplasmic reticulum is the most important cellular stress sensor, and can thus withhold protein synthesis and metabolism in order to first restore cellular homeostasis. ER stress results in the unfolded protein response (UPR), which if severe enough can initiate PCD (56). The UPR results in the activation of caspase 12 mediated through the Bcl-2 family, which results in downstream caspases being triggered (56). The ER stress can also induce MOMP and can thus also activate apoptosis via the mitochondrial pathway. The Bcl-2 family of proteins can thus coordinate the cross talk between the mitochondria and the ER to initiate caspase dependent intrinsic mediated apoptosis (56).

In addition, crosstalk also exists between the extrinsic and the intrinsic pathways, both at the initiation and execution levels. This occurs despite the difference in pathway activation, as both pathways converge at the activation of the executioner caspase 3 (56). DNA damage results in intrinsic apoptotic molecules such as Bax to activate the mitochondrial pathway, as well as the upregulation of genes such as FasL, in the extrinsic pathway.

a) Initiator Caspases

Cellular enzymes that degrade proteins, biologically known as proteases, are synthesized as inactive pro-enzymes in cells. Triggers within the specific cell activate them, and upon activation, the enzymes elicit their actions (57). Caspases (**C**ysteine **Asp**artyl-specific Prote**ases**) are a family of cellular proteases present essentially in all animal cells in their inactive form (8). The initiator caspases are caspase 8, 9, and 10. One of the first actions of the initiator caspases is to start to degrade the cytoskeletal proteins (55). Degradation of these proteins result in the shrinkage of the cell, as the rigidity is lost. The degradation results in the formation of membrane "blebs" (Fig 6). Blebs are defined as fluid-filled structures, devoid of organelles, arising from the cell membrane that has pinched off from the cell and floated away (50). In addition to bleb formation, initiator caspases initiate the translocation of phosphatidylserine and the release of cytochrome c (49).

b) Phosphatidylserine (PS)

The plasma membrane of a cell is comprised mainly of phospholipids. These phospholipids play an important role in both the construction and functioning of the cell membrane (35). The phospholipid PS is normally confined to the inner layer of the plasma membrane (see Fig 6, which shows the PS flip). The activated initiator caspases induces the action of the enzyme scramblase and inactivation of the enzyme translocase (58). Translocase, which normally inhibits the spontaneous PS flip to the outer membrane, is thus inhibited. Scramblase activation mediates the outward flip of PS to the outer membrane (55). The significance of PS being flipped to the outer membrane is to serve as a cell-cell recognition signal, in order to alert the surrounding cells and macrophages that the cell is undergoing death. The macrophages and neighbouring cells thus prepare themselves for phagocytosis of the cell after it has undergone apoptosis (55, 58).

c) Mitochondria

The mitochondria of a cell play an integral role in cellular death. This results from the cells ability to release signals, which either trigger or inhibit cell death. The

mitochondrion is stimulated by the initiation caspases to release apoptosis activating factor (APAF) and cytochrome c (Fig 6) (49). The bcl-2 family of gene products, within the mitochondrion, controls the release of mitochondrial molecules. The gene bax promotes release of molecules, and the gene bcl-2 inhibits molecule release (51, 55). The fine balance of these two genes, however, remains unclear. What is known is that, upon release of APAF and cytochrome c from the mitochondrion, the two molecules form a complex, which initiates the activation of the execution caspases (23, 55).

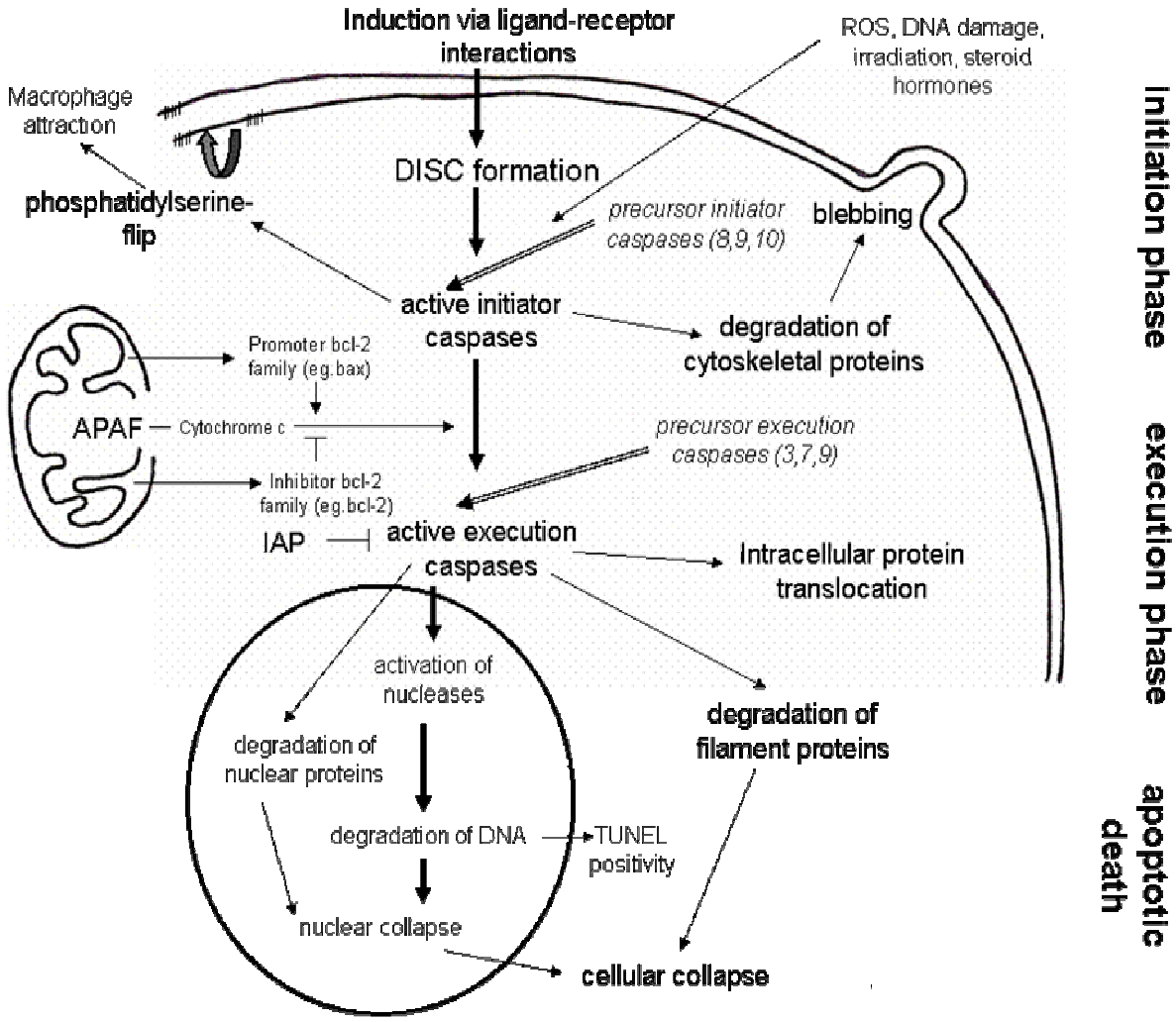


Fig. 6**:** The caspase mediated apoptotic cascade (55). (With kind permission from Springer Science + Business Media: Journal of Anatomy and Embryology, The apoptosis cascade – morphological and immunohistochemical methods for its visualization, issue 200, 1999, pg 2, Huppertz B, Frank H-G, Kaufmann F, figure 1).

2.6.2.2. Execution Phase

The inhibitors of apoptosis (IAP) can inhibit the activation of execution caspases, by binding directly to the activated caspases or by blocking caspase activation (7, 49). If no inhibition has been exerted, the execution caspases are then activated and the cell has irrevocably committed itself to undergo death (55).

a) Execution caspases

Activation of execution caspases (caspase 3, 7, and 9), in particular caspase 3, initiates a host of cascades (Fig 7), including the translocation of proteins from the nucleus to the cytoplasm (8). In addition to protein translocation, proteins critical to cell survival are cleaved through activation of various proteases, or directly by the executionary caspases (55). Degradation of cytoskelatal proteins, are responsible for the apoptotic characteristics, such as blebbing and collapse of the nucleus and ultimately the cell (23).

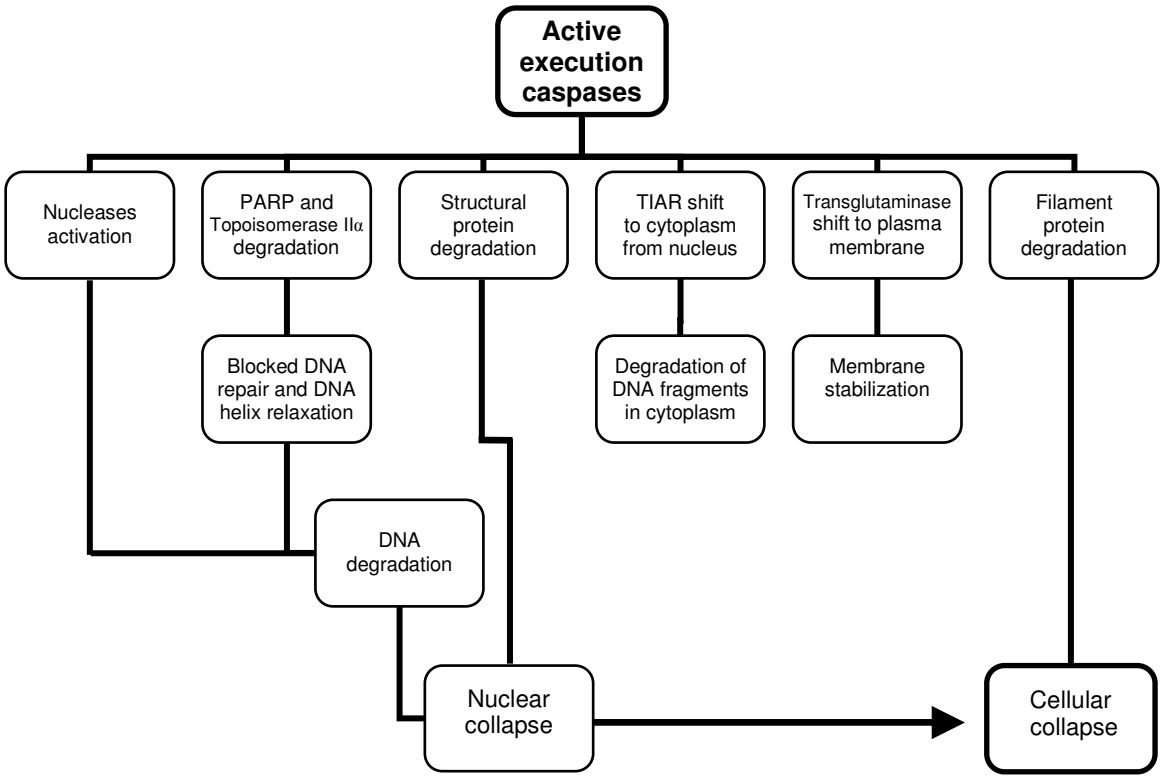


Fig 7: Schematic representation of the action of execution caspases.

b) Protein translocation

The translocation of proteins is not fully elucidated, but translocation serves as useful markers for the determination of the execution stages of apoptosis. Ordinarily the protein TIAR (T-cell restricted intracellular antigen related protein) is restricted to the nucleus, where it acts as a nuclease, cleaving DNA (64). During the activation of execution caspases, TIAR is gradually translocated from the nuclease (Fig 7) to the cytoplasm (55). The enzyme transglutaminase II is evenly distributed throughout the cytoplasm in normal, non-apoptotic cells. During later stages of apoptosis transglutaminase II, is activated and is translocated to beneath the plasma membrane. In this new sub-plasmalemmal location transglutaminase II forms crosslinks between the cytoplasmic proteins (59). The crosslink results in the formation of extensive protein scaffolds, which are postulated to inhibit the apoptotic cell from releasing its cytoplasmic contents into the interstitium, and creating inflammation (10, 59).

c) Nuclear protein degradation

Active execution caspases target the proteins that are involved in DNA maintenance and repair, such as PARP (Poly-(ADP-ribose) polymerase) and topoisomerase IIα (Fig 7). PARP assists in the repair of DNA damage, and is thus important for maintaining DNA integrity in normal cells (55, 59). Thus, PARP is a target for execution caspases, which, after cleavage, reduces DNA repair and amplified chromatin damage. Seeing as the protein topoisomerase IIα, in the normal cell binds chromatin, it is thus also a target for execution caspases (10). PARP and topoisomerase IIα are rendered inactive by a combination of caspase-dependent activated endonucleases, leading to single strand breaks of the DNA (55, 59). Lamins are found along the inner surface of the nuclear membrane. They are the major structural proteins of the nuclear envelope and are involved in maintaining the structure of the nucleus (55, 59). Apoptotic degradation of the lamins leads to structural changes within the nucleus, resulting in nuclear collapse and fragmentation.

2.5.2.3. Apoptotic death

a) DNA degradation

The role of DNA fragmentation in apoptotic cell death has been a contentious issue since it was first linked with apoptosis (10, 59). It has been suggested that it plays a role in suppressing the inflammation response (10) by possibly no release of kinins. The first reported biochemical evidence of apoptosis was when irradiated lymphocyte DNA was fragmented into specific nucleosomal-length fragments *in vivo* (10). It was later, in 1980, that Wylie and Kerr introduced the notion that endonucleolytic DNA degradation was a component of apoptosis (10). Interestingly, no mitochondrial DNA fragmentation is noted in apoptosis, indicating that DNA fragmentation is a specific apoptotic event (6). DNA fragmentation has since this, been linked to endonuclease activity, and has subsequently been used as the biochemical marker of apoptosis (59).

Activation of endonucleases, such as DFF (DNA Fragmentation Factor) leads to the discovery that DNA fragmentation is a component of cellular death (23, 55). This stemmed from the observation that in almost all situations of morphologically characterized apoptosis, the biochemical event of internucleosomal DNA fragmentation is prominent, leading to its utility as the maker of apoptosis (10). Biochemically, DNA is fragmented by endonucleases in the linker regions of between histones on the chromosomes, in 180-200 bp (6, 50). Series of nucleosomal chains of 180-200 bp multiples are characteristic to apoptotic cell death, and seen as the ‘apoptotic ladder’, and used as a marker in biochemical assays (10, 23).

b) Apoptotic Bodies

Following the breakup of the nucleus, the cell emits processes, known as buds; these buds contain condensed nuclear fragments (55). These budding processes tend to break off, and are referred to as apoptotic bodies. These apoptotic bodies are then phagocytized by aggregated macrophages or surrounding cells, attracted by the PS externalization (50).

2.6. Apoptosis and Mitogen Activated Protein Kinases (MAPKs)

Environmental factors, including growth factors, intracellular responses, physical and chemical stress, initiate MAPKs (60). The MAPKs are signalling molecules that transduce signals, upon activation, from the plasma membrane to the cell nucleus. The MAPKs play an integral role in controlling cell growth, survival, proliferation, differentiation, migration, and development (60, 61, 62). The activation, specificity, and regulation of MAPKs are tightly controlled within the body (68). The MAPK proteins are comprised of three subfamilies, namely: extracellular kinases (ERK), the c-Jun amino-terminal kinases (JNK), and the p38 kinases (62).

When cells are exposed to a host of stimuli including bacterial pathogens, certain growth factors and other environmental stress, the activity of p38 is upregulated (63). These up-regulations result in p38 mediating the transmission, amplification and diversification of the extracellular signal, thus, initiating various cellular responses (61, 63).

Seeing as the MAPKs are regarded as being an essential component in signal transduction (61, 64), a study investigated the effect that organochlorines may potentially have on induction of MAPK cascades (62). Their study investigated whether or not there is an increase in MAPK phosphorylation in murine Sertoli cells, during phagocytosis of apoptotic cells. The Sertoli cells were incubated with apoptotic mouse thymocytes which displayed PS on the outer membrane surface, and where subsequently analyzed for MAPK phosphorylation. After 10 min of incubation, the phosphorylation of all the MAPKs testes, ERK, JNK, and p38, were briefly enhanced (62). This observation leads to the suggestion that MAPKs are involved in the PS flip during the initiation phase, and the phagocytosis, in the apoptotic phase.

2.7. Immunohistochemistry (IHC)

The fact that cells within a population may begin apoptosis at various times after a cell death signal has been initiated, is a major and often overlooked, and unappreciated aspect of cellular death (65). The duration of apoptosis varies from cell to cell, and from species

to species. Thus the accurate detection of apoptosis, and the subsequent interpretation of an IHC assay, is crucial to the efficacy of a particular study (66).

The concept of IHC has been employed in numerous biological research fields, since its inception in the 1940s (67). This method is based on the affinity between an antigen (Ag) and antibody (Ab). The definition of an Ag is a substance, which upon induction by a specific stimulus has the capability of stimulating the immune system to produce a response that is solely directed at that specific stimulus. This response by the immune system to the Ag is referred to as an Ab. Thus, the Ag has a mutual affinity for the Ab, whose formation it caused (68). This affinity is an attractive force between these molecules, which enables them to form a bond (69). For the usage of scientific research Ab are produced and available for antigen identification.

Various types of IHC techniques exist (67), but the fundamental concept of using an Ag directed against an Ab in a tissue, and the subsequent visualization of this formed complex, has remained fairly unchanged.

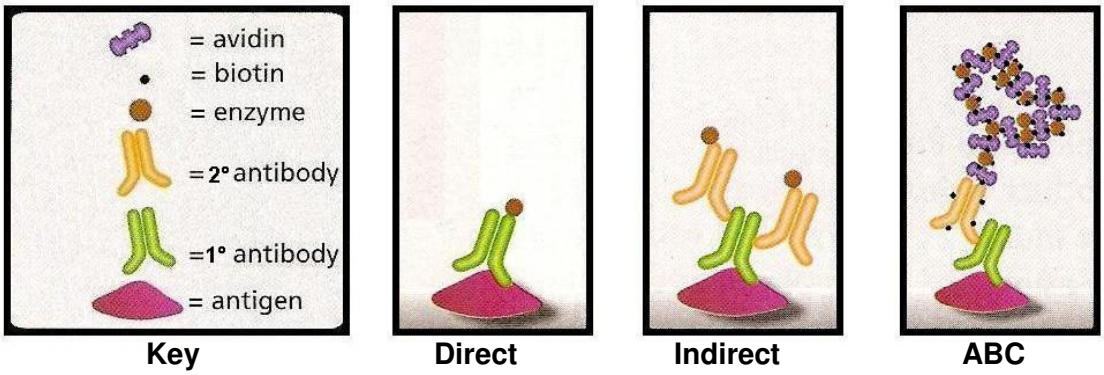


Fig. 8: Schematic representation of the major immunohistochemical methods (70). Figure used with copyright permission from Vector Laboratories, CA, USA.

2.7.1. The direct method

The direct method (Fig 8) allows for the specific, primary (1°) Ab to bind to a specific Ag within the tissue. This one-to-one binding however yields a poor sensitivity when detecting a low level of Ag, as optimal complex formation must occur (67).

2.7.2. The indirect method

The direct method piloted the development of an indirect method (Fig 8), in which an unlabelled 1° Ab directed against the Ag was used. After the application of an unlabelled primary Ab, a labeled secondary (2°) Ab, directed against the 1° Ab, was applied (68). Thus a magnification in the reactivity resulted as numerous 2° Ab are able to react with various Ag on the 1° Ab, increasing complex formation (67).

