CHAPTER 3

3.1 INTRODUCTION

Breeding the two selected species in captivity, under controlled conditions, has a number of important advantages that can assist in ultimately defining the specimens as baseline or reference organisms: (1) exposure to pollutants and/or harmful substances can be minimised or avoided; (2) water quality – physical parameters and chemical components – can be monitored throughout the lifespan of the specimen; (3) a healthy diet with a specific nutritional value can be administered; (4) and the exact age of the fish is known. These four aspects combined allow the researcher to document a detailed history of each individual specimen.

The practical component of the project consisted of five successive phases. These were executed during 2004 through 2006 at the Department of Zoology's Aquatic Research Facilities, University of Johannesburg, South Africa.
The methodology used during these five phases included various techniques, procedures and protocols regarding the breeding of the selected species and tissue sampling of those specimens (phases 1, 2, and 3), a qualitative assessment of the histological structure of the selected target organs (phase 4) and an assessment of the histological integrity of those organs using a quantitative assessment protocol (phase 5). All materials used and all techniques, procedures and protocols employed in each phase will be discussed in detail in the following sections.

3.2 PHASE 1: BREEDING OF BASELINE SPECIMENS

The objective of the first phase of the project was to successfully breed *Clarias gariepinus* and *Oreochromis mossambicus* specimens in a laboratory setup, under controlled conditions, and then rear the fish until sexually mature. In order to use these fish as baseline specimens, the criteria mentioned below formed an integral part of the breeding process regarding the materials used and methods followed:

**Criteria for breeding and rearing of baseline specimens**

- Fish must be reared on a balanced, high quality diet.
- Exposure to harmful substances must be avoided.
- Water quality must be maintained at optimal levels, monitored at regular time intervals, and the chemical content of the water should be known.

3.2.1 Breeding process

To breed fish under controlled conditions and to adhere to the criteria as stated above, a correct and suitable physical research environment was essential. This included a proper breeding setup, suitable breeding medium (water), specific breeding methods for the selected species, and an appropriate feeding regime.
3.2.1.1 – Breeding setup
The breeding of the two selected species was executed in an environmental room (Figure 3.1(1)). This room allows for controlled conditions throughout the research period (e.g. specific temperature and day/night photoperiod). An automatic timer controlled the day/night conditions of the room to simulate summer conditions (breeding season: 13 - 14 hours of day light). The room, fitted with an air conditioning system (Figure 3.1(4)), allowed for the water temperature to be maintained at the desired level of 26 ± 1°C.

The environmental room was fitted with six, 800ℓ (7ft) glass tanks (Figure 3.1(2)). Each tank was labelled according to its contents: C1, C2, and C3 indicating tanks 1 – 3 containing C. gariepinus and O1, O2 and O3 indicating tanks 1 – 3 containing O. mossambicus. Each tank was aerated (Figure 3.1 (6)) and was connected to a ViaAqua 750 canister filter (Figure 3.1(3)), subsequently producing six effective circulating systems (Figure 3.2) with an approximate circulatory capacity of 700ℓ of water per hour per tank. The density of fish per volume of water was calculated to be ± 35ℓ water per O. mossambicus specimen and 80ℓ water per C. gariepinus specimen.

It was essential to avoid possible contamination by potential harmful substances in the form of apparatus and/or chemicals used. To minimise any contamination within the breeding setup, all tools and equipment used during the breeding process like fishnets, glassware, filter media etc., were washed and rinsed with distilled water before introduction to the breeding setup.

3.2.1.2 - Breeding medium
The choice of water (breeding medium) was reconstituted reverse osmosis water (RRO-water). Tap water was deionised using a reverse osmosis process (Figure 3.1(5)). However, by doing so, necessary trace elements are also removed from the water. These elements need to be replaced or reconstituted. This was done

The necessary trace elements were acquired from Merck, South Africa and included: Calcium chloride dehydrate (CaCl$_2$.2H$_2$O), Magnesium sulphate heptahydrate (MgSO$_4$.7H$_2$O), Sodium hydrogen carbonate (NaHCO$_3$) and Potassium chloride (KCl) (Figure 3.1 (7)).

Once all these trace elements were added to the reverse osmosis water, the solution was aerated for 24 hours in the environmental room to stabilise and allowed to attain the desired temperature. The water was then introduced to the breeding tanks. Two thirds of the water was replaced every 14 days during the one year growth period. Water quality was monitored on a regular basis (Section 3.2.2).
Figure 3.2  Circulating system: This figure shows the setup of the circulating system used for each tank in the environmental room. Each tank was fitted with a ViaAqua canister filter which consisted of various filter trays and allowed water to circulate through the system.
3.2.1.3 - Breeding methods

In captivity, *C. gariepinus* and *O. mossambicus* require different breeding methods and therefore, the methodology for breeding these two species will be discussed separately.