2.7.3. Avidin-Biotin Complex (ABC) method

The efficacy of this method lies in the affinity that the protein avidin, found in egg white, has for the vitamin biotin (67). Avidin possesses an extraordinarily high binding affinity for biotin; this affinity is over one million times stronger than that of Ab for most Ag. As a result, this bond is irreversible, unlike the Ab-Ag bond, which is reversible. The ABC (Fig 8) can thus successfully be utilized as avidin has four binding sites for biotin, thus antibodies and antigens can be conjugated with several molecules of biotin, forming a biotinylated complex (71).

IHC methods are valuable assessment tools in cellular research. The durability and specificity of the reaction products and their utility in fixed tissue sections make these methods and especially the ABC method, highly efficient diagnostic tools.

2.7.4. Immunohistochemical Assays

Caspase-mediated apoptosis should ideally be assessed using more than one method, since cells first express caspases, and then fragment their DNA upon death. Thus, immunohistochemical techniques to assess the expression of caspase-3, the main execution caspase, and DNA fragmentation, will serve as valuable tools to assess apoptosis (9).

The caspase-3 assay has found utility in the field of scientific research as the detector of the first sign of caspase-mediated cellular death. Frigo *et al* (11) demonstrated the utility

of a caspase 3/7 assay in identification of apoptosis inducted through apoptotic ligand activation by DDT. Caspase-3 is present in its inactive form in normal cells. It is only through the initiation of apoptosis that caspase-3 is cleaved and activated, thus an immunohistochemical reaction can be visualized (9).

Following executioner caspase activation, the cell undergoes apoptotic cascades, resulting in ultimate DNA fragmentation (10). The TUNEL assay has become the most widely used histochemical method to DNA fragments of apoptotic cells (10, 72). The TUNEL assay labels the 3'OH ends of the DNA with TdT (terminal transferase) using dUTP. In an environmental risk assessment, it was reported that exposure to various toxic chemicals may cause strand breaks in the DNA, directly or indirectly (12).

McClusky *et al* (26) compared testicular apoptosis in *C. gariepinus*, reared in an aquarium to *C. gariepinus* inhabiting the Rietvlei urban nature reserve in South Africa, which is polluted with industrial effluents. The study found a surprising low incidence of TUNEL-labeling as compared to caspase-3 labeling, resulting form inadequate DNA fragmentation. However, they found that reactivity was specific and abundant, suggesting that upstream events, such as caspase-3 activation, are prolonged. Thus, they concluded that, the TUNEL assay greatly underestimates the apoptotic events occurring within the cell, and that caspase-3 immunostaining is superior in quantifying apoptosis.

Based on these findings, a decision was made to assess the utility of these two immunohistochemical assays in a DDT-sprayed area to determine the extent of testicular apoptosis in *C. gariepinus* as well as *O. mossambicus*. Seeing as a difference in quantification of apoptosis was observed in the study by McClusky *et al* (26), the use of three different fixatives was employed, to determine the optimal fixative that would yield the greatest amount of positive cells. With all the above-mentioned points, it would be possible to compare the degree of apoptosis between the two species, as well as to compare and confirm the efficacy of the IHC assays in apoptotic quantification, based on the findings of McClusky *et al.* (26). Thus it would be possible to determine which fixative has greater efficacy in yielding superior immunostaining, to facilitate optimal quantification of apoptotic cells. In addition, the degree of observed positive cells can be

correlated with the concentrations DDT, in order to establish to what extent the DDT is eliciting a hormonal disrupting response on the spermatogenic process.

CHAPTER 3: MATERIALS AND METHODS

3.1. Study area

In the Limpopo Province, the Soutpansberg Mountains gives rise to the perennial Luvuvhu River, which flows through approximately 200 km of a diverse array of landscapes, before joining the Limpopo River in the Kruger National Park. The Luvuvhu River catchment (5941 km^2) forms part of the Limpopo River system, which flows downstream into Mozambique. The present study was undertaken in the Luvuvhu River catchment in the Vhembe District Municipality in the Limpopo Province, South Africa (16). This area, close to Thohoyandou, was selected for the study as DDT is currently being utilized there for malaria vector control (fig 9).

The study sites investigated include (fig 9):

Albasini Dam GPS; (758 m; S 23º05.974'; E 30º05.998'):

The AD is situated 45 km west of the DDT sprayed area, and was used as the control site.

Nandoni Dam GPS; (490m,S 22º88.945'; E 30º35.745'):

The ND was selected as the first exposed site, as it lays 15 km from Thohoyandou, at the start of the DDT sprayed area.

Xikundu Weir GPS; (450m; S 22º48.506'; E 30º47.924'):

The XW further east towards the Kruger National Park was used as the second exposed site (fig 9). The XW receives water that has gone through approximately 70 km of DDT sprayed area, and is regarded as the greater exposed site within the current study.

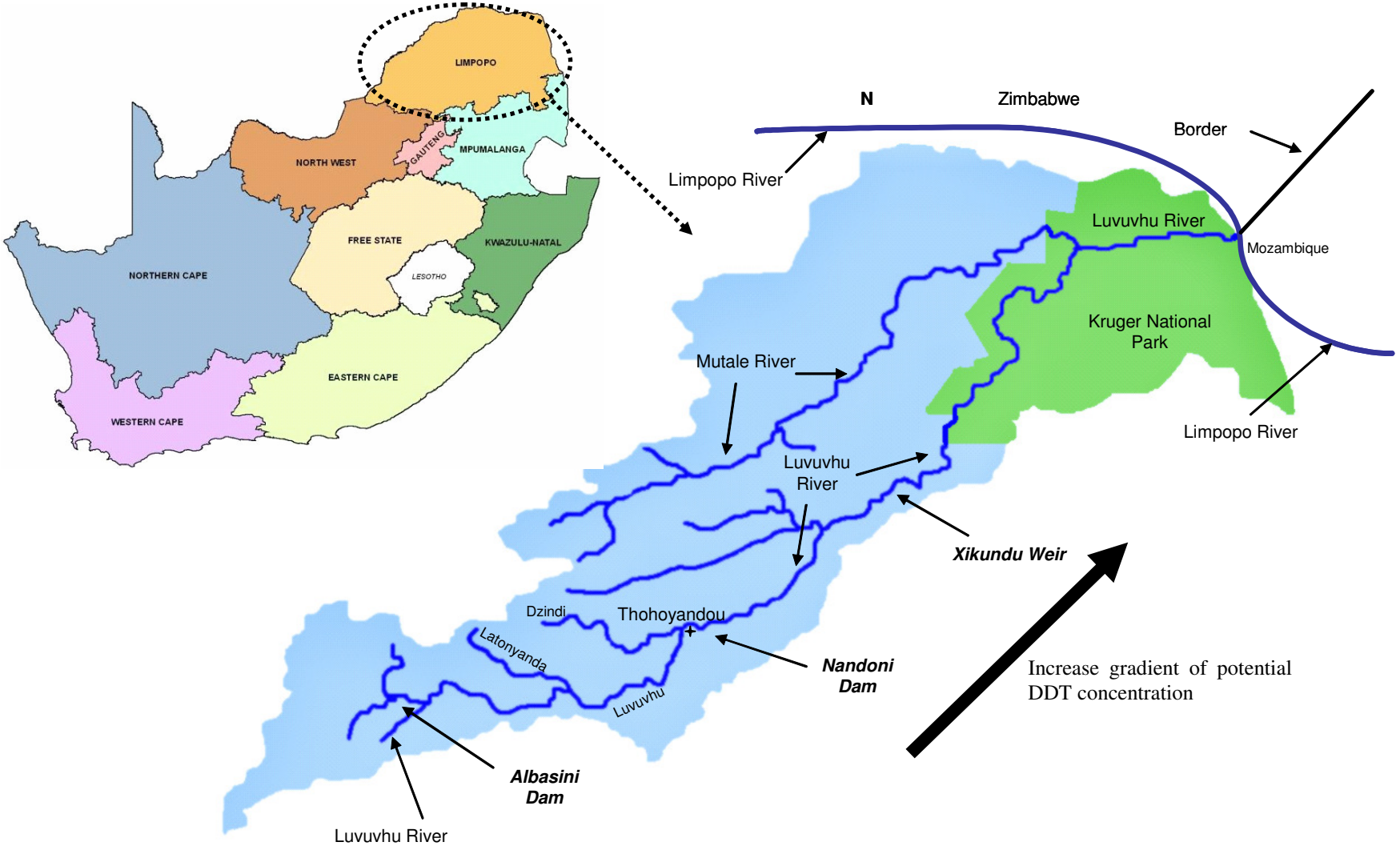


Fig 9: Map of the Luvuvhu River catchment, indicating the three sampling sites (16). Copyright permission obtained from RHP, CSIR, South Africa.

3.2. Fish as an experimental organism

Fish have been utilized in various studies as a model system to evaluate pollution in the aquatic environment, caused by metals and persistent organic pollutants (12, 73). The effectiveness of fish as a test organism stems from their sensitivity to toxicants as well as their all-year-round availability, thus making them prime candidates for assessing aquatic pollution (74).

3.2.1. Animals

Mozambique Tilapia (*Oreochromis mossambicus* – Peters 1852) is a highly adaptable, fast maturing species widely used in aquaculture, fisheries, and angling. Naturally distributed from East Africa and the lower Zambezi River system, *O. mossambicus* also occurs southwards, to the Boesman River system in the Eastern Cape. In Southern Africa, distribution is throughout the Limpopo River system, and coastal rivers including the Lower Orange and other Namibian rivers (75, 76). Although thriving in standing waters, *O. mossambicus* occurs in all but fast-flowing waters, tolerating fresh, marine, and brackish waters (75, 76). Spawning occurs during summer, with the mouth-brooding female raising 3-4 broods per season. The *O. mossambicus* fry grow rapidly and may reach maturity and breed within one year (76). Primarily foraging on algae, particularly diatoms, insects, and invertebrates are preyed upon by larger individuals (75).

Sharptooth catfish (*Clarias gariepinus* – Burchell 1822) is a resilient tropical catfish species generally considered as one of the most important tropical catfish species for aquaculture, angling, and as a food source. It has an almost Pan-African distribution from the Nile to West Africa and from Algeria to the Zambezi River System, and rivers to the south as far as the Orange River. Catfish favours mainly quiet waters, flood plains, large sluggish rivers, and dams (75, 76). Spawning takes place during the summer months, after the summer rains, in flooded grassy verges with high oxygen content. Catfish may reach maturity within one year, but the majority after two years or more. A nocturnal, omnivorous scavenger that feeds at the bottom or in thickly weeded areas and occasionally feeds at the surface (76). Being natural air-breathers makes *C. gariepinus*

easily susceptible to insecticide and molluscicide sprays, because they frequently rise to the surface for air (76).

3.3. Sample collection procedure

3.3.1. Fish collection procedure

Fish were collected during the low-flow season, 22 October - 2 November 2007, and the high-flow season 12 – 29 February 2008. Six gill nets were cast at various points at each of the three sites, increasing the probability of collecting fish. The fish were retained in two aerated containers filled with tap water, prior to dissection.

3.3.2. Testes preparation procedure

Each male specimen was weighed and the total length measurements were recorded (Table 2). The fish were subsequently sacrificed by severing the spinal cord posterior to the opercula, and the testes were removed from each specimen (73). The right and left testes lengths and mass were measured individually, and the total gonad mass was used to calculate the gonadosomatic index. The mid-piece of the excised testes was removed, and divided equally among three fixatives (See Section 3.5).

3.3.3. The Gonadosomatic Index (GSI)

The gonadosomatic index (GSI) was calculated for each of the *O. mossambicus* and *C. gariepinus* specimens. The GSI represents the total gonadal weight relative to the total body weight, expressed as a percentage:

$$\textbf{GSI} = \frac{\textbf{Total gonad weight}}{\textbf{Total body weight}} \times 100$$

The GSI, an indicator of gonadal growth and maintenance (77, 78), has been used extensively in the past, as early as 1927, and is currently still used as a tool to aid

histological assessment. The establishment of a correlation, in male fish, between the inhibition of testicular growth and the potency of estrogenic compounds (79, 80), has lead to the utilization of GSI as a biomarker for the exposure of aquatic wildlife to environmental estrogens (77, 78, 79).

Table 2: Length and mass measurements of the collected fish

	n	Total Length (mm)			Mass (g)		
		Ave	± SD	Median	Ave	± SD	Median
October 2007							
O. mossambicus							
Albasini Dam	9	310.00	62.30	275.00	422.22	856.48	270.00
Nandoni Dam	0	0.00	0.00	0.00	0.00	0.00	0.00
Xikundu Weir	10	324.73	26.23	325.00	591.00	107.03	605.00
C. gariepinus							
Albasini Dam	10	632.50	92.38	625.00	1762.00	856.48	1550.00
Nandoni Dam	3	725.00	61.44	750.00	2533.33	550.76	2500.00
Xikundu Weir	6	590.83	73.72	567.50	1666.60	280.48	1600.00
February 2008							
O. mossambicus							
Albasini Dam	5	260.60	12.50	260.00	292.00	20.49	290.00
Nandoni Dam	10	285.30	49.16	274.50	405.90	301.48	329.50
Xikundu Weir	10	318.90	29.91	315.00	521.00	137.96	535.00
C. gariepinus							
Albasini Dam	10	534.10	110.57	599.00	1389.00	803.28	1360.00
Nandoni Dam	4	776.25	40.55	768.50	2850.00	1078.58	2850.00
Xikundu Weir	6	601.67	68.39	607.50	1408.33	405.48	1475.00

n = Number of fish

Ave = Average

SD = Standard Deviation

3.5. Fixatives for histology and Immunohistochemistry (IHC)

As mentioned in chapter 1, in order to establish an optimal fixative for both histological and immunohistochemical analysis, three different fixatives were employed to test their adequacy and efficacy. Following normal histological practices, Bouin's Fluid (BF) (Table 3), 10% Neutrally Buffered Formalin (NBF) (Table 4), and the IHC fixative Paraformaldehyde (PFA) (Table 5), were used for fixation. BF is routinely used for histological analysis as it produces sections with great histological clarity and excellent cellular preservation. However, BF is a stringent fixative, and is not usually recommended for immunohistochemical analysis, as it tends to mask the antibody binding sites, thus yielding poorer results (81, 82). NBF yields poorer histological clarity than BF, but it is not as stringent a fixative as BF. Thus, NBF is also recommended for immunohistochemical analysis, as the antibody binding sites are not as masked as fixation in BF (82).

Paraformaldehyde (PFA), which contains no methanol, as in NBF, was used as the third fixative as it is recommended for IHC analysis (81). Methanol is implicated in the formation of strong bonds, which inhibits optimal antigen exposure post-fixation (69). All fixatives were prepared one-week prior to start of sample collection period.

3.5.1. Bouin's Fluid (BF)

Table 3: Standard operating procedures for BF preparation (81)

Chemical substance	Quantity (ml)
Saturated aqueous picric acid	750
Formalin (40% HCHO)	250
Glacial acetic acid	50

Testes were fixed in Bouin's Fluid for 24 hours at room temperature. After 24 hours, samples were washed in tap water for 1 hour, and then dehydrated stepwise in 30% ethanol for 1 hour, followed by 1 hour in 50% ethanol, and finally stored in 70% ethanol until further tissue processing.

3.5.2. 10 % Neutrally Buffered Formalin (NBF)

Table 4: Standard operating procedures for NBF preparation (81)

Chemical substance	Quantity
Sodium phosphate monobasic ($NaH_2PO_4.H_2O$)	4.0 g
Sodium phosphate dibasic (Na_2HPO_4)	6.5 g
Formalin (40% HCHO)	100 ml

The two buffer salts, sodium phosphate mono- and dibasic, are dissolved in 900 ml distilled water, to form solution A. Solution A's pH is adjusted to 7.3. The 100 ml 37-40% formaldehyde was added to A, prior to sample collection in the field (81). Testes were fixed for 24 hours in NBF at room temperature. After 24 hours, samples were washed in buffer (A) for 1 hour, then dehydrated stepwise in 30% ethanol for 1 hour, followed by 1 hour in 50% ethanol, and finally stored in 70% ethanol until tissue processing

3.5.3. 4% Paraformaldehyde (PFA)

Table 5: Standard operating procedures for PFA preparation (81)

Chemical substance	Quantity (g)
Sodium phosphate monobasic ($NaH_2PO_4.H_2O$)	4.0
Sodium phosphate dibasic (Na_2HPO_4)	6.5

The buffer salts were dissolved in distilled water to 1000 ml and heated to 60-70°C in fume hood. Once temperature was reached, 40 g paraformaldehyde powder was added and continuously stirred until solution was clear. The solution was cooled and thereafter filtered, and the pH was adjusted to 7.3 (81).

Testes were fixed for 24 hours in PFA at 4 °C. After 24 hours, the samples were washed in tap water for 1 hour, and then dehydrate stepwise in 30% ethanol for 1 hour, followed by 1 hour in 50% ethanol, and finally stored in 70% ethanol until tissue processing.

3.6. Histological assessment

Histology is the study of the structure of a given cell in order to understand the composition of the cell, and therefore be able to understand the processes that occur within the cell. In order to obtain a point of reference, a histological assessment was conducted on the testes of both the testes of *O. mossambicus* and *C. gariepinus.* In order to visualize the different cells that constitute the testes and to aid in identifying abnormalities, the haematoxylin and eosin technique was employed.

3.6.1. Haematoxylin and Eosin (H&E) staining technique

Haematoxylin is a chemical base, which stains acids, thus it binds to the DNA within the cell, and results in staining the nucleus blue. Eosin, which is an acidic substance, binds to bases, and thus imparting a pink staining to the protein-rich cytoplasm (81). The Department of Pathology at the Onderstepoort Veterinary Institute embedded blocks of *O. mossambicus* and *C. gariepinus* testes in paraffin wax, made sections of 4µm thick which were subsequently collected on Superfrost slides (Menzel-Glaser, Germany). The slides were then stained with H&E for histological assessment, using light microscopy. Using a Nikon BH-2 microscope fitted with a CC-2 digital camera, which is coupled to a computer with image analyzing software, the histological sections were analyzed at a magnification of 40X. Based on the visualization of the various cells, it was possible to identify the gonadal reproductive stage of each specimen.

3.6.2. Gonadal reproductive stages

The present investigation utilized the classification system used by Pieterse (2004) (83), which is based on the Goodbred classification system (84). This classification system recognizes four developmental stages based on a gonadal histopathological study into the effects of endocrine disruption in the male common carp (Table 6).

Table 6: Histological characteristics of the testes (83)

Stage	Characteristics of testis
0	*Immature Phase* Sparse or no spermatogenic activity in the interstitial tissue; spermatocytes dominate; no spermatozoa present
1	*Early spermatogenic Phase* Predominantly thin interstitial tissue with scattered spermatogenic activity; spermatocytes to spermatids dominate; few spermatozoa are observed
2	*Mid-spermatogenic Phase* Moderate thickness of interstitial tissue; moderate spermatozoa proliferation, maturation and equal mix of spermatocytes; spermatids and spermatozoa present
3	*Late spermatogenic Phase* Thick interstitial tissue; scattered spermatozoa proliferation and maturation regions; all stages of development observed; spermatozoa dominate

3.7. Immunohistochemical Assessment

3.7.1. Tissue preparation

The Department of Pathology at the Onderstepoort Veterinary Institute embedded testes of *O. mossambicus* and *C. gariepinus* testes in paraffin wax blocks, and prepared all the slides for histological and IHC analysis.

Sections of 4µm thick were floated onto a distilled water bath, (45 °C), and collected on Superfrost slides (Menzel-Glaser, Germany). Each slide was comprised of three tissue sections, representing the three fixatives, obtained from the same specimen, as indicated in Fig. 10. The slides were then prepared for two IHC assays, the Caspase assay and the TUNEL assay.

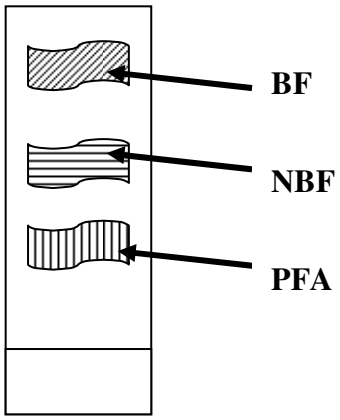


Fig. 10: Layout of testicular section on slides for histological and IHC processing. (BF=Bouin's Fluid, NBF=Neutrally Buffered Formalin, PFA=Paraformaldehyde).

3.7.2. Caspase Immunohistochemistry

The caspase assay was conducted on *O. mossambicus* and *C. gariepinus* as follows: Slides were deparaffinized with Xylene and then rehydrated through two washes in 100% EtOH and 95% EtOH in distilled water, each, for 10 min per wash. Epitopes masked by fixation were revealed by an antigen retrieval procedure. This involved heating the slides, immersed in an Antigen Unmasking Solution (Vectorlabs, Burlingame, CA, USA), in a domestic microwave at 100% power until boiling, and then at 30% power for 10 min. The slides were then cooled for 30 min at room temperature (RT).

Endogenous peroxidases were then quenched with 0.3% hydrogen peroxide in distilled water in darkness for 10 min. Following a rinse in distilled water, the slides were placed in 0.1% (v/v) Tween 20 (Sigma) in phosphate buffered saline (PBS-A: 137 mM NaCl, 29 mM $NaH_2PO_4 \cdot H_2O$, 9 mM Na_2HPO_4, pH 7.4) for 5 min. Slides were then incubated with blocking solution (5% normal goat serum in 0.1% Tween 20-PBS-A) for 1 hour at RT. Slides were then incubated (4 °C) overnight with the cleaved caspase-3 antibody (Asp175) (Cell Signaling Technology, Beverly, MA, USA) at 1:200 dilution at 4 °C.

The following day the slides were washed in three washes of 0.1% Tween 20/PBS-A for 5 min each. The secondary antibody (biotinylated antirabbit IgG) was added to each of the slides, ensuring that the entire section is covered, and incubated at RT for 30 min. Following three PBS-A washes for 5 min each, the slides were incubated with the

Vectastain avidin-biotin-complex (Vectorlabs, Burlingame, CA, USA) for a further 30 min at RT, after which slides were washed in three washes of PBS-A for 5 min each.

Immunoreactions were detected by incubating the slides with 3, 3´-diaminobenzidine (DAB) (Vectorlabs, Burlingame, CA, USA) solution prepared according to the suppliers instructions, for 1-2 min in darkness. After the DAB reaction, slides were rinsed in glass-distilled water for 5 min. Counterstained was done with Hematoxylin QS (Vectorlabs, Burlingame, CA, USA), a modification of Mayer's Hematoxylin, specifically developed for immunohistochemistry, with less than 45 seconds staining time.

After staining, slides were finally rinsed in running tap water until water was colourless. Slides were dehydrated in 3 washes of 100% butanol, cleared in 3 washes of 100% Xylene, and mounted with Entellan (Merck, Darmstadt, Germany).

3.7.3. Terminal deoxynecleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) Immunohistochemistry

The TUNEL assay was conducted on *O. mossambicus* and *C. gariepinus* as follows:
Slides were deparaffinized with Xylene and then rehydrated through two washes in 100% EtOH in distilled water for 3 min each, one wash of 95% EtOH in distilled water for 5 minutes and one wash in 75% EtOH for 5 min. Permeabilization of the tissue was achieved by incubation of the slides in 0.5% Triton X-100 for 10 min at RT.

Endogenous peroxidases were then quenched with 0.3% hydrogen peroxide in distilled water in darkness for 10 min. Quenching was followed by two washes in phosphate buffered saline (PBS-B: 50mM sodium phosphate, pH 7.4; 200mM NaCl). Subsequent steps for TUNEL staining were carried out using the ApopTag-Peroxidase Kit according to the supplier's instructions (Chemicon, Temecula, CA). DNA fragmentation was detected by incubating the slides with the TdT-mediated dUTP nick end-labelling reaction mixture (71) in a humidified chamber at 37 °C for 60 min. Following incubation, the slides were treated with the antidigoxigenin-peroxidase complex for 30 min at RT, and washed in three PBS-B washes for 5 minutes each.

Immunoreactions were detected by incubating the slides with 3, 3´-diaminobenzidine (DAB) (Vectorlabs, Burlingame, CA, USA) solution prepared according to the suppliers instructions, for 1-2 min in darkness. After the DAB reaction, slides were rinsed in glass-distilled water for 5 minutes. Counterstained was done with Hematoxylin QS (Vectorlabs, Burlingame, CA, USA), a modification of Mayer's Hematoxylin, specifically developed for immunohistochemistry, with less than 45 seconds staining time.

After staining, slides were finally rinsed in running tap water until water was colourless. Slides were dehydrated in 3 washes of 100% butanol, cleared in 3 washes of 100% Xylene, and mounted with Entellan (Merck, Darmstadt, Germany).

3.7.4. Microscopic quantification

To quantify the number of apoptotic cells labeled during the IHC process, each entire tissue section was evaluated and all the lobules in a given section was counted and scored as caspase-3-positve or caspase-3- negative or TUNEL-positive or TUNEL-negative. A positive cell is stained brown after the reaction with DAB. A lobule was designated as positive if it contained at least one cluster of three positively stained cells at a 20x magnification. The number of positive lobules was expressed as a percentage.

In each of the caspase-3-positve or TUNEL-positive lobules, the lobule was scored for the number of caspase-3-positive or TUNEL-positive cells within that lobule. In addition the type of germ cell displaying positivity was indicated, i.e. spermatocytes, spermatids and spermatozoa.

3.8. Analytical chemical analysis

Fat samples from each fish specimen and water samples from each sampling site were collected to determine the levels of *o,p'*- and *p,p'*-DDT, -DDD and –DDE.

3.8.1. Water collection procedure

Water samples were collected in October 2007 and in February 2008 at a depth of 5-15 cm under the water surface, in glass bottles pre-washed with methanol. These samples were stored at 4˚C until further analysis was conducted. At each of the three sampling sites, pH, electrical conductivity (EC) (µS/cm), Total Dissolved Salts (TDS) (ppm), water temperature (°C) and the oxygen percentage (%) was measured during both season 1 and season 2. These parameters were measured using the PH Scan 2 pH meter (Eutech Instruments), the Cyberscan CON 110 conductivity/TDS/°C meter RS 232 (Eutech Instruments) and the Cyberscan DO 100 meter (for dissolved oxygen).

3.8.1.1. Water analysis

The water samples were filtered through a glass fibre filter before analysis ensued. Using standard methods of gas chromatography – mass spectophotometry (GC/MS) 003 (85), the samples were analysed at the FDA laboratories, Pretoria, South Africa. OCs were extracted from the water samples using solid phase extraction. Using a C18 cartridge, a clean up was performed, and then the analytes were eluted with Hexane-diethyl ether. Quantification of results was determined using a fortified calibration curve. The OCs were detected at a level of detection (LOD) of 0.5 µg/l, using a gas chromatography coupled to a mass spectrometry detector (GC/MS).

3.8.2. Sediment collection procedure

Sediment samples were collected in October 2007 and in February 2008 from the banks of the sampling site, at a level below the water surface in pre-washed methanol glass bottles, which were stored at 4 °C until further analysis.

3.8.2.1. Sediment analysis

All sediment samples were dried in an oven at 110 °C, eluted with Hexane-petroleum ether and extraction via reflux was conducted at the FDA Laboratories, Pretoria, South Africa. Samples were allowed to cool and dry under vacuum at 35 °C. Using a C18 cartridge, a clean up was performed and then the analytes were eluted with Hexane-diethyl ether. The OCs were detected at a LOD of 0.5 µg/kg, using a GC/MS detector.

3.8.3. Tissue preparation for chemical analysis

When available, fat was collected for each specimen. The fat was wrapped in foil and stored at -20 °C until processing for target chemical analysis in the laboratory (73).

3.8.3.1. Tissue analysis

Using standard methods of GC/MS 008 (86), fish fat samples were analysed at the FDA Laboratories, Pretoria, South Africa. The OCs were extracted and the clean up was first conducted on a C18 cartridge, and then followed by florisil solid phase extraction (SPE). The analytes were eluted using petroleum ether-diethyl ether. The OCs were detected at a LOD of 0.010 mg/kg, using a GC/MS detector.

3.9. Statistical analysis

Statistical analysis was conducted to compare results between the three sampling sites in order to:

- Assess if the two assays yield similar results, with regard to a representation of apoptotic cellular death.

- Assess which of the three fixatives yields a superior immunoexpression, whilst retaining tissue structure and clarity.

A chi-squared (Goodness of Fit) Test was used to enable the comparison of the

distribution between the samples from each study site, as well as to test the associations between the variables, such as fixatives, seasons, assays, and dams. The differences in selected water and tissue samples were calculated using the Microsoft software package, Excel.

CHAPTER 4: RESULTS

4.1. Water quality assessment

The water quality parameters recorded during both seasons, are represented in Table 7. The measured pH values from AD and ND were above the suggested upper value, a pH of 8, for South African inland waters (87). The EC ranged from 139.2 µS/cm in season 2 to 825 µS/cm in season 1. Similarly, the TDS ranged from 69.50 ppm in season 2 to 413 ppm in season 1. The water temperature ranged from 18 °C to 24.7 °C during season 1 and from 28 °C to 29.5 °C in season 2. The percentage oxygen saturation ranged from 113.7% to 136.1% spanning the two seasons.

Table 7: Physical water quality parameters measured at the three sampling sites

	Albasini Dam		**Nandoni Dam**		**Xikundu Weir**		**DWAF**
Season	*1LF*	*2 HF*	*1LF*	*2 HF*	*1LF*	*2 HF*	
pH	8.48	8.61	8.93	8.28	7.3	6.96	6.0 – 8.0
Electrical conductivity (EC) (µS/cm)	825	305	424	150.5	182.3	139.2	**
Total Dissolved Salts (TDS) (ppm)	413	153	214	75.2	-	69.50	**
Temperature (°C)	24.7	28	22.4	29.5	18	28.9	5 - 30
Oxygen (mg/L)	8.74	10	9.81	10.38	12	9.27	> 4*
Oxygen concentration (%)	114.9	127.3	113.7	136.1	135.2	123.3	> 40 % *

1 LF = Season 1 Low Flow

2 HF = Season 2 High Flow

* *Lethal below this value*

** *Refer to DWAF (1996) for guidelines* (87)

4.2. Target chemical analysis

4.2.1. Chemical analysis of collected water samples

The pesticide DDT and its metabolites were measured in the collected water from the AD, the ND, and the XW. The results of the chemical target analysis for both seasons are presented in Table 8 as mean values (µg/l). While all the concentrations of DDT and its isomers were all below 0.10 µg/l in season 1 in all three sites, there is a general increase in DDT and its isomers in the second season. In season 2 *o,p´*-DDE has a value of 1.2 µg/l at the XW and *p,p´*-DDE which has a value of 0.6 µg/l at both AD and XW. It would thus appear that XW is the site that has the greatest levels of the persistent DDT metabolite DDE in the water.

Table 8: DDT and its metabolites in water samples collected at the three sites (mean)

	Albasini Dam		**Nandoni Dam**		**Xikundu Weir**	
Season	**Oct 07**	**Feb 08**	**Oct 07**	**Feb 08**	**Oct 07**	**Feb 08**
Target EDC (µg/l)						
o,p´-DDT	<0.10	<0.50	<0.10	<0.50	<0.10	<0.50
p,p´-DDT	<0.10	<0.50	<0.10	<0.50	<0.10	<0.50
o,p´-DDD	<0.10	<0.50	<0.10	<0.50	<0.10	<0.50
p,p´-DDD	<0.10	<0.50	<0.10	<0.50	<0.10	<0.50
o,p´-DDE	<0.10	<0.50	<0.10	<0.50	<0.10	1.20
p,p´-DDE	<0.10	0.60	<0.10	<0.50	<0.10	0.60

4.2.2. Chemical analysis of collected sediment samples

The pesticide DDT and its metabolites were measured in the collected sediment from the AD, the ND and the XW. The results of the chemical target analysis for both seasons are represented in Table 9 as mean values (µg/kg). While all the concentrations of DDT and its isomers were all below 0.10 µg/kg in season 1 in all three sites, there is a general increase in DDT and its isomers in the second season. In season 2 *p,p´*-DDT has a value of 1.00 µg/kg at ND and 4.00 µg/kg at the XW, which indicates recent exposure to DDT. In the ND *p,p´*-DDE has a value of 4.00 µg/kg in season 2, while at the XW *o,p´*-DDE has a value of 1.00 µg/kg and *p,p´*-DDE a value of 13.00 µg/kg. It would thus appear that XW is the site that has the greatest levels of the persistent DDT metabolite DDE in the sediment.

Table 9: DDT and its metabolites in sediment samples collected at the three sites (mean)

	Albasini Dam		**Nandoni Dam**		**Xikundu Weir**	
Season	**Oct 07**	**Feb 08**	**Oct 07**	**Feb 08**	**Oct 07**	**Feb 08**
Target EDC (µg/kg)						
o,p´-DDT	<0.10	<0.50	<0.10	<0.50	<0.10	<0.50
p,p´-DDT	<0.10	<0.50	<0.10	1.00	<0.10	4.00
o,p´-DDD	<0.10	<0.50	<0.10	<0.50	<0.10	<0.50
p,p´-DDD	<0.10	<0.50	<0.10	<0.50	<0.10	<0.50
o,p´-DDE	<0.10	<0.50	<0.10	<0.50	<0.10	1.00
p,p´-DDE	<0.10	<0.50	<0.10	4.00	<0.10	13.0

4.2.3. Chemical analysis of fat tissue

The pesticide DDT and its metabolites were measured in the fat of *O. mossambicus* (Table 10) are represented as mean (µg/kg) ± SD. While there was no fat available in the specimens from season 1, the concentrations of DDT and its isomers were below the value of 0.50 µg/kg in season 2 at AD, except for *p,p´*-DDE which had a value of 350.0 µg/kg. Interestingly, in season 2 *o,p´*-DDE has the lowest values in both *O. mossambicus* from the ND (84.00 µg/kg) and XW (340.00 µg/kg), with *p,p´*-DDD having the highest values at ND (4300.00 µg/kg) and the XW 13000.00 µg/kg). The high levels of both isomers of DDD and DDE indicate that greatest levels DDT isomers occur in *O. mossambicus* from ND and XW, with the *O. mossambicus* at the XW being the most affected.

Table 10: DDT and its metabolites in *O. mossambicus* fat tissue (mean ± SD)

	Albasini Dam		**Nandoni Dam**		**Xikundu Weir**	
Season	**Oct 07**	**Feb 08**	**Oct 07**	**Feb 08**	**Oct 07**	**Feb 08**
Specimen sample size	**(0 of n=9)**	**(1 of n=5)**	**(0 of n=0)**	**(10 of n=10)**	**(0 of n=10)**	**(9 of n=10)**
Target EDC (µg/kg)						
o,p´-DDT	*	< 50	*	84.00 ± 16.00	*	340.00 ± 150.00
p,p´-DDT	*	< 50	*	445.00 ± 440.00	*	7000.00 ± 3300.00
o,p´-DDD	*	< 50	*	98.00 ± 54.00	*	190.00 ± 41.00
p,p´-DDD	*	352.00	*	4300.00 ± 5200.00	*	13000.00 ± 4100.00
o,p´-DDE	*	< 50	*	130.00 ± 85.00	*	270.00 ± 64.00
p,p´-DDE	*	< 50	*	1400.00 ± 1200.00	*	6300.00 ± 1700.00

* No fat available in collected fish

The pesticide DDT and its metabolites were measured in the fat of *C. gariepinus* (Table 11) are represented as mean (µg/kg) ± SD. An interesting pattern is observed in the concentrations of DDT and its isomers in the fat tissue of *C. gariepinus.* In season 1, the concentrations of *o,p´*-DDT *p,p´*-DDT and *o,p´*-DDD are much higher than the concentrations measured in season 2. In season 2 *p,p´*-DDD (35000.00 µg/kg) and *o,p´*-DDE (560.00 µg/kg) have much higher values as compared with season 1 (8000.00 µg/kg and 88.00 µg/kg, respectively in XW). However the concentrations of *p,p´*-DDE (37000.00 µg/kg season 1 and 8200.00 µg/kg season 2 in XW), shows a similar pattern to that of of *o,p´*-DDT *p,p´*-DDT and *o,p´*-DDD, in that the highest values were measured during season 1. The high levels of DDT measured in season 1 coincide with the application of DDT in the surrounding areas during the sampling period. However the presence of both isomers of DDD and DDE indicates that historic DDT exposure also occurs in *C. gariepinus* as is seen in *O. mossambicus* from ND and XW, with both species at the XW being the most affected.

Table 11: DDT and its metabolites in *C. gariepinus* fat tissue in (mean ± SD)

	Albasini Dam		**Nandoni Dam**		**Xikundu Weir**	
Season	**Oct 07**	**Feb 08**	**Oct 07**	**Feb 08**	**Oct 07**	**Feb 08**
Specimen sample size	**(5 of n=10)**	**(2 of n=10)**	**(3 of n=3)**	**(2 of n=4)**	**(3 of n=6)**	**(6 of n=6)**
Target EDC (µg/kg)						
o,p´-DDT	95.00 ± 16.00	62.00 ± 5.00	200.00 ± 42.00	77.00 ± 7.00	2300.00 ± 180.00	420.00 ± 260.00
p,p´-DDT	210.00 ± 100.00	75.00 ± 9.00	1400.00 ± 610.00	260.00 ± 39.00	18000.00 ± 1400.00	4500.00 ± 4300.00
o,p´-DDD	< 50	< 50	170.00 ± 43.00	100.00 ± 5.00	540.00 ± 190.00	280.00 ± 150.00
p,p´-DDD	290.00 ± 100.00	2400.00 ± 590.00	2200.00 ± 830.00	9400.00 ± 1600.00	8100.00 ± 960.00	35000.00 ± 27000.00
o,p´-DDE	< 50	< 50	< 50	93.00 ± 10.00	88.00 ± 31.00	570.00 ± 320.00
p,p´-DDE	2500.00 ± 2100.00	310.00 ± 100.00	9300.00 ± 5100.	2200.00 ± 210.00	37000.00 ± 3700.00	8200.00 ± 3900.00

4.3. Histological assessment

The utility of three different fixatives was employed to determine the extent to which each could be used to give the optimal histological representation of the testicular section. Optimal fixation would make it possible to identify any structural or cellular abnormalities in the tissue sections. Through the assessment of all the tissue sections, no structural abnormalities were observed.

The three different fixatives yielded varying results. In both season 1 and season 2, BF proved to produce the optimal clarity, in both *O. mossambicus* and *C. gariepinus,* making it easier to identify the cells. In comparison, the fixative NBF did not yield a great clarity as compared to BF, and the identification of cells became difficult in some instances. The fixative PFA, however, yielded better clarity and cellular identification than NBF, but still yields a weaker clarity than BF.

The following photomicrographs represent the haematoxylin-and-eosin staining of the testicular sections fixed in the three different fixatives. Each set of photomicrographs represent both *O. mossambicus* and *C. gariepinus* tissue sections, fixed in, BF, NBF or PFA. On each photomicrograph, the different cellular stages of spermatogenesis will be indicated, as mentioned in chapter 2 (see section 2.2).