(i) *Clarias gariepinus*

*Clarias gariepinus* specimens were bred through hormone induced spawning according to the methodology described by Ross (2001). This method was used as this species do not reproduce spontaneously in captivity. It must be noted that the potential effects of the hormone-induced treatment on normal gonadal development is not known at this stage and that it is assumed that it did not affect the normal gonadal development of the fish. A breeding pair of healthy, adult *C. gariepinus* specimens were selected from a temperature controlled aquarium at the University of Johannesburg’s Aquatic Research Facilities (Figure 3.3 (1-3)). The male and female specimens were placed in separate pre-breeding porcelain baths and allowed to acclimatise for 24 hours (Figure 3.3 (4-5)). The baths were covered with netting to prevent the fish from jumping out. Fresh pituitary glands from the same species were stored in ethanol to prevent contamination and to preserve potency. Both the male and the female were then injected twice (twelve hours apart) with an appropriate dosage (1 ml 0.9% saline and one gland per specimen depending on the size of the specimen (± 2 kg)) over a 24 hour period. The female, showing plumpness of the abdomen, were then carefully sampled for eggs to confirm sexual maturity.

The male was sacrificed and the testes were removed. Milt was collected in a small, dry glass beaker. The eggs were then hand stripped from the female by applying firm but gentle downward movements to the abdomen. The milt was added to the eggs and gently stirred. A small amount of water was then added to the mixture, activating the sperm and allowing fertilisation of the eggs. The eggs were then placed in a hatchery system filled with aged tap water (Figure 3.3 (6)). Twenty-four hours later, the eggs hatched (Figure 3.3 (7)). The *C. gariepinus* offspring were kept in the
hatchery system for a week and then transferred to the 800ℓ glass tanks (containing RRO-water) in the environmental room. Glass shelters were put in every tank to minimise stress (Figure 3.3 (9-10). The fry was then allowed to grow until sexually mature. Sexually mature specimens selected for the assessments were obtained from two breeding processes as described above to obtain the desired sample size (eggs hatched 8 May 2004 and 28 July 2004). The specimens were sampled over three sampling periods for the histological assessment (25 February 2005, 5 April 2005 and 25 May 2005) depending on their size and sexual maturity.

(ii) *Oreochromis mossambicus*

This species is easily maintained under laboratory conditions and specimens were allowed to breed spontaneously by manipulating optimal breeding conditions. Before the breeding process, the genetic purity of the resident breeding pair population were confirmed as it has been documented that crossbreeding between *O. mossambicus* and *O. niloticus* can occur (Agnèse, 1998; Moralee *et al.*, 2000). The aquarium stock of *O. mossambicus* specimens was confirmed to be of pure strain (Moralee *et al.*, 2000). Sexually mature *O. mossambicus* specimens were selected from a temperature controlled aquarium at the University of Johannesburg’s Aquatic Research Facilities (one adult male and three adult females) (Figure 3.4 (1-2)). These specimens were placed in the 800ℓ glass tanks filled with RRO-water in the environmental room (Figure 3.4 (3)).

*Oreochromis mossambicus* is a mouth-brooder species. After the eggs are laid by the female and fertilised by the male, they are incubated in the mouth of the female (Skelton, 1993). The selected breeding females were monitored on a daily basis for any sign of possible eggs indicated by a dark colouration of the mouth area. After approximately two weeks, *O. mossambicus* eggs hatched. The male was immediately removed from the tank. After an additional seven day period the fry were released from the female’s mouth and the female was removed from the tank. The fry were allowed to grow for a period of ± 1 year. This species was bred once and the
eggs hatched on 27 May 2004. The specimens were sampled on 23 September 2005.

(iii) F2 Group
Early in the one year growth period, some of the *O. mossambicus* females became sexually mature and an unsuspected F2 generation was produced (from hereon referred to as the F2 group). These specimens were then transferred to a separate growth tank (Figure 3.4 (4-5)). It was decided to include these young specimens (once 4 months old) in the study as whole-mount histological preparations. However, they did not form part of the qualitative and quantitative histological assessment, but were included to better understand the general histology and anatomical orientation of the selected target organs used in the histological assessment. The F2 group specimens hatched 15 February 2005. The specimens were sampled on 21 June 2005.

3.2.1.4 – Feeding methods
The choice of high quality food and the subsequent feeding methods used were specifically chosen to favour good fish health. All *C. gariepinus* specimens were fed twice daily throughout their growing period (± 4% of body mass per serving which corresponds with the percentage of food typically used for farmed fish (Craig, 2002)). Initially, three days after the small *C. gariepinus* eggs hatched, the specimens were fed on egg yolk. Once the fish had grown to a suitable size (± 15 mm), they were fed on TetraMin Growth Complete Flaked Food, TetraPrima Complete Granular Food (Tetra, Germany - © 2006 Spectrum Brands) and Campcon frozen blood worms (Figure 3.4 (6)). All *O. mossambicus* specimens were fed twice daily (± 4% of body mass per serving) throughout the growing period. The fish were exclusively fed on TetraMin Growth Complete Flaked Food and TetraPrima Complete Granular Food (Tetra, Germany - © 2006 Spectrum Brands) (Figure 3.4(6)). The nutritional value of the above mentioned food products are listed in Table 3.1 as indicated by the manufacturer.
### Table 3.1: Nutritional analyses of food used during the breeding process.