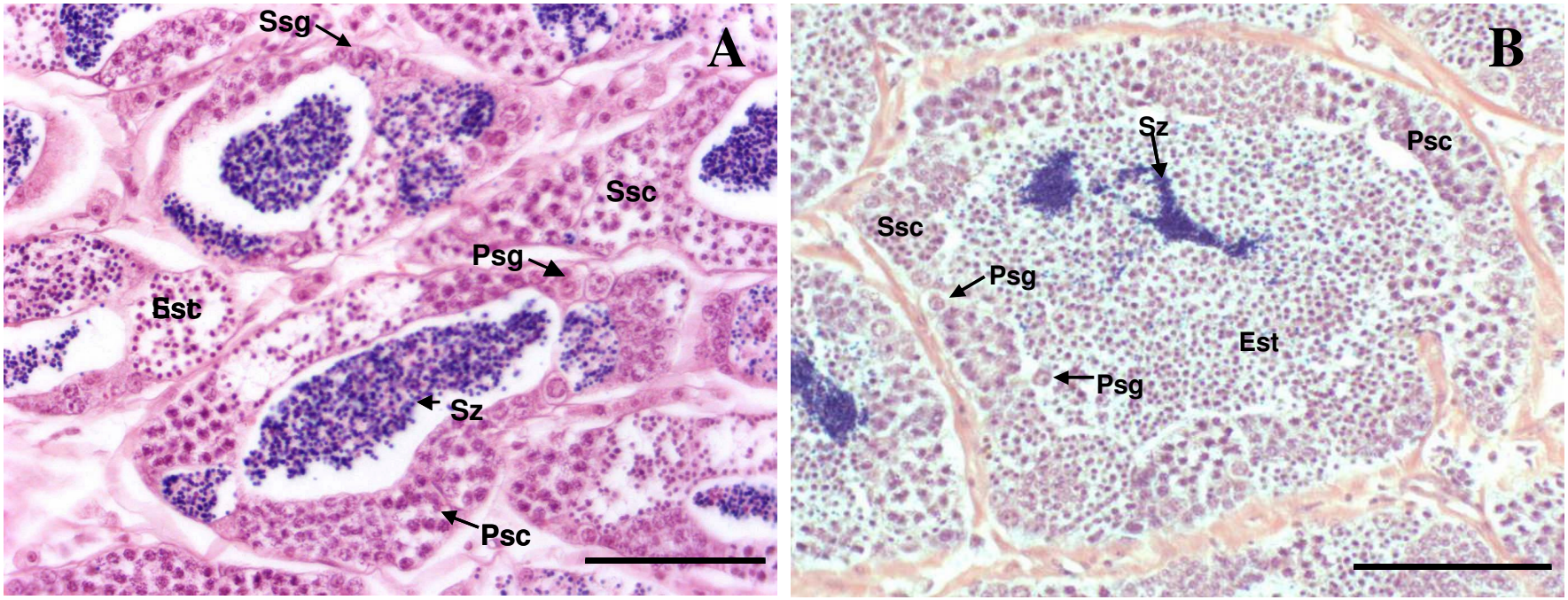


Fig. 11: Photomicrographs of haematoxylin and eosin-stained testicular sections of *O. mossambicus* (A) and *C. gariepinus* (B) fixed in BF. Psg: primary spermatogonia; Ssg: secondary spermatogonia; Psc: primary spermatocytes; Ssc: secondary spermatocytes; Est: Early spermatids; Sz: spermatozoa. Magnification 40 X. Scale bar = 20µm.

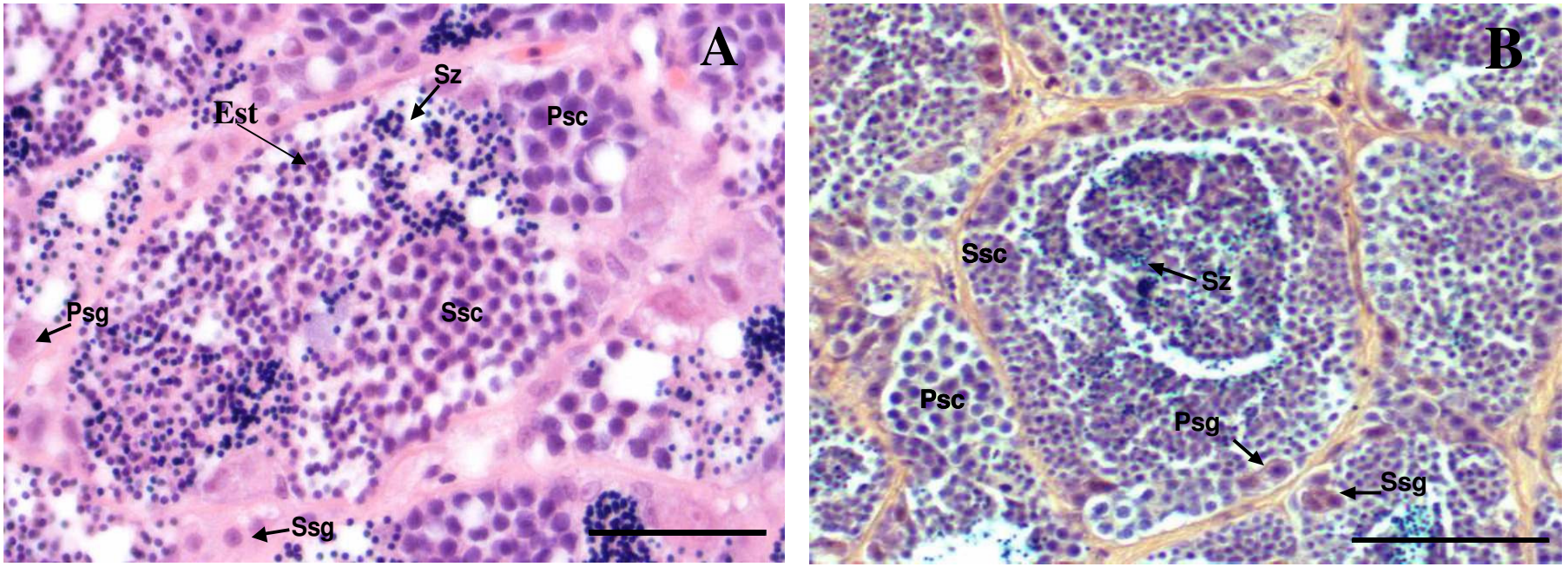


Fig. 12: Photomicrographs of haematoxylin and eosin-stained testicular sections of *O. mossambicus* (A) and *C. gariepinus* (B) fixed in NBF. Psg: primary spermatogonia; Ssg: secondary spermatogonia; Psc: primary spermatocytes; Ssc: secondary spermatocytes; Sz: spermatozoa. Magnification 40 X. Scale bar = 20µm.

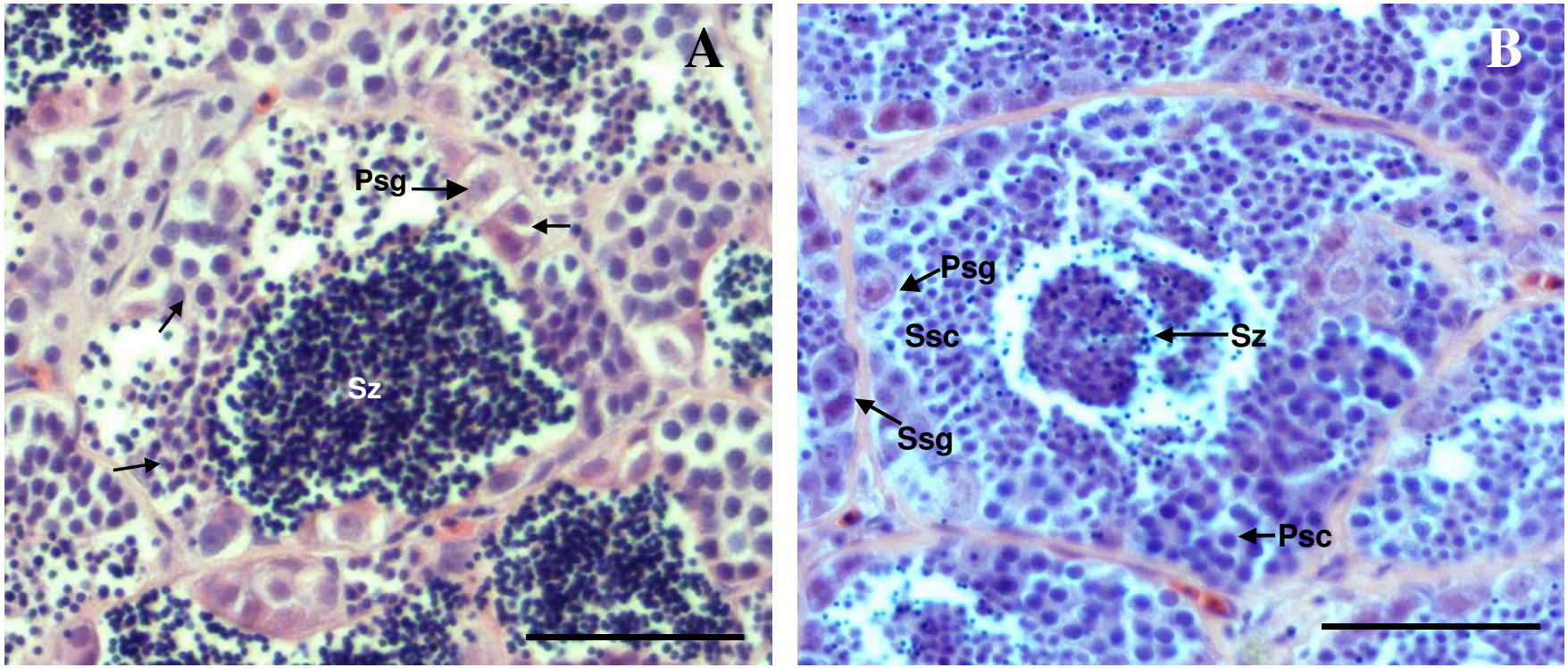


Fig. 13: Photomicrographs of haematoxylin and eosin-stained testicular sections of *O. mossambicus* (A) and *C. gariepinus* (B) fixed in PFA. Psg: primary spermatogonia; Ssg: secondary spermatogonia; Psc: primary spermatocytes; Ssc: secondary spermatocytes; Sz: spermatozoa. Magnification 40 X. Scale bar = 20µm.

4.3.1. The Gonadosomatic Index

The GSI, when compared between season 1 and season 2, differs marginally, with the GSI from season 2 being lower than that of season 1, in both *O. mossambicus* (fig 14) *and C. gariepinus* (fig 15).

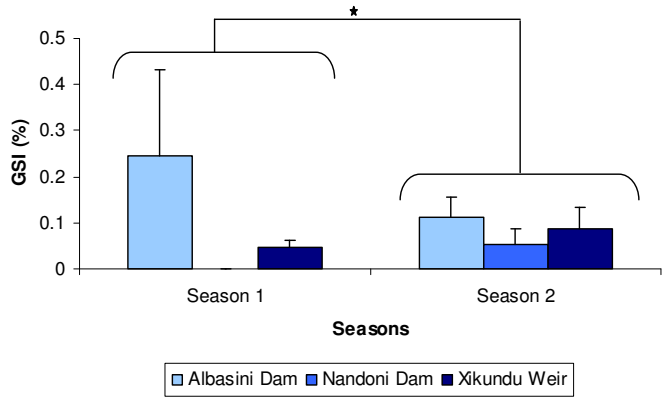


Fig. 14: Comparison of GSI in season 1 and 2 in *O. mossambicus.* Significant (* $p<0.05$) differences observed between season 1 and season 2.

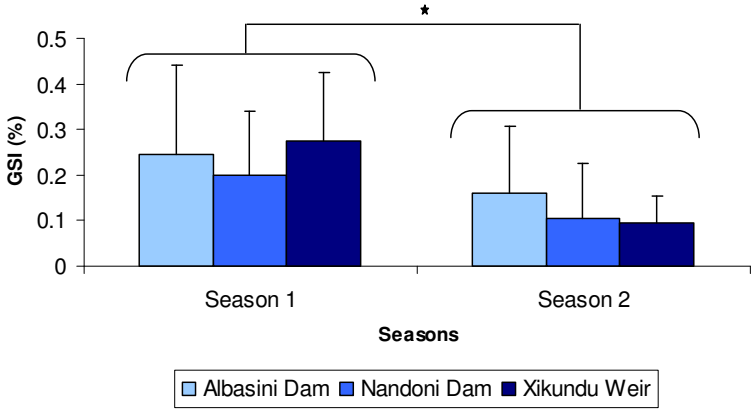


Fig. 15: Comparison of GSI in season 1 and 2 in *C. gariepinus.* Significant (* $p<0.05$) differences observed between season 1 and season 2.

Statistically ($p<0.05$) the seasons differ in both the GSI and caspase-3 and TUNEL positive cells. When taking into consideration that GSI is an indicator of gonadal growth, the developmental stage of the collected fish is relevant. During season 1, the majority of the collected specimens, were in the developed stage, with a few being in the developing

stage. In contrast, in season 2, the majority of the collected specimens were in mature stage (mid-spermatogenic) of development. Thus, being reproductively active the amount of sperm will decrease, thus possibly affecting gonadal weight, and hence GSI. The decrease in the GSI in season 2 may therefore possibly attributed to normal reproductive cycles, rather than be caused by an influence from various external factors.

Table12: The developmental stages in the testes of both *O. mossambicus* and *C. gariepinus* from season 1 and 2

	Developmental stage		
	Early spermatogenic	Mid-spermatogenic	Late Spermatogenic
Season 1			
Albasini Dam			
O. mossambicus	0	11	0
C. gariepinus	0	3	7
Nandoni Dam			
O. mossambicus	0	0	0
C. gariepinus		3	0
Xikundu Weir			
O. mossambicus	1	8	1
C. gariepinus	0	0	6
Season 2			
Albasini Dam			
O. mossambicus	2	3	0
C. gariepinus	5	5	0
Nandoni Dam			
O. mossambicus	1	9	0
C. gariepinus	4	1	0
Xikundu Weir			
O. mossambicus	0	10	0
C. gariepinus	3	1	0

4.4. Immunohistochemical assessment

The extent of apoptotic death is characterized by the amount of positive cells that are stained brown, as a result of DAB (see sections 3.7.2 and 3.7.3). In order to visualize, and thus being able to optimally quantify the extent of cellular apoptotic death, three fixatives, were employed (see section 3.5). The following photomicrographs serve as an indication of the extent to which the type of fixative was able to stain the apoptotic cells, thus enabling quantifying.

Each set of photomicrographs represent either the caspase-3 or the TUNEL assay, for each one of the three sites investigated. In addition, the amount of positive cells that each fixative yielded will be represented. At the end of the section, please find Tables 12 and 13 (pg 59-61) presenting the average amount of cells found per assay, per species, in each one of the three sites that were investigated. For a complete table of the quantification of all the positive cells in the entire tissue section, please consult the appendix (Tables 1-22, pg 85-96).

4.4.1. The Caspase-3 Assay – Comparing the efficacy of fixation of *O. mossambicus* tissue sections, in the three sites, the Albasini Dam, the Nandoni Dam and the Xikundu Weir, during both season 1 and season 2.

a) Albasini Dam

In the AD, during season 1, the greatest number of positive cells is yielded by BF (n=2673), compared to NBF (n=1790) and PFA (n=934). In season 2, a similar pattern is observed with the yielding greatest amount of positive cells yielded by BF (n=65), as compared to PFA (n=12). There is a greater amount of positive cells in season 1, as compared to season 2.

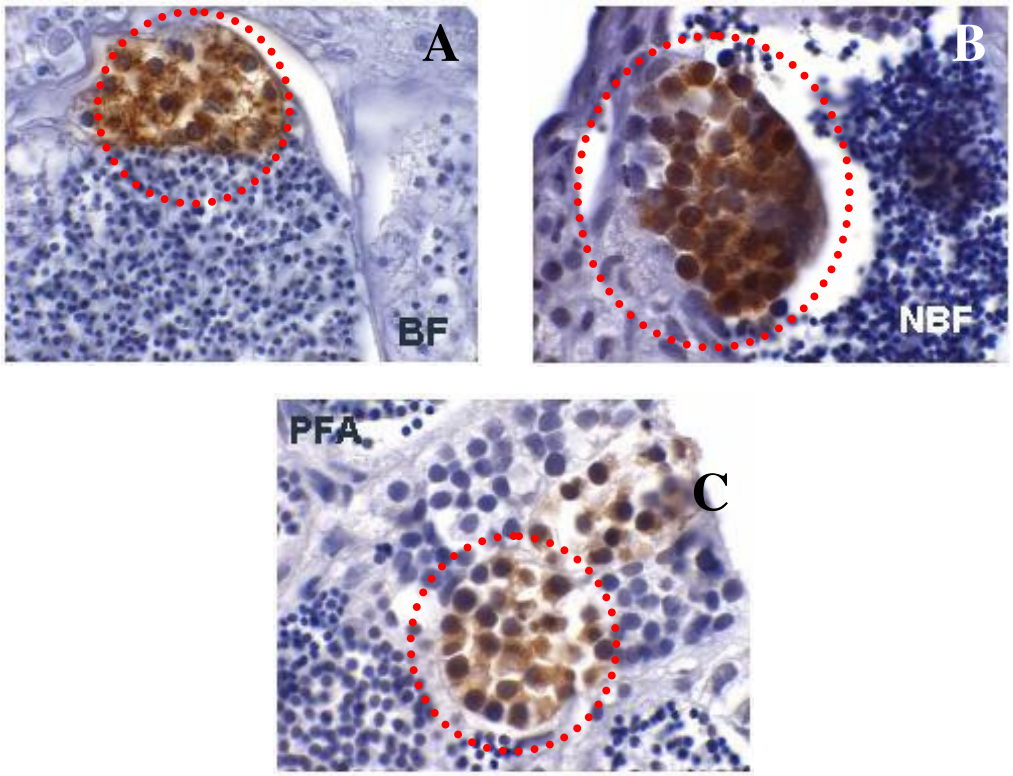
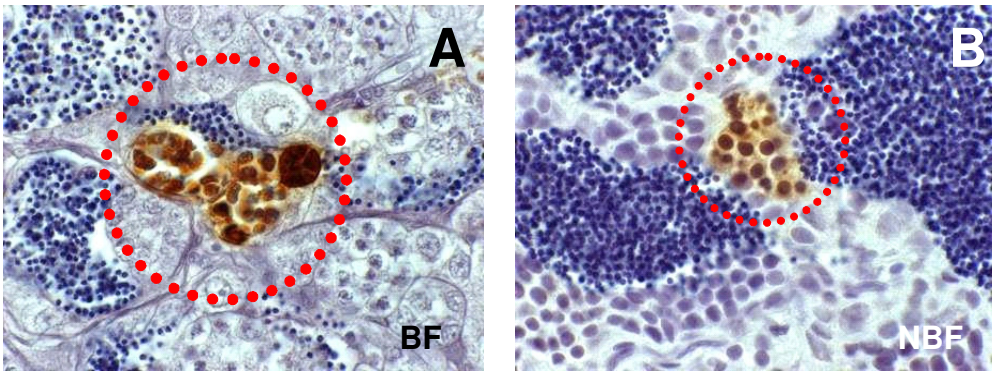


Fig. 16: Caspase-3 immunoreactivity in the testes of *O. mossambicus* from the AD. Note the cluster formation indicated by the red dashed circle.

b) Nandoni Dam

In the ND, during season 1, no *O. mossambicus* was sampled, despite numerous efforts. In season 2, similar to the situation in AD, the greatest number of positive cells is yielded by BF (n=2673) as compared to NBF (n=277), and PFA (n=254). It is noteworthy that the same cluster formation that was observed at AD is also present in ND.



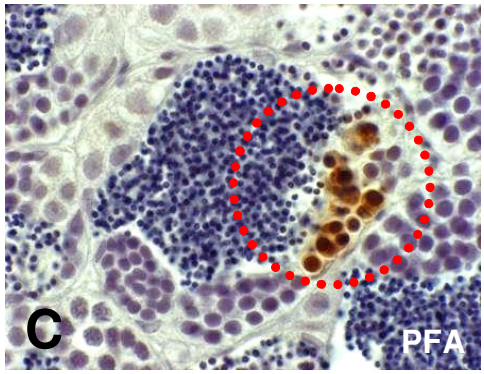
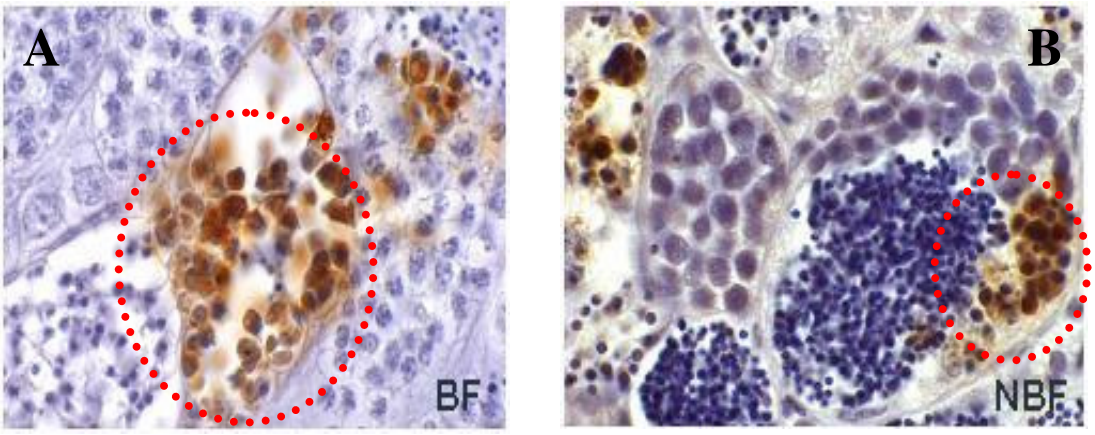


Fig. 17: Caspase-3 immunoreactivity in the testes of *O. mossambicus* from the ND (2HF). Note the cluster formation indicated by the red dashed circle.

c) Xikundu Weir

During season 1, in XW, the greatest amount of positive cells was yielded by BF (n=6745), compared to PFA (n=5245), and NBF (n=3643). In season 2 the greatest amount of positive cells was yielded by BF (n=161), followed by NBF (n=39), but PFA having no positively stained caspase-3 cells. The caspase-3 immunoreactivity was present in a cluster formation, as observed in the AD and ND.



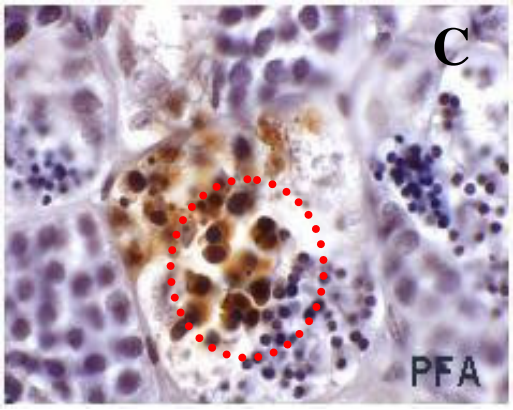
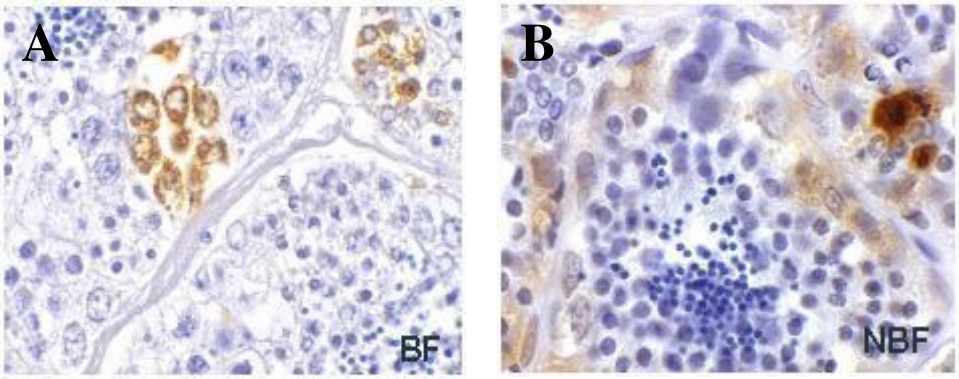


Fig. 18: Caspase-3 immunoreactivity in the testes of *O. mossambicus* from the XW. Note the cluster formation depicted by red dashed circle.

4.4.2. The Caspase-3 Assay – Comparing the efficacy of fixation of *C. gariepinus* tissue sections, in the three sites, during both season 1 and season 2.

a) Albasini Dam

In the AD, during season 1, the greatest number of positive cells was yielded by NBF (n=108) compared to PFA (n=92), and BF (n=23). In season 2, a similar pattern is observed with greatest amount positive cells yielded by NBF (n=392) as compared to PFA (n=387) and BF (n=118). There is a greater amount of positive cells in season 2, as compared to season 1.



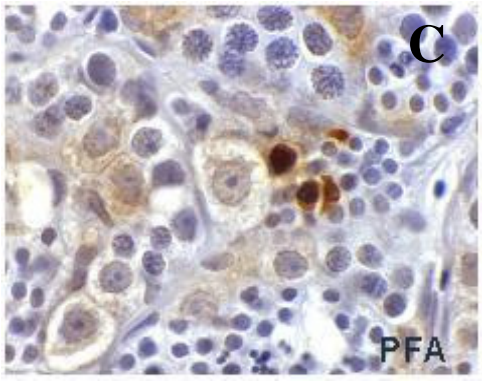


Fig. 19: Caspase-3 immunoreactivity in the testes of *C. gariepinus* from the AD.

b) Nandoni Dam

In the ND, during season 1, the greatest number of positive cells was yielded by PFA (n=1926), compared to NBF (n=453), and BF (n=33). In season 2 only NBF (n=20) yielded positive cells, with BF and PFA having no positively stained caspase-3 cells.

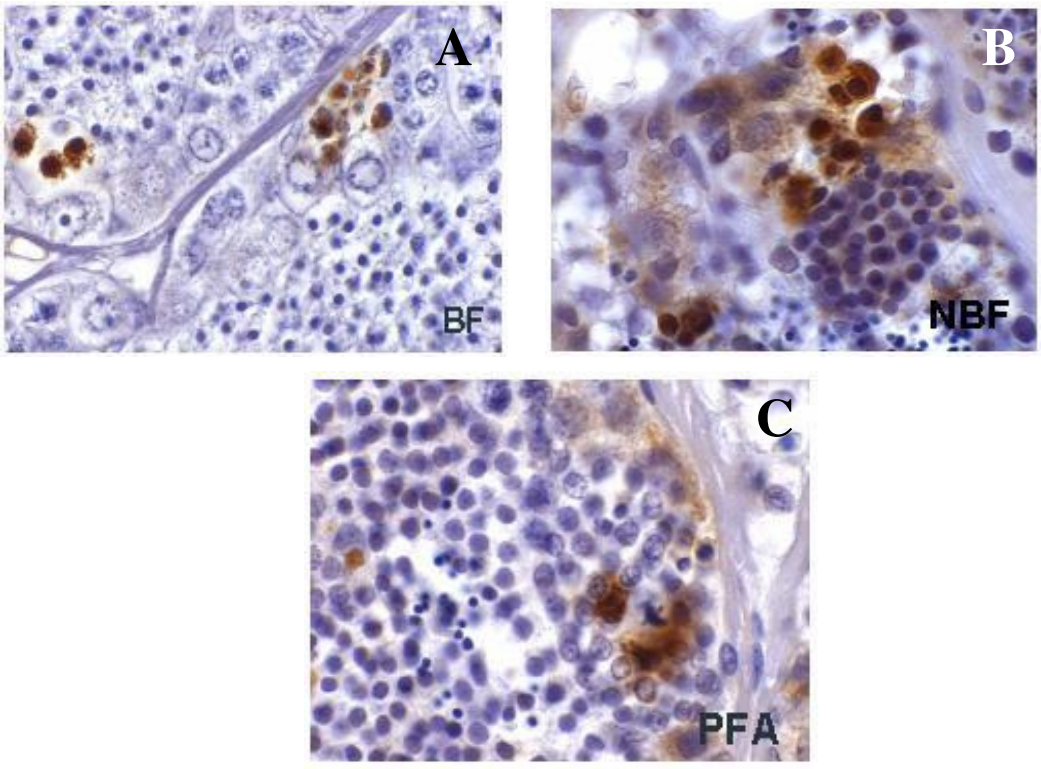


Fig. 20: Caspase-3 immunoreactivity in the testes of *C. gariepinus* from the ND.

c) Xikundu Weir

During season 1, the low flow season, the greatest amount of positive cells was yielded by NBF (n=733), compared to PFA (n=685), and BF (n=31). In season 2, the high flow season, only NBF (n=39) yielded positive cells, with BF and PFA having no positively stained caspase-3 cells.

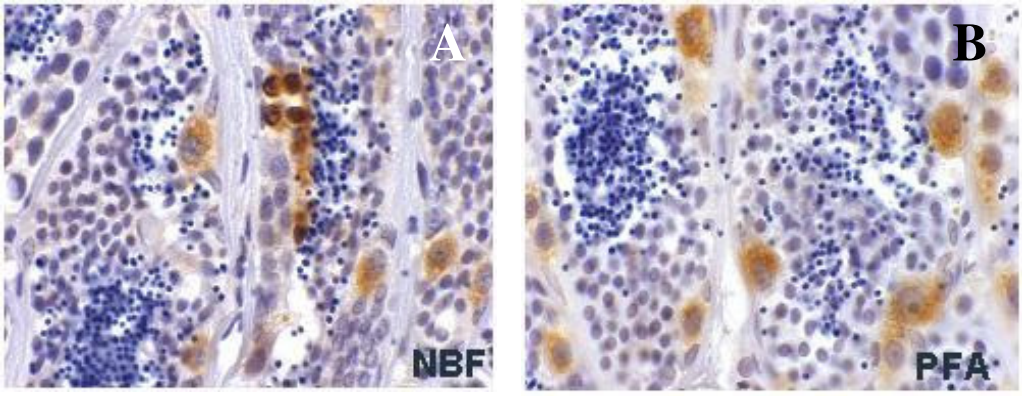


Fig. 21: Caspase-3 immunoreactivity in the testes of *C. gariepinus* from the XW.

4.4.3. The TUNEL Assay – Comparing the efficacy of fixation of *O. mossambicus* tissue sections, in the three sites, during both season 1 and season 2.

a) Albasini Dam

During season 1, the greatest amount of positive cells was yielded by BF (n=15712) compared to PFA (n=3519) and NBF (n=2903). This was the site with the greatest number of positive cells observed in season 1. In season 2, the greatest amount of positive cells was yielded by NBF (n=45), followed by PFA (n=28) and BF yielding no positive cells. The cluster formation is again observed, as in the caspase-3 assay.

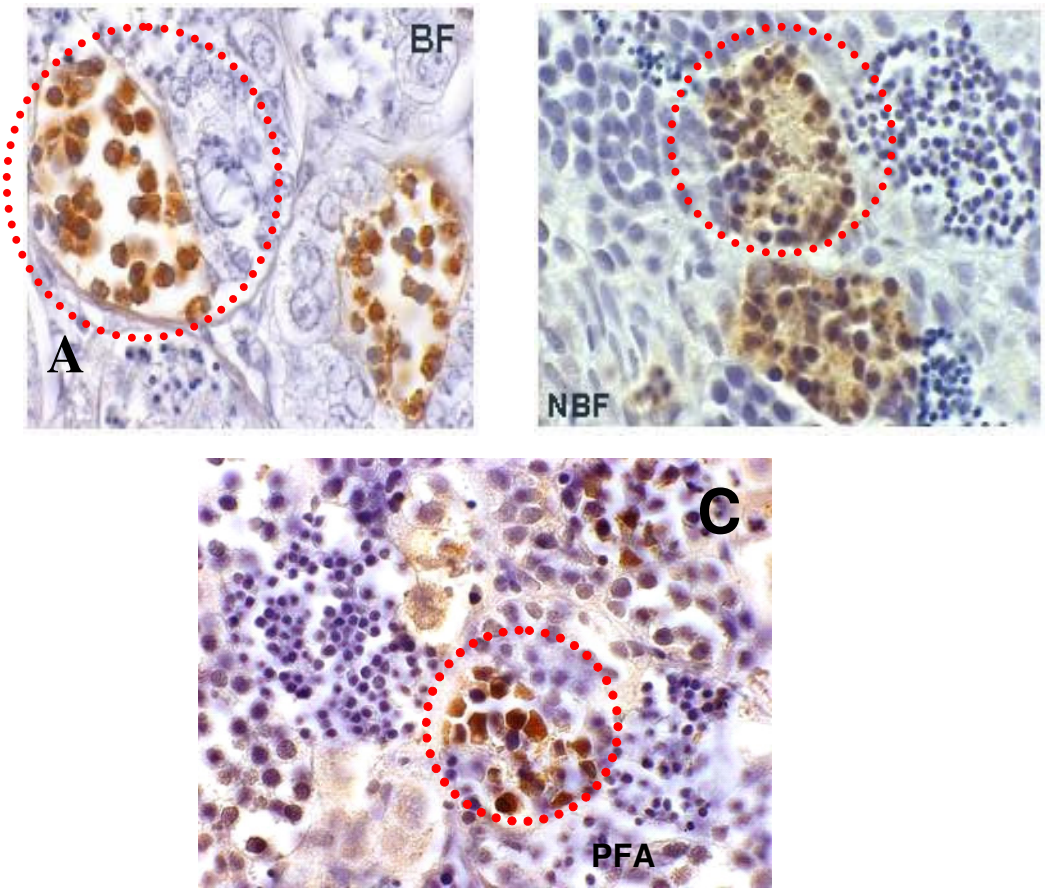


Fig. 22: TUNEL immunoreactivity in the testes of *O. mossambicus* from the AD.

b) Nandoni Dam

In the ND, during season 1, no *O. mossambicus* was sampled, despite numerous efforts. In season 2, similar to the situation in XW, where the greatest number of positive cells was yielded by NBF (n=53), compared to BF (n=46), and PFA (n=38).

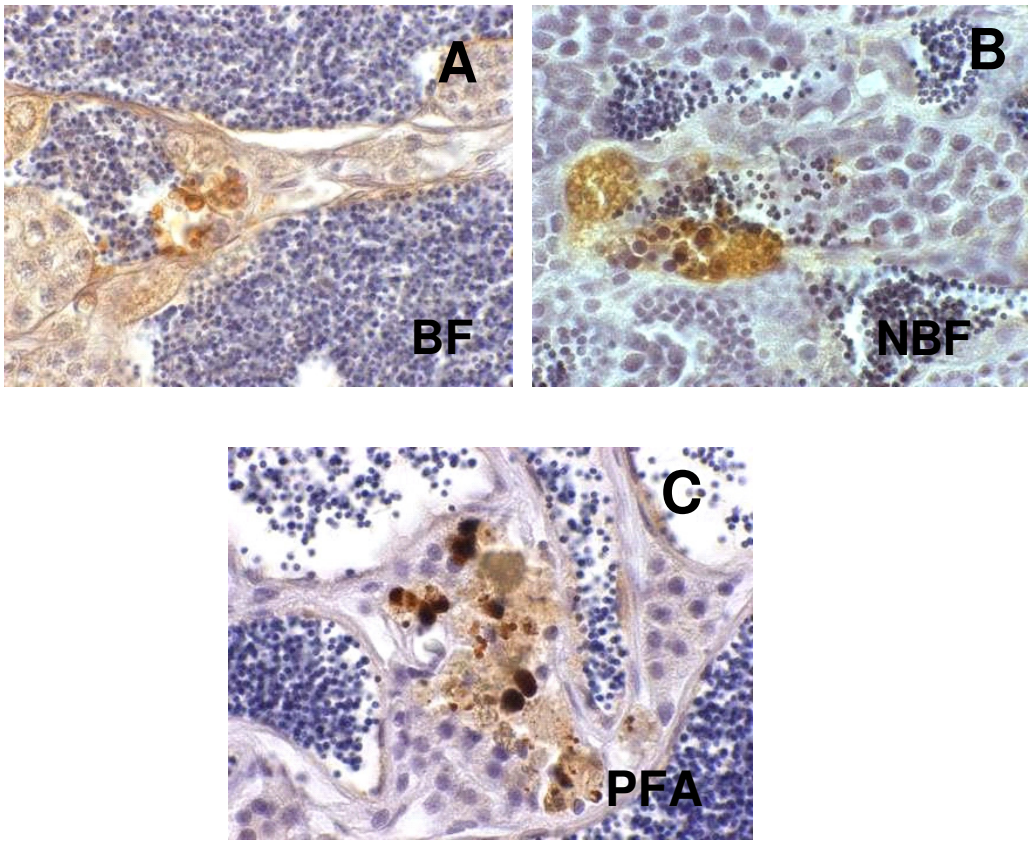


Fig. 23: TUNEL immunoreactivity in the testes of *O. mossambicus* from the ND (2HF).

c) Xikundu Weir

During season 1, in XW, the greatest amount of positive cells was yielded by BF (n=267), as compared to PFA (n=266), and NBF (n=200). In season 2, the greatest amount of positive cells was yielded by NBF (n=16), followed by BF (n=8), but PFA having no positively stained caspase-3 cells. Similar to the caspase-3 immunoreactivity, the was also TUNEL immunoreactivity was present in a cluster formation.

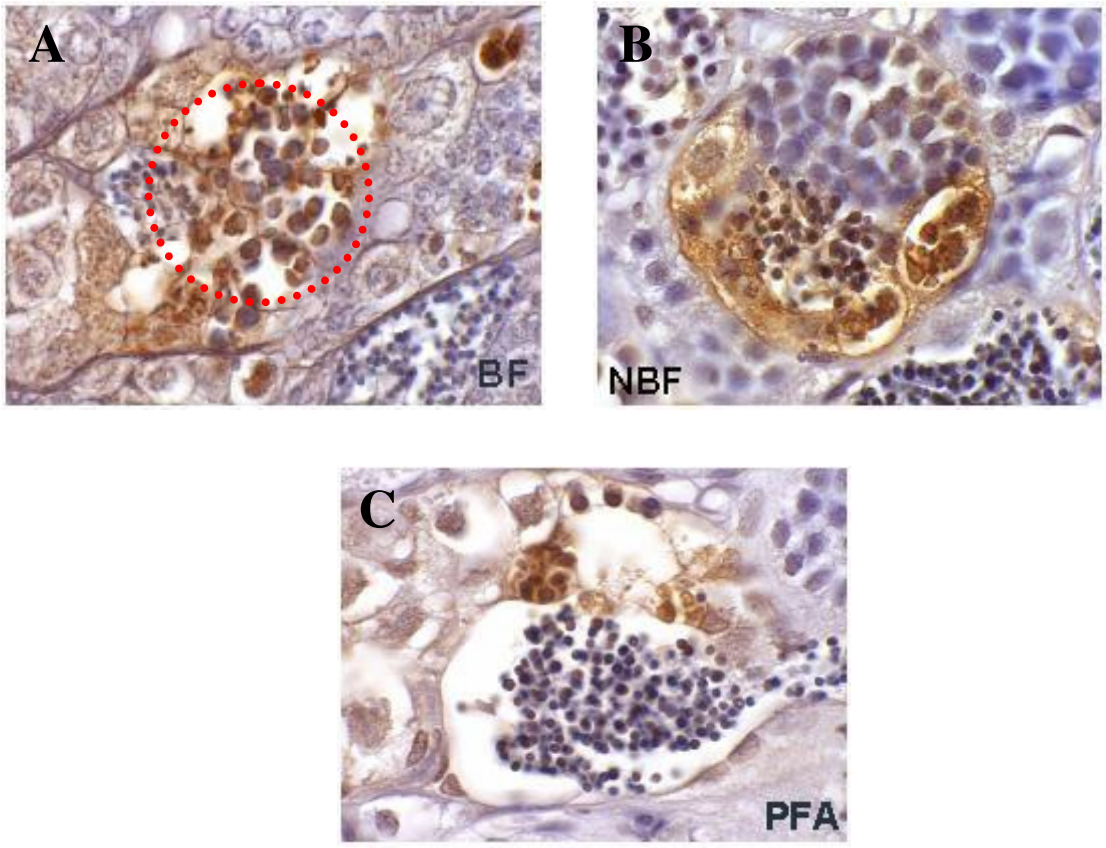


Fig. 24: TUNEL immunoreactivity in the testes of *O. mossambicus* from the XW. Note the cluster formation indicated by the red dashed circle.

4.4.4. The TUNEL Assay - Comparing the efficacy of fixation of *C. gariepinus* tissue sections, in the three sites, during both season 1 and season 2.

a) Albasini Dam

During season 1, the greatest amount of positive cells was yielded by PFA (n=488) followed by NBF (n=392) and then BF (n=246). In season 2, the greatest amount of positive cells was yielded by NBF (n=50), followed by PFA (n=9) and BF (n=6).

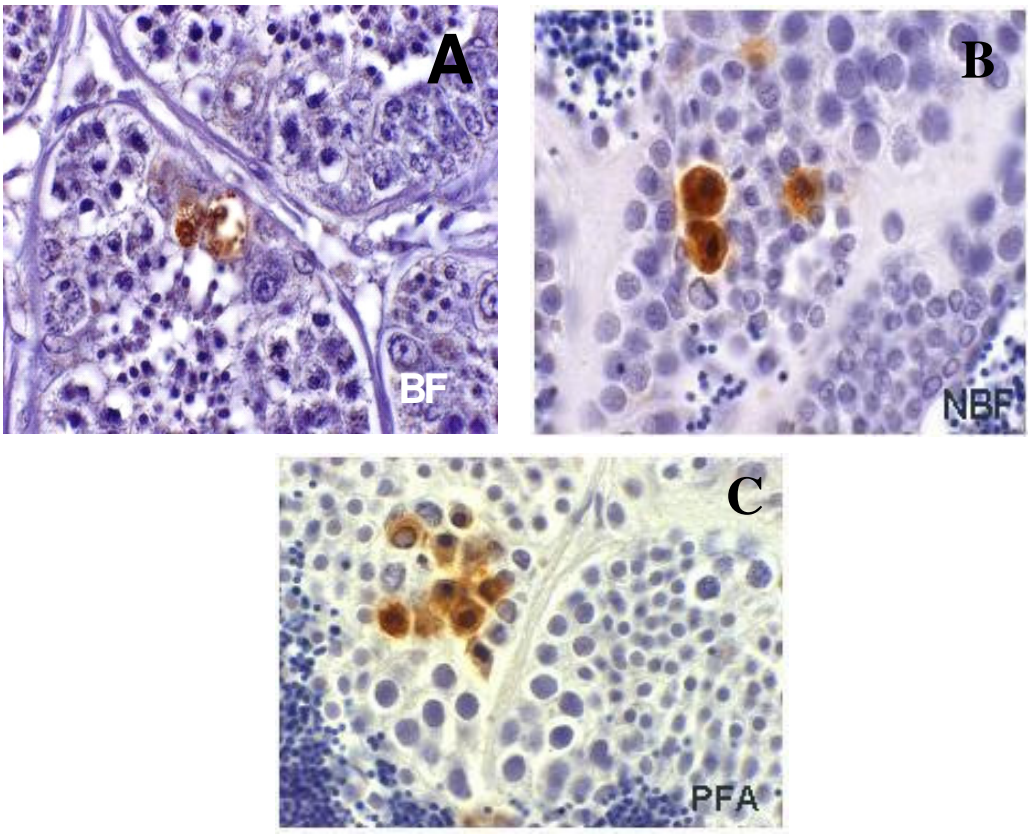
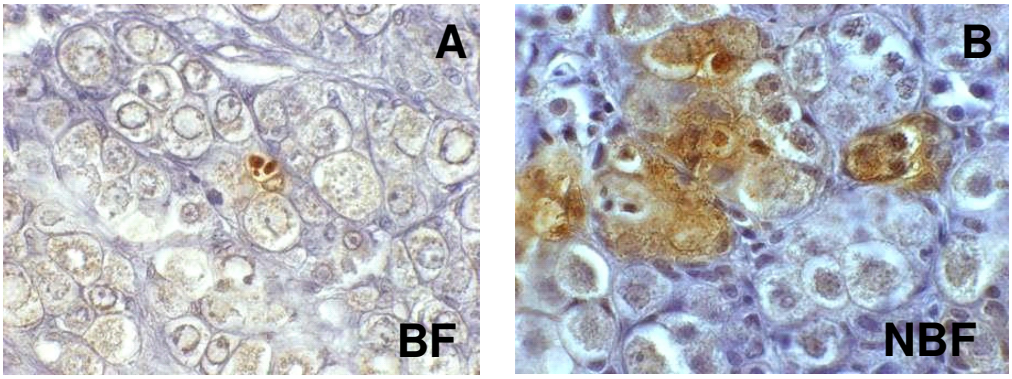


Fig. 25: TUNEL immunoreactivity in the testes of *C. gariepinus* from the AD.

b) Nandoni Dam

During season 1, the greatest amount of positive cells was yielded by NBF (n=29) followed by PFA (n=26) and then BF (n=4). In season 2, all three fixatives yielded no observed TUNEL positive cells. Thus a similar result is observed as in AD.



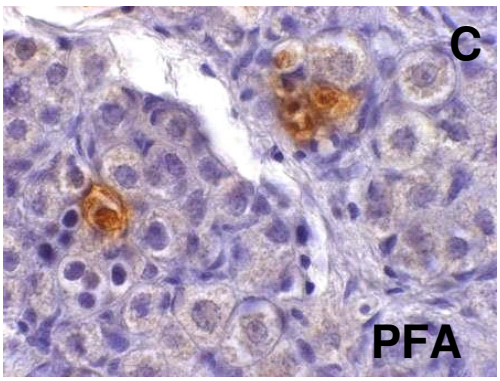


Fig 26: TUNEL immunoreactivity in the testes of *C. gariepinus* from the ND.

c) Xikundu Weir

During season 1, the low flow season, the greatest amount of positive cells was yielded by NBF (n=31) followed by PFA (n=14). The fixative BF yielded no positively stained cells. In season 2, the high flow season, only NBF (n=50) yielded positive cells, with BF and PFA having no positively stained TUNEL cells.

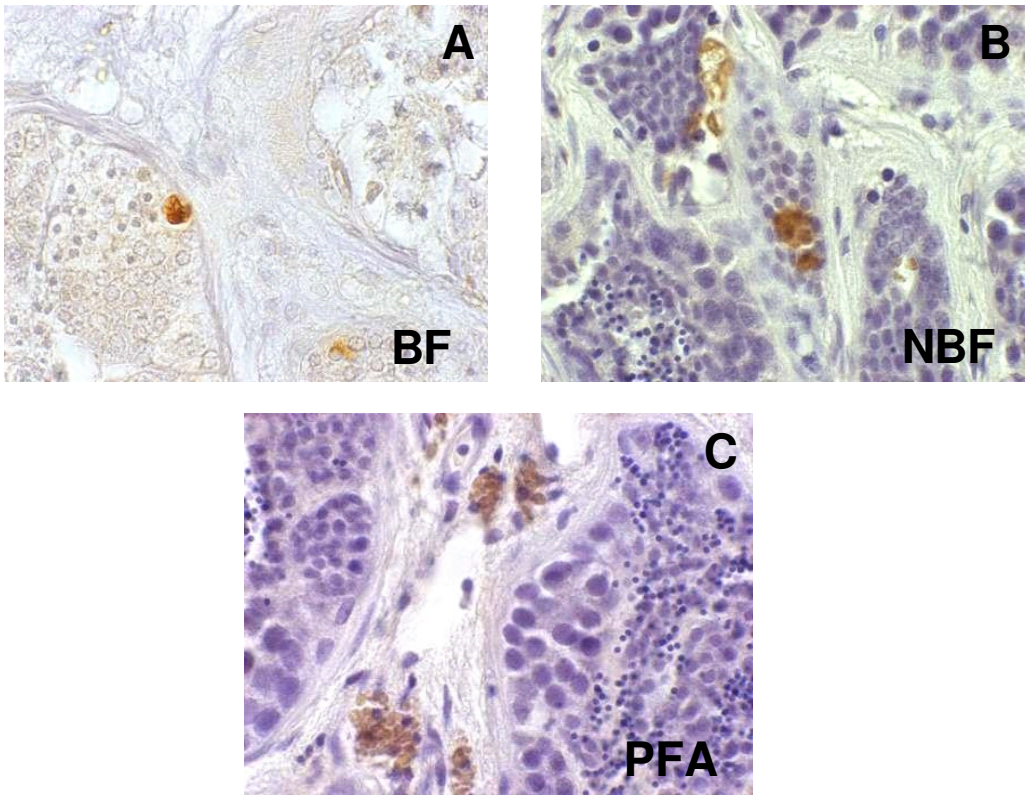


Fig. 27: TUNEL immunoreactivity in the testes of *C. gariepinus* from the XW.

Table 13: Summary of quantification data for the October 2007 collection period

		Total Lobules			Positive Lobules			% Positive Lobules			Positive Cells			Positive Cells/Lobule		
Albasini Dam		**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**
Caspase Assay	**Ave**	1350	899	838	28	18	11	2.72	2.79	1.61	297	199	104	9	10	8
O. mossambicus	**SD**	1010	626	433	29	17	17	3.30	3.61	2.47	361	252	154	5	6	5
TUNEL Assay	**Ave**	1350	899	838	100	23	28	9.94	3.22	3.95	1746	323	391	8	9	9
O. mossambicus	**SD**	1010	626	433	211	42	41	19.82	4.86	5.78	3956	658	691	5	6	4
Caspase Assay	**Ave**	1375	1085	1160	1	2	2	0.04	0.26	0.19	2	11	9	0	2	2
C. gariepinus	**SD**	615	451	520	2	5	3	0.12	0.52	0.32	7	22	14	1	2	2
TUNEL Assay	**Ave**	1375	1085	1160	5	7	8	0.31	0.59	0.62	25	39	49	4	4	6
C. gariepinus	**SD**	615	451	520	4	11	13	0.26	0.97	0.95	25	54	75	3	4	5
Nandoni Dam																
Caspase Assay	**Ave**	676	938	1205	2	20	107	0.15	1.77	6.84	11	151	642	2	5	5
C. gariepinus	**SD**	783	640	694	4	25	173	0.26	1.60	10.61	19	217	1049	3	3	1
TUNEL Assay	**Ave**	676	938	1205	1	2	2	0.67	1.16	0.58	1	10	9	1	1	1
C. gariepinus	**SD**	783	640	694	1	4	4	1.15	2.00	1.00	2	17	15	1	2	2
Xikundu Weir																
Caspase Assay	**Ave**	951	1030	1058	59	44	47	6.90	5.57	4.14	675	364	525	10	10	11
O. mossambicus	**SD**	467	506	490	32	37	40	3.89	5.34	2.67	522	326	541	4	6	6
TUNEL Assay	**Ave**	951	1030	1058	3	3	3	0.40	0.41	0.38	27	27	20	5	6	6
O. mossambicus	**SD**	467	506	490	4	4	2	0.47	0.44	0.45	35	30	21	4	4	3
Caspase Assay	**Ave**	1673	1557	1692	1	27	31	0.04	1.69	1.87	5	122	114	1	4	4
C. gariepinus	**SD**	437	316	620	2	35	41	0.09	2.05	2.50	13	157	126	3	2	2
TUNEL Assay	**Ave**	1673	1557	1692	0	1	1	0.00	0.05	0.04	0	5	2	0	1	1
C .gariepinus	**SD**	437	316	620	0	2	1	0.00	0.13	0.09	0	13	6	0	2	2

BF= Bouin's Fluid, NBF= Neutrally Buffered Formalin, PFA= Paraformaldehyde, Ave= Average, SD= Standard Deviation

Table 14: Summary of quantification data for the February 2008 collection period

		Total Lobules			Positive Lobules			% Positive Lobules			Positive Cells			Positive Cells/Lobule		
Albasini Dam		**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**
Caspase Assay	**Ave**	454	533	622	1	0	0	0.38	0.00	0.05	13	0	2	0	0	0
O. mossambicus	**SD**	222	213	105	2	0	1	0.57	0.00	0.12	18	0	5	0	0	0
TUNEL Assay	**Ave**	454	533	622	0	2	1	0.00	0.32	0.15	0	9	6	0	0	0
O. mossambicus	**SD**	222	213	105	0	2	2	0.00	0.47	0.33	0	14	13	0	0	0
Caspase Assay	**Ave**	842	779	830	2	6	5	0.22	0.96	0.67	12	39	39	3	3	3
C. gariepinus	**SD**	322	381	229	3	10	8	0.36	1.69	0.94	27	65	62	3	3	3
TUNEL Assay	**Ave**	842	779	830	0	1	0	0.01	0.23	0.03	1	5	1	1	0	1
C. gariepinus	**SD**	322	381	229	0	4	1	0.04	0.73	0.08	2	16	3	2	1	2
Nandoni Dam																
Caspase Assay	**Ave**	938	1039	983	0	1	0	0.00	0.06	0.00	0	5	0	0	2	0
C. gariepinus	**SD**	613	362	462	0	2	0	0.00	0.12	0.00	0	10	0	0	4	0
TUNEL Assay *C. gariepinus*	**Ave**	938	1039	983	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
	SD	613	362	462	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
Caspase Assay	**Ave**	636	649	523	5	3	3	0.76	0.60	0.52	40	27	25	8	5	3
O. mossambicus	**SD**	312	441	246	4	4	5	0.62	0.89	0.81	33	42	53	4	4	4
TUNEL Assay	**Ave**	636	649	523	1	1	1	0.08	0.12	0.10	5	5	4	1	2	1
O. mossambicus	**SD**	312	441	246	2	1	1	0.25	0.22	0.23	15	10	8	2	3	3

BF= Bouin's Fluid, NBF= Neutrally Buffered Formalin, PFA= Paraformaldehyde, Ave= Average, SD= Standard Deviation

Table 14 *continued*: Summary of quantification data for the February 2008 collection period

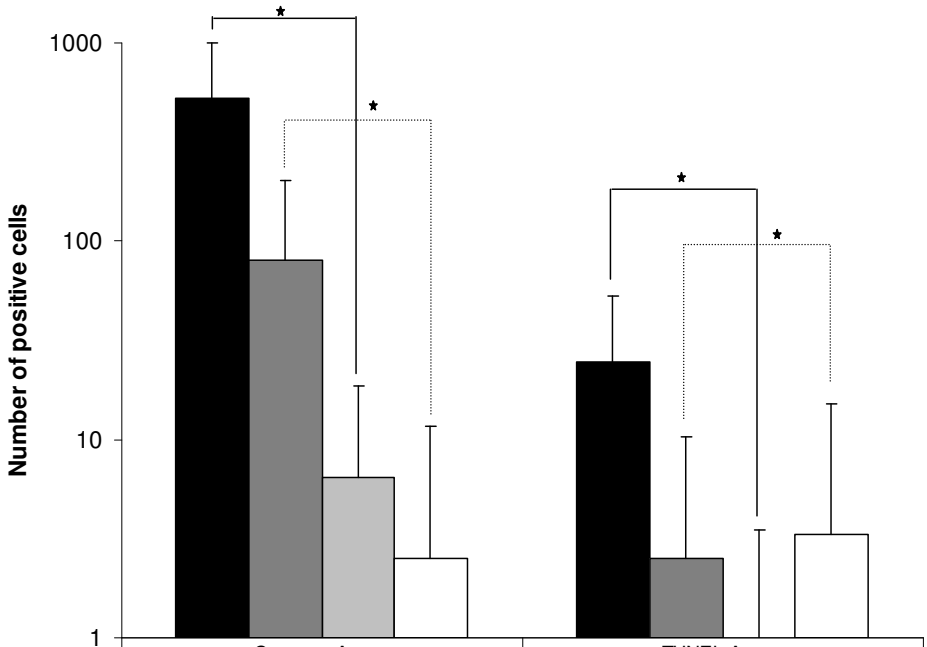
		Total Lobules			Positive Lobules			% Positive Lobules			Positive Cells			Positive Cells/Lobule		
Xikundu Weir		**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**
Caspase Assay	**Ave**	706	556	679	2	1	0	0.44	0.21	0.00	16	4	0	4	1	0
O. mossambicus	**SD**	249	185	334	2	2	0	0.51	0.52	0.00	15	9	0	4	2	0
TUNEL Assay	**Ave**	706	556	679	0	1	0	0.03	0.07	0.00	1	2	0	0	1	0
O. mossambicus	**SD**	249	185	334	1	1	0	0.08	0.15	0.00	3	4	0	1	1	0
Caspase Assay	**Ave**	839	905	757	0	1	0	0.00	0.06	0.00	0	8	0	0	3	0
C. gariepinus	**SD**	468	470	459	0	2	0	0.00	0.09	0.00	0	16	0	0	4	0
TUNEL Assay *C.*	**Ave**	839	905	757	0	3	0	0.00	0.20	0.00	0	10	0	0	0	0
gariepinus	**SD**	468	470	459	0	6	0	0.00	0.34	0.00	0	20	0	0	0	0

BF= Bouin's Fluid, NBF= Neutrally Buffered Formalin, PFA= Paraformaldehyde, Ave= Average, SD= Standard Deviation

4.5 Analysis of quantitative results

a) Abasini Dam

The comparison of the caspase-3 assay and the TUNEL assay in the efficacy of determining apoptotic death is reflected by the amount of positive cells (fig 28). Thus, based on the quantification, an interesting pattern observed between the two seasons. The amount of caspase-3 and TUNEL positive cells observed in *O. mossambicus*, are significantly ($p<0.05$) different in season 2 as compared to season 1. The opposite observation, however, is seen in *C. gariepinus* where a significantly ($p<0.05$) different amount of caspase-3 and TUNEL positive cells are observed in season 1 as compared to season 2.



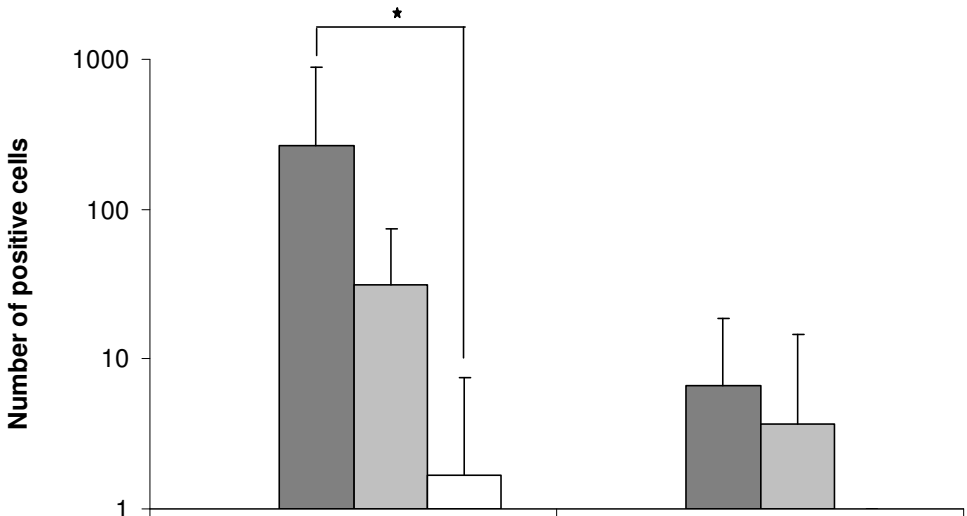
	Caspase Assay	TUNEL Assay
O S1	521.1	24.43
C S1	80.5	2.5
O S2	6.43	1
C S2	2.5	3.33

Fig. 28**:** Graphical representation of the total positive cells in both species in the Albasini Dam (O S1 = *O. mossambicus* season 1, C S1 = *C. gariepinus* season 1, O S2 = *O. mossambicus* season 2, C S2 = *C. gariepinus* season 2). Significant (* $p<0.05$)

differences observed between season 1 and season 2.

b) Nandoni Dam

In the ND, there was no *O. mossambicus* collected in season 1, thus there is no recording on the graph (refer to figure 29). However, the amount of caspase-3 positive cells observed in *O. mossambicus* in season 2 is the highest, when compared to XW and AD. The amount of caspase-3 positive cells in *C. gariepinus* is significantly ($p<0.05$) different in season 1, when compared to XW and AD.

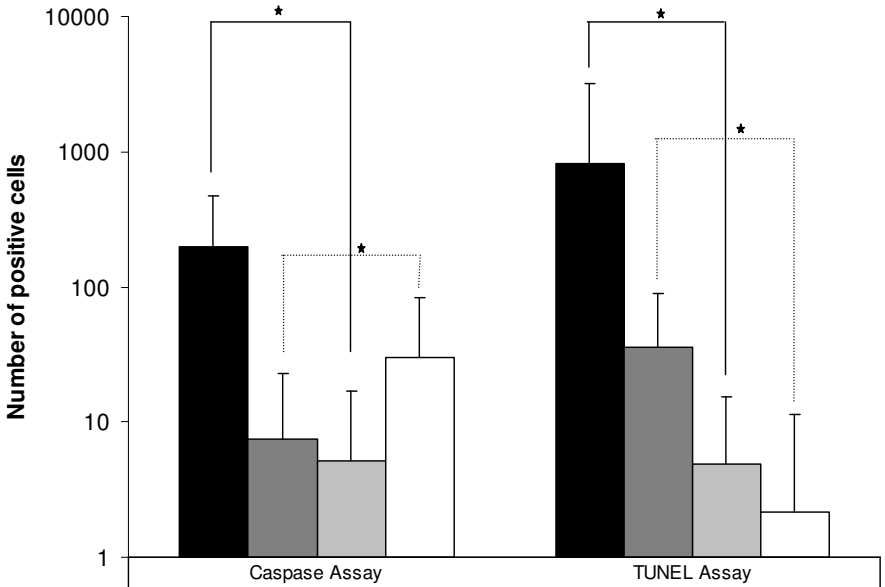


	Caspase Assay	TUNEL Assay
O S1	0	0
C S1	268	6.56
O S2	31	3.7
C S2	1.67	0

Fig. 29: Graphical representation of the total positive cells in both species in the Nandoni Dam (O S1 = *O. mossambicus* season 1, C S1 = *C. gariepinus* season 1, O S2 = *O. mossambicus* season 2, C S2 = *C. gariepinus* season 2). Significant (* $p<0.05$) differences observed between season 1 and season 2.

c) Xikundu Weir

In season 1, the caspase-3 and TUNEL positive cells of *C. gariepinus* sections was significantly ($p<0.05$) different than in season two (fig 30). However, in *O. mossambicus*, the amount of positive cells is significantly ($p<0.05$) different in season 2, from in season 1. The TUNEL positive cells, however, are greater in season 1, than in season two.



	Caspase Assay	TUNEL Assay
O S1	199.89	819.78
C S1	7.43	35.73
O S2	5.13	4.87
C S2	29.9	2.17

Fig. 30: Graphical representation of the total positive cells in both species in the Xikundu Weir (O S1 = *O. mossambicus* season 1, C S1 = *C. gariepinus* season 1, O S2 = *O. mossambicus* season 2, C S2 = *C. gariepinus* season 2). Significant (* $p<0.05$) differences observed between season 1 and season 2.

4.6. Correlation between DDT concentration and observed positive cells

The decision to correlate the amount of positive cells with the concentrations of DDT and its isomers measured in the fat of the collected fish, stems from the findings that high

levels of *p,p´*-DDT, *p,p´*-DDD and *p,p´*-DDE were found in the fat of the collected fish, as well as *o,p´*-DDE and *p,p´*-DDE in the water and sediment samples.

The table (Table 14) below presents the Pearson correlation co-efficients, which give an indication of the strength with which two variables are associated. A value between -1 and 0 indicates a negative relationship, whereas a value between 0 and +1 indicates a positive relationship. If a value lies between -0.7 and -1, the relationship between the variables is considered to be strongly negative and hence strongly positive if the value lies between +0.7 and +1. In the current scenario (Table 13) there appears to be no strong positive or strong negative correlation.

Table 15: Pearson rank co-efficient r values: indicating the correlation between the observed amount of positive cells and the concentrations of DDT and its isomers found in fat tissue of collected fish

	Amount of positive cells observed		
	Caspase- 3 assay	**TUNEL assay**	**Total (Caspase + TUNEL)**
Target EDC (µg/L)			
o,p´-DDT	0.093	-0.082	0.013
p,p´-DDT	0.024	-0.100	-0.054
o,p´-DDD	-0.040	-0.142	-0.120
p,p´-DDD	-0.052	-0.166	-0.143
o,p´-DDE	-0.189	-0.166	-0.239
p,p´-DDE	0.010	-0.120	-0.070
Total DDT	0.005	-0.134	-0.082
Total DDE	0.008	-0.120	-0.072
DDT:DDE	-0.068	0.012	-0.040

The r values ranged from -0.239 for the correlation between *o,p´*-DDE and the total amount of positive cells in the caspase-3 and TUNEL assay, to +0.093 for the correlation between *o,p´*-DDT and the amount of positive cells in the caspase-3 assay. Thus there is no strong positive or negative correlation and no significant differences ($p>0.05$) between the isomers of DDT and the amount of positive cells in either one of the assays. In an ideal statistical situation we would have used Multiple Imputation (MI) where a set of values are drawn using a statistical distribution based on the observed values. A mean of the drawn values is used then instead (Conversation with Dr. Samuel Manda). Instead due to an incomplete data set with values below the detection limit, the current study utilizes

a naïve adhoc process of selecting a random value, and thus a complete case analysis was conducted on the data. It is thus possible that the data may not give an exact account of the correlation between the variables, due to the concentrations of DDT and its isomers being below the detection limit.

CHAPTER 5: DISCUSSION

5.1. Target chemical analysis of collected water and sediment samples

The physical water quality parameters were within the acceptable range as stipulated by the South African water quality guidelines (87) at all three sampling sites in both seasons, even though previous studies conducted in the Luvuvhu River have noted a slight elevation in the pH, which may cause eutrophication at a later stage (16, 19). The chemical analyses of water samples and sediment showed that DDT and its metabolites in the AD, ND and XW, were below the detection limit of 0.50 µg/l (water) and 0.50 µg/kg. sediment) in season 1. This finding is similar to that found by a previous study n the same area (19). In season 2, *o,p'*-DDE and *p,p'*-DDE was present in the water and sediment from XW, These findings suggest that in the region, the continual spraying of DDT over the past 60 years, has been metabolized and is no present in the water and sediment.

5.2. Histological assessment

The histological assessment of the testes of *O. mossambicus* and *C. gariepinus* showed no histological abnormalities or histological lesions. The H&E section of the testes fixed in BF, showed better clarity and ease of identification of the stages of spermatogenesis compared to samples fixed in NBF and PFA. This corresponds with the recommendation that BF is the optimal fixative for histology (68, 81). The GSI showed significant differences between both species from all three sites between season 1 (higher GSI) and season 2 (lower GSI). The higher GSI levels could be associated with the developmental stages identified, as only fish from season 1 was mid- spermatogenic and the majority of developing (early spermatogenic) fish were collected during season 2. Even though the GSI may be used as a crude tool to aid histological assessment (79, 80) in the current study, no correlation could be made between the decreases in the GSI in season 2 to influences of external factors. These changes in GSI may be attributed to normal reproductive cycles.

5.3. Immunohistochemical assessment

The present study describes testicular apoptosis in *C. gariepinus* and *O. mossambicus* inhabiting a DDT sprayed area. Although the caspase-3 and TUNEL assays have been used in identifying and quantifying testicular apoptosis in *C. gariepinus* (26), this is the first time that these assays have been used in identifying and quantifying testicular apoptosis in *O. mossambicus*. Comparing the number of caspase-3 positive cells between the three sampling sites, *O. mossambicus* from the XW and AD had the highest amount of positive cells compared to *C. gariepinus* in both season 1 and 2. In ND, no *O. mossambicus* was collected in season 1, thus no comparison can be made with the amount of positive cells in season 2. When comparing the number of positive cells in the TUNEL assay in *O. mossambicus* between the three sampling sites in season 1, the highest amount of positive cells was found in fish from the AD compared to fish from the XW. In *C. gariepinus* specimens from both season 1 and 2, the highest amounts of positive cells were found in fish from the AD. Consequently, no similar patterns were identified when comparing the amount of TUNEL positive cells in the two species between the three sampling sites. A pattern could be identified in AD and XW, where corresponding changes occur in each dam using the caspase-3 assay. When identifying the stage of spermatogenesis that is affected, the spermatocytes are the cells that were stained positive for caspase-3, but further investigation is required to identify the possible mechanism acting on the spermatocytes. What is more interesting is that the caspase-3 and TUNEL positive cells appear in clusters in the testes of *O. mossambicus* fixed in all three fixatives. McClusky *et al* (26) found a similar result in the testes of *C. gariepinus* testes fixed in NBF, in this study clusters were identified in NBF as well as PFA, but not in BF. This coalescence of germ cells is a common feature of apoptosis in fishes (88), and may be a consequence of cytoplasmic bridges, joining germ cells. If there is an adequate apoptotic stimulus to one cyst, the stimulus may be carried to the adjacent cysts, via the cytoplasmic bridges, which are present in a species of catfish (89), but unknown in the tilapia species. The formation of the germ cell coalescence in the testes of *O. mossambicus* is a novel finding, as immunohistochemical assays, such as the caspase-3

and TUNEL, have not been used before in assessing the effect of fish species inhabiting a DDT sprayed area.

Evaluating the efficacy of the two assays by comparing the total amount of caspase-3 and TUNEL positive cells from all three sites and both seasons, the caspase-3 assay yielded the highest amount of positive cells. Thus, the result of this current study indicates that the TUNEL assay underestimates the extent of apoptosis. McClusky *et al* (26) found a similar result in a study on *C. gariepinus* from an urban nature reserve in South Africa, polluted with industrial effluent. The variation in the immunostatining patterns of the caspase-3 and TUNEL assay have been previously observed in ovarian follicles of both buffalo and cattle (24) as well as in humans (25), to which no specific conclusions were drawn. The underestimation of the TUNEL assay is directly related to the fragmentation of DNA that occurs during apoptotic death (10). The TUNEL assay only yields a positive result when double strand DNA fragments are formed (90). In order to enhance TUNEL expression, more stringent antigen revival techniques are recommended such as adequate fixation and enhanced antibody-antigen binding to ensure effective preservation without over-fixation (72, 82).

5.4. Fixative assessment

The ability of an assay to yield optimal results greatly depends on the binding of the antibody to the antigen, which allows the active enzyme of the assay to generate a positive result (69). Even though there are numerous techniques available to enhance antibody-antigen binding (71, 72, 90), the effectiveness of an assay depends on the ability of a fixative to preserve the antigens, without over-fixation of the tissue section which will make the retrieval of the antigens as well as antibody binding difficult (67, 69, 91). In both seasons, the fixation of *O. mossambicus* testes in BF yielded the highest amount of caspase-3 positive cells while the testes fixed in NBF yielded the highest amounts of TUNEL positive cells. The fixation of *C. gariepinus* testes in NBF, yielded the highest amount of caspase-3 and TUNEL positive cells in both seasons. This variation in the efficacy of the fixative is due to the different properties the fixative has i.e. the ability to

form cross bridges (69). BF is recommended as a fixative for histology as it preserves cellular integrity, while possibly masking antigen sites making IHC detection difficult (68, 81, 82). The fact that the testes of *O. mossambicus* fixed in BF yielded a higher amount of positive cells as compared to NBF, is a novel finding. However, in the current study it is recommended that NBF be used as it yields the most positive results in testicular sections, where BF and PFA yielded no positive cells.

5.5. Correlation of DDT metabolites with observed positive cells

The concentrations of DDT and its metabolites in *O. mossambicus* could not be determined in season 1 in fish from all three sampling sites as no fat was available from these specimens. In season 2, in the fat samples of *O. mossambicus* from the AD, ND and XW, levels of *p,p'*-DDD was the highest. Lower levels of *p,p'*-DDE and *p,p'*-DDT were detected in fish from the ND and XW while in the AD, *p,p'*-DDE and *p,p'*-DDT were below the detection limit of 50 µg/kg.

In fat samples of *C. gariepinus* from the AD and ND collected during season 1, *p,p'*-DDE levels were the highest, followed by *p,p'*-DDD and *p,p'*-DDT. In season 2, *p,p'*-DDD levels were the highest followed by *p,p'*-DDE, and *p,p'*-DDT. In season 1, fat samples of fish from the WX had the highest levels of *p,p'*-DDE followed by *p,p'*-DDT and *p,p'*-DDD while in season 2, *p,p'*-DDD levels were the highest, followed by *p,p'*-DDE, and *p,p'*-DDT. However, no strong correlation was found between the amount of caspase-3 positive cells and levels of *p,p'*-DDE (Pearson: r = 0.010) and *p,p'-DDD* (Pearson: r = -0.189), as well as the amount of TUNEL positive cells and *p,p'-DDE* (Pearson: r = -0.120) and *p,p'-DDD* (Pearson: r = -0.166). In addition, no strong correlation was found between the total amount of positive cells (caspase-3 + TUNEL) and *p,p'-DDE* (Pearson: r = -0.070) and *p,p'-DDD* (Pearson: r = -0.143).

Even though no strong positive or negative correlation could be found, one must keep in mind that the DDT is applied to the area two months prior to sampling, as well as continually for the past 60 years (14). Thus, the effect that is observed may be

accumulatory and thus a lag effect of the continual spraying occurs. This may explain why the levels of *p,p'*-DDE were found to be the highest in the collected water, sediment and fat tissue. Various studies investigate the potential that the exposure of the persistent metabolite *p,p'*-DDE may have on the testes. A study investigating the effects that DDT has on the expression of apoptosis-activating pathways in human endometrial Ishikawa and embryonic kidney cells (11), found that DDT induces TNFα expression, which leads to the activation of caspase-3 mediated apoptosis, which was confirmed by using the caspase-3 assay. The Sertoli cells in the testes are the nursing cells, which provide an optimal environment for germ cell growth and maturation (34). The protein transferrin is one of the Sertoli cells' major secretory products that supports developing germ cells, whereas ABP binds to testosterone, and thus maintain high testosterone concentrations, which facilitates spermatogenesis. In a study that investigated the effect that *p,p'-DDE* has on transferrin and androgen-binding protein in the cultured rat Sertoli cells, it was suggested that *p,p'*-DDE induced apoptosis of cultured rat Sertoli cells which may be activated via the caspase-3 mediated mitochondria-mediated pathway (92).

In so doing, the metabolite may decrease the survival rate of the cultured Sertoli cells, thus inhibiting transferrin expression, which maintains high testosterone concentrations, leading to possible abnormal spermatogenic maturation (92). This inability of the Sertoli cells to function, will lead to the activation of apoptosis. However, prolonged experimental exposure to *p,p'*-DDE leads to a feedback mechanism, enabling certain functions of the Sertoli cells to be taken over by the androgen binding protein (93). This is however only an intermediary solution that keeps apoptosis activation at bay for a short period of time. It is thus possible that the *p,p'*-DDE levels measured had an effect on the Sertoli cells of *O. mossambicus* and *C. gariepinus*, but not necessarily induced apoptosis of the germ cells at the stage of sampling.

5.6. Studies indicating effects of *p,p'*-DDE on various target sites

Since the metabolite *p,p'*-DDE has been shown to have a direct effect on the Sertoli cells and thus an indirect effect on the germ cells, various studies investigated the effect *p,p'*-

DDE may have on other target sites. Numerous studies have investigated the possible effect that *p,p'*-DDE has on, the steroid producing ability of the liver, kidneys and brain (43) as well as aromatase inhibition of testosterone (94) in different organisms. Steroid hormones, such as estrogen and testosterone, require a host of enzymes and pathways in order to ensure its adequate synthesis and regulation. The protein StAR and P450*scc* are expressed in all steroid producing organs in the body. StAR regulates the amount of cholesterol movement across the membrane so that P450*ssc* cleavage initiates steroidogenesis. Upon exposure to *p,p'*-DDE a study found that there is a significant ($p<0.05$) up-regulation of StAR and P450*scc* mRNA expression in the kidney, the liver and the brain, and concluded that the same result could be seen in the testes. This up-regulation is suggested to be the initial site of endocrine disruption and as a result, hormone production may be altered and cause cellular disturbances, which may lead to apoptosis (43).

Benachour *et al* (94) investigated the possible hormonal disturbances in a study investigating the cytotoxic effects of *p,p'*-DDE on aromatase inhibition. Aromatization of testosterone into estrogens is an important step in the maintenance of optimal steroid hormone balance. The study concluded that *p,p'*-DDE results in cytotoxic effects on aromatase inhibition, and long-term exposure to *p,p'*-DDE experimentally, not only induces aromatase inhibition, but may possibly affect cell signaling mechanisms. This alteration in cell signaling may result in the cell not being able to send an appropriate apoptotic signal, hence even though *p,p'*-DDE may be present, apoptosis may thus not be activated (93). It may be possible that even though no correlation was observed between the measured levels of DDT metabolites and positive cells, a possible indirect effect does exist, but requires further investigation.

The majority of studies, focus on the effects that one specific hormone may have at a particular time on the organism concerned. However, aquatic organisms, such as fish, are in daily contact with, and exposed to, a mixture of numerous xenobiotic substances and environmental changes. Thus, perhaps it would be more plausible that a host of chemicals may have an affect on an organism as opposed to a single chemical (94).

CHAPTER 6: CONCLUSIONS

The current study investigated the utility of immunohistochemical techniques for the assessment of the modulation of testicular apoptosis in fish from a DDT-polluted area.

Objective 1:
To compare the degree of testicular apoptosis between the fish from the three sites.

The study concluded that the XW yielded the greatest amount of positive cells in the caspase-3 assay. In season 1, there was a greater amount of both caspase-3 and TUNEL positive cells, as compared to season 2, in all three the sampling sites. The reference site, the AD, had a similar pattern to that the XW. This indicates that the reference site may have been the optimal reference site for DDT, but not for other EDCs, that may be present in the other sampling sites, as the amount of positive cells at the AD and the XW are comparable. In conclusion, in season 1 there is a significant ($p<0.05$) higher amount of up-regulation of apoptosis as compared to season 2.

Objective 2:
To compare the efficacy of the TUNEL and cleaved caspase-3 immunostaining of apoptosis.

The utility of the caspase-3 and TUNEL assays plays a pivotal role in the quantification of the extent of apoptotic up-regulation. Even though the two assays identify different stages of cellular death, the caspase-3 assay yielded the greatest amount of positive cells. This study shows that the TUNEL assay greatly underestimated the degree of positive cells, however, there is utility for both the caspse-3 and TUNEL assay to give an indication of modulation of normal cellular apoptosis.

Objective 3:

To compare the efficacy of three different fixatives for optimal immunostaining.

This study also concluded that optimal fixation is essential to allow for the optimal immunostaining of cellular apoptotic death. The important aspect of fixation should not be overlooked, as the choice of fixative may either determine the extent to which an accurate quantification may be possible. It would be recommended that all three fixatives be used, as BF, PFA and NBF have utility in IHC.

Objective 4:

To assess the concentrations of DDT in the fat tissue of both *O. mossambicus* and *C. gariepinus,* and to correlate these values with the degree of observed testicular apoptosis.

The current study found that the levels of *p,p'*-DDE to be the highest in the sampled water and sediment at the XW and also in the fat tissue of both *O. mossambicus* and *C. gariepinus* from the XW. In addition, the current study showed no strong positive or negative correlation between the increased number of apoptotic cells and the concentrations of DDT and its metabolites in the fat samples. There may be other factors that have contributed to the up-regulation of apoptosis and further investigations are required.

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Appendix

Table 1: Quantification of caspase-3 assay of *O. mossambicus* sampled at Albasini Dam – October 2007

	Total Lobules			Positive Lobules			% Positive Lobules			Positive Cells			Positive Cells/Lobule		
	BF	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**
AO1	760	496	630	76	57	27	10.00	11.49	4.29	1134	721	284	15	13	11
AO2	1093	326	694	65	17	50	5.95	5.21	7.20	482	128	440	7	8	9
AO3	1454	514	550	2	14	0	0.14	2.72	0.00	6	22	0	3	2	0
AO4	839	928	730	13	12	5	1.55	1.29	0.68	128	119	90	10	10	18
AO5	1604	2080	1353	9	12	2	0.56	0.58	0.15	81	74	11	9	6	6
AO6	205	248	301	7	1	3	3.41	0.40	1.00	88	16	19	13	16	6
AO7	677	687	968	0	1	3	0.00	0.15	0.31	0	2	18	0	2	6
AO8	3673	1168	1689	51	19	7	1.39	1.63	0.41	424	185	29	8	10	4
AO9	1849	1644	624	27	27	3	1.46	1.64	0.48	330	523	43	12	19	14

Table 2: Quantification of caspase-3 assay of *O. mossambicus* sampled at Albasini Dam – February 2008

	Total Lobules			Positive Lobules			% Positive Lobules			Positive Cells			Positive Cells/Lobule		
	BF	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**
AO1	310	350	751	4	0	2	1.29	0.00	0.27	38	0	12	0	0	0
AO2	751	897	605	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
AO3	481	420	589	3	0	0	0.62	0.00	0.00	27	0	0	0	0	0
AO4	175	510	689	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
AO5	553	489	475	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0

Table 3: Quantification of caspase-3 assay of *O. mossambicus* sampled at Nandoni Dam – February 2008

	Total Lobules			Positive Lobules			% Positive Lobules			% Positive Lobules			Positive Cells/Lobule		
	BF	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**
NO1	559	433	421	7	4	5	1.25	0.92	1.19	58	37	30	8	9	6
NO2	458	507	375	8	14	5	1.75	2.76	1.33	83	134	27	10	10	5
NO3	547	501	648	7	7	15	1.28	1.40	2.31	49	62	170	7	9	11
NO4	1432	931	1178	6	1	4	0.42	0.11	0.34	42	8	27	7	8	7
NO5	475	1791	475	2	2	0	0.42	0.11	0.00	16	10	0	8	5	0
NO6	379	477	403	0	2	0	0.00	0.42	0.00	0	10	0	0	5	0
NO7	572	241	354	1	0	0	0.17	0.00	0.00	3	0	0	3	0	0
NO8	897	702	521	13	2	0	1.45	0.28	0.00	98	13	0	8	7	0
NO9	568	471	459	1	0	0	0.18	0.00	0.00	10	0	0	10	0	0
NO10	473	436	395	3	0	0	0.63	0.00	0.00	43	0	0	14	0	0

Table 4: Quantification of caspase-3 assay of *O. mossambicus* sampled at Xikundu Weir – October 2007

	Total Lobules			Positive Lobules			% Positive Lobules			Positive Cells			Positive Cells/Lobule		
	BF	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**
XO1	1304	707	1517	72	26	31	5.52	3.68	2.04	763	234	409	11	9	13
XO2	715	712	366	70	104	22	9.79	14.61	6.01	685	1003	281	10	10	13
XO3	863	1551	1843	112	95	97	12.98	6.13	5.26	1397	126	1150	12	1	12
XO4	944	498	423	37	8	7	3.92	1.61	1.65	499	134	120	13	17	17
XO5	2080	1845	1529	29	15	112	1.39	0.81	7.33	261	264	196	9	18	2
XO6	685	466	1216	41	42	83	5.99	9.01	6.83	609	729	1368	15	17	16
XO7	431	1306	1015	8	12	9	1.86	0.92	0.89	35	57	90	4	5	10
XO8	1041	513	1161	100	72	69	9.61	14.01	5.94	1709	631	1343	17	9	19
XO9	575	1285	708	63	59	38	10.96	4.59	5.37	592	428	285	9	7	7
XO10	873	1417	801	61	5	1	6.99	0.35	0.12	195	37	3	3	7	3

Table 5: Quantification of caspase-3 assay of *O. mossambicus* sampled at Xikundu Weir – February 2008

	Total Lobules			Positive Lobules			% Positive Lobules			Positive Cells			Positive Cells/Lobule		
	BF	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**
XO1	448	512	879	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
XO2	240	375	358	4	6	0	1.67	1.60	0.00	47	29	0	12	5	0
XO3	1048	854	509	2	0	0	0.19	0.00	0.00	9	0	0	5	0	0
XO4	798	755	1098	3	4	0	0.38	0.53	0.00	12	10	0	4	3	0
XO5	648	354	379	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
XO6	958	608	975	3	0	0	0.31	0.00	0.00	23	0	0	8	0	0
XO7	607	587	105	5	0	0	0.82	0.00	0.00	15	0	0	3	0	0
XO8	948	359	603	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
XO9	758	405	897	4	0	0	0.53	0.00	0.00	31	0	0	0	0	0
XO10	609	751	989	3	0	0	0.49	0.00	0.00	24	0	0	8	0	0

Table 6: Quantification of caspase-3 assay of *C. gariepinus* sampled at Albasini Dam – October 2007

	Total Lobules			Positive Lobules			% Positive Lobules			Positive Cells			Positive Cells/Lobule		
	BF	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**
AC1	1798	1606	1802	3	1	0	0.00	0.06	0.00	0	3	3	0	3	0
AC2	1497	1620	1890	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
AC3	905	1012	1157	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
AC4	2198	1375	1207	0	1	0	0.00	0.07	0.00	0	3	0	0	3	0
AC5	1987	1125	1375	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
AC6	1589	1405	1035	6	0	5	0.38	0.00	0.48	23	0	26	4	0	5
AC7	1202	947	875	0	11	5	0.00	1.16	0.57	0	43	26	0	4	5
AC8	905	826	808	0	11	7	0.00	1.33	0.87	0	59	37	0	5	5
AC9	108	127	75	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
AC10	1557	809	1375	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0

Table 7: Quantification of caspase-3 assay of *C. gariepinus* sampled at Albasini Dam – February 2008

	Total Lobules			Positive Lobules			% Positive Lobules			Positive Cells			Positive Cells/Lobule		
	BF	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**
AC1	852	795	846	2	5	12	0.23	0.63	1.42	6	21	74	3	4	6
AC2	504	457	758	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
AC3	704	589	825	1	0	0	0.14	0.00	0.00	6	0	0	6	0	0
AC4	1137	905	1031	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
AC5	1527	1791	1337	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
AC6	473	597	827	1	33	21	0.21	5.53	2.54	3	208	177	3	6	8
AC7	873	667	473	10	0	0	1.15	0.00	0.00	89	0	0	9	0	0
AC8	679	521	643	3	5	4	0.44	0.96	0.62	14	33	13	5	7	3
AC9	1037	672	758	0	9	2	0.00	1.34	0.26	0	65	9	0	7	5
AC10	638	797	804	0	9	15	0.00	1.13	1.87	0	65	114	0	7	8

Table 8: Quantification of caspase-3 assay of *C. gariepinus* sampled at Nandoni Dam – October 2007

	Total Lobules			Positive Lobules			% Positive Lobules			Positive Cells			Positive Cells/Lobule		
	BF	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**
NC1	361	1254	1604	0	9	11	0.0	0.72	0.69	0	47	60	0	5	5
NC2	100	202	403	0	2	3	0.00	0.99	0.74	0	6	13	0	3	4
NC3	1567	1359	1607	7	49	307	0.45	3.61	19.10	33	400	1853	5	8	6

Table 9: Quantification of caspase-3 assay of *C. gariepinus* sampled at Nandoni Dam – February 2008

	Total Lobules			Positive Lobules			% Positive Lobules			Positive Cells			Positive Cells/Lobule		
	BF	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**
NC1	405	610	404	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
NC2	1352	1256	1405	0	3	0	0.00	0.24	0.00	0	20	0	0	7	0
NC3	421	879	820	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
NC4	1575	1409	1302	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0

Table 10: Quantification of caspase-3 assay of *C. gariepinus* sampled at Xikundu Weir – October 2007

	Total Lobules			Positive Lobules			% Positive Lobules			Positive Cells			Positive Cells/Lobule		
	BF	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**
XC1	1043	1713	1708	0	86	105	0.00	5.02	6.15	0	384	304	0	4	3
XC2	2034	1551	1430	0	53	53	0.00	3.42	3.71	0	247	238	0	5	4
XC3	1766	1501	1500	4	0	0	0.23	0.00	0.00	31	0	0	8	0	0
XC4	1547	1836	916	0	4	3	0.00	0.22	0.33	0	15	22	0	4	7
XC5	1403	968	1812	0	10	11	0.00	1.03	0.61	0	50	51	0	5	5
XC6	2246	1772	2787	0	8	11	0.00	0.45	0.39	0	37	70	0	5	6

Table 11: Quantification of caspase-3 assay of *C. gariepinus* sampled at Xikundu Weir – February 2008

	Total Lobules			Positive Lobules			% Positive Lobules			Positive Cells			Positive Cells/Lobule		
	BF	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**
XC1	508	660	637	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
XC2	1731	1805	1508	0	4	0	0.00	0.22	0.00	0	39	0	0	10	0
XC3	875	951	920	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
XC4	854	875	764	0	1	0	0.00	0.11	0.00	0	6	0	0	6	0
XC5	560	605	105	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
XC6	508	532	610	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0

Table 12: Quantification of TUNEL assay of *O. mossambicus* sampled at Albasini Dam – October 2007

	Total Lobules			Positive Lobules			% Positive Lobules			Positive Cells			Positive Cells/Lobule		
	BF	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**
AO1	760	496	630	3	13	6	0.36	2.62	0.95	12	106	52	4	8	9
AO2	1093	326	694	629	27	103	57.55	8.28	14.84	11803	490	1862	19	18	18
AO3	1454	514	550	12	10	6	0.83	1.95	1.09	72	90	35	6	9	6
AO4	839	928	730	224	132	97	26.70	14.22	13.29	3646	2031	1310	16	15	14
AO5	1604	2080	1353	7	10	10	0.44	0.48	0.74	36	60	67	5	6	7
AO6	205	248	301	5	0	7	2.44	0.00	2.33	34	0	59	7	0	8
AO7	677	687	968	4	2	6	0.59	0.29	0.62	26	9	38	7	5	6
AO8	3673	1168	1689	9	8	4	0.25	0.68	0.24	55	38	38	6	5	10
AO9	1849	1644	624	6	7	9	0.32	0.43	1.44	28	79	58	5	11	6

Table 13: Quantification of TUNEL assay of *O. mossambicus* sampled at Albasini Dam – February 2008

	Total Lobules			Positive Lobules			% Positive Lobules			Positive Cells			Positive Cells/Lobule		
	BF	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**
AO1	310	350	751	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
AO2	751	897	605	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
AO3	481	420	589	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
AO4	175	510	689	0	3	5	0.00	0.59	0.73	0	14	28	0	0	0
AO5	553	489	475	0	5	0	0.00	1.02	0.00	0	31	0	0	0	0

Table 14: Quantification of TUNEL assay of *O. mossambicus* sampled at Nandoni Dam – February 2008

	Total Lobules			**Positive Lobules**			**% Positive Lobules**			**Positive Cells**			**Positive Cells/Lobule**		
	BF	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**
NO1	559	433	421	0	2	3	0.00	0.46	0.71	0	11	15	0	6	5
NO2	458	507	375	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
NO3	547	501	648	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
NO4	1432	931	1178	0	2	3	0.00	0.21	0.25	0	10	23	0	5	8
NO5	475	1791	475	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
NO6	379	477	403	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
NO7	572	241	354	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
NO8	897	702	521	7	4	0	0.78	0.57	0.00	46	32	0	7	8	0
NO9	568	471	459	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
NO10	473	436	395	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0

Table 15: Quantification of TUNEL assay of *O. mossambicus* sampled at Xikundu Weir – October 2007

	Total Lobules			Positive Lobules			% Positive Lobules			Positive Cells			Positive Cells/Lobule		
	BF	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**
XO1	1304	707	1517	3	2	4	0.23	0.28	0.26	22	22	38	7	11	10
XO2	715	712	366	4	8	5	0.56	1.12	1.37	38	79	50	10	10	10
XO3	863	1551	1843	0	1	1	0.00	0.06	0.05	0	3	3	0	3	3
XO4	944	498	423	0	3	2	0.00	0.60	0.47	0	27	13	0	9	7
XO5	2080	1845	1529	3	2	2	0.14	0.11	0.13	19	24	6	6	12	3
XO6	685	466	1216	5	0	2	0.73	0.00	0.16	25	0	10	5	0	5
XO7	431	1306	1015	0	1	1	0.00	0.08	0.10	0	3	3	0	3	3
XO8	1041	513	1161	13	5	3	1.25	0.97	0.26	114	30	21	9	6	7
XO9	575	1285	708	6	11	7	1.04	0.86	0.99	49	78	56	8	7	8
XO10	873	1417	801	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0

Table 16: Quantification of TUNEL assay of *O. mossambicus* sampled at Xikundu Weir – February 2008

	Total Lobules			Positive Lobules			% Positive Lobules			Positive Cells			Positive Cells/Lobule		
	BF	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**
XO1	448	512	879	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
XO2	240	375	358	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
XO3	1048	854	509	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
XO4	798	755	1098	2	3	0	0.25	0.40	0.00	8	10	0	4	3	0
XO5	648	354	379	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
XO6	958	608	975	0	2	0	0.00	0.33	0.00	0	6	0	0	3	0
XO7	607	587	105	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
XO8	948	359	603	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
XO9	758	405	897	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
XO10	609	751	989	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0

Table 17: Quantification of TUNEL assay of *C. gariepinus* sampled at Albasini Dam – October 2007

	Total Lobules			Positive Lobules			% Positive Lobules			Positive Cells			Positive Cells/Lobule		
	BF	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**
AC1	1798	1606	1802	10	8	10	0.56	0.50	0.55	53	42	82	5	5	8
AC2	1497	1620	1890	9	7	8	0.60	0.43	0.42	65	60	41	7	9	5
AC3	905	1012	1157	6	7	11	0.66	0.69	0.95	12	24	52	2	3	5
AC4	2198	1375	1207	9	11	5	0.41	0.80	0.41	55	82	16	6	7	3
AC5	1987	1125	1375	4	36	44	0.20	3.20	3.20	19	168	250	5	5	6
AC6	1589	1405	1035	0	0	1	0.00	0.00	0.10	0	0	19	0	0	19
AC7	1202	947	875	0	0	2	0.00	0.00	0.23	0	0	7	0	0	4
AC8	905	826	808	2	2	3	0.22	0.24	0.37	10	16	21	5	8	7
AC9	108	127	75	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
AC10	1557	809	1375	7	0	0	0.45	0.00	0.00	32	0	0	5	0	0

Table 18: Quantification of TUNEL assay of *C. gariepinus* sampled at Albasini Dam – February 2008

	Total Lobules			Positive Lobules			% Positive Lobules			Positive Cells			Positive Cells/Lobule		
	BF	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**
AC1	852	795	846	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
AC2	504	457	758	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
AC3	704	589	825	1	0	0	0.14	0.00	0.00	6	0	0	6	0	0
AC4	1137	905	1031	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
AC5	1527	1791	1337	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
AC6	473	597	827	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
AC7	873	667	473	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
AC8	679	521	643	0	12	0	0.00	2.30	0.00	0	50	0	0	4	0
AC9	1037	672	758	0	0	2	0.00	0.00	0.26	0	0	9	0	0	5
AC10	638	797	804	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0

Table 19: Quantification of TUNEL assay of *C. gariepinus* sampled at Nandoni Dam – October 2007

	Total Lobules			Positive Lobules			% Positive Lobules			Positive Cells			Positive Cells/Lobule		
	BF	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**
NC1	361	1254	1604	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
NC2	100	202	403	2	7	7	2.00	3.47	1.74	4	29	26	2	4	4
NC3	1567	1359	1607	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0

Table 20: Quantification of TUNEL assay of *C. gariepinus* sampled at Nandoni Dam – February 2008

	Total Lobules			Positive Lobules			% Positive Lobules			Positive Cells			Positive Cells/Lobule		
Fixative	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**
NC1	405	610	404	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
NC2	1352	1256	1405	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
NC3	421	879	820	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
NC4	1575	1409	1302	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0

Table 21: Quantification of TUNEL assay of *C. gariepinus* sampled at Xikundu Weir – October 2007

	Total Lobules			Positive Lobules			% Positive Lobules			Positive Cells			Positive Cells/Lobule		
	BF	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**
XC1	1043	1713	1708	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
XC2	2034	1551	1430	0	5	3	0.00	0.32	0.21	0	31	14	0	6	5
XC3	1766	1501	1500	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
XC4	1547	1836	916	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
XC5	1403	968	1812	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
XC6	2246	1772	2787	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0

Table 22: Quantification of TUNEL assay of *C. gariepinus* sampled at Xikundu Weir – February 2008

	Total Lobules			Positive Lobules			% Positive Lobules			Positive Cells			Positive Cells/Lobule		
	BF	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**	**BF**	**NBF**	**PFA**
XC1	508	660	637	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
XC2	1731	1805	1508	0	4	0	0.00	0.22	0.00	0	39	0	0	10	0
XC3	875	951	920	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
XC4	854	875	764	0	1	0	0.00	0.11	0.00	0	6	0	0	6	0
XC5	560	605	105	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0
XC6	508	532	610	0	0	0	0.00	0.00	0.00	0	0	0	0	0	0