<table>
<thead>
<tr>
<th>FOOD PRODUCT</th>
<th>INGREDIENTS</th>
<th>ANALYSIS</th>
</tr>
</thead>
</table>
| TetraMin Growth Complete Flaked Food | • Fish  
• Fish derivatives  
• Cereals  
• Yeasts  
• Vegetable protein extracts  
• Molluscs  
• EEC permitted colourants and preservatives | Protein 47%  
Oil 8.0%  
Fiber 2.0%  
Ash 10.0%  
Moisture 6.0% |
|  |  | Added vitamins per kg  
Vitamin A 15 500 IE  
Vitamin D3 970 IE  
Vitamin E 50 mg  
L-ascorbyl-2-polyphosphate 122 mg |
| Campcon frozen blood worms | • Bloodworm  
• Water  
• Vitamins | Crude protein 6.50%  
Crude fat 0.57%  
Crude fiber 0.50%  
Moisture 90.7% |
|  |  |  |
| TetraPrima Complete Granular Food | • Fish  
• Fish derivatives  
• Cereals  
• Yeasts  
• Vegetable protein extracts  
• Molluscs and crustaceans  
• EEC permitted colourants and preservatives  
• Derivatives of vegetable origin  
• Minerals  
• Algae  
• Oils and fats  
• L-lysine monochlorhydrate  
• Lecithin  
• Citric acid | Protein 47.5%  
Oil 6.5%  
Fiber 2.0%  
Ash 10.5%  
Moisture 6.0% |
|  |  | Added vitamins per kg  
Vitamin A 29 770 IU  
Vitamin D3 1 860 IU  
Vitamin E 200 mg  
L-ascorbyl-2-polyphosphate 137 mg |

### 3.2.2 Water quality monitoring

Water quality is a term used to describe the chemical, physical and biological characteristics of water (DWAF, 2002). Water quality parameters should be monitored on a regular basis. This record of water quality should demonstrate that conditions in the aquatic system are stable and unlikely to stress the organisms.
MATERIAL & METHODS

CHAPTER 3

3.2.2.1 - Physical water quality monitoring

Physical water quality parameters for each breeding tank were monitored on a weekly basis at random time intervals. Readings were recorded for five physical parameters: (1) temperature; (2) conductivity; (3) total dissolved salts (TDS); (4) dissolved oxygen (DO); (5) and pH. The readings were taken using a Cyberscan DO 100-dissolved oxygen and conductivity meter as well as a WTW InoLab pH meter (Figure 3.1 (9)).

(i) **Temperature** expressed in degrees Celsius (°C) is an important parameter regarding its affect on the solubility of oxygen in water, the rate of photosynthesis by algae and higher plants, the metabolic rates of aquatic organisms, and the sensitivity of organisms to toxic wastes, parasites and diseases (Ida, 1997).

As mentioned in Skelton (1993), the water temperature range for *O. mossambicus* is estimated between 15°C and 42°C, but prefers warm temperatures of 22°C and above. According to Teugels (1986), *C. gariepinus* can tolerate temperatures ranging from 8°C to 35°C but their optimal temperature for growth is 28 - 30°C. As both species were bred in the same environmental room, for the purpose of this study, water temperature was kept constant at 26 ± 1°C during the breeding period.

(ii) **Conductivity** is a numerical expression of the ability of water to conduct an electrical current, resulting from the presence of charged species in solution (DWAF, 1993). Conductivity is influenced by an array of factors, for example the concentration and nature of the solutes, the degree of dissociation into ions, the electric charge, the mobility of the ions, and the temperature of the solution (DWAF, 1993). Conductivity was measured in micro Siemens per centimetre (µS/cm) using a Cyberscan DO 100-dissolved oxygen and conductivity meter.
(iii) **Total Dissolved Salts (TDS)** is a measure of the amount of soluble material in a sample of water, which represents the total quantity of dissolved matter, both organic and inorganic, and ionised or un-ionised (Dallas and Day, 1993). This parameter was expressed as parts per million (ppm).

(iv) **Dissolved Oxygen (DO)** measures the amount of gaseous oxygen \( (O_2) \) dissolved in an aqueous solution. The maintenance of adequate dissolved oxygen (DO) concentrations is critical for the survival and functioning of the aquatic biota. This parameter is commonly expressed as a concentration in terms of milligrams per litre (mg/l) and/or as a percentage (%). Dissolved oxygen concentrations for surface water ranges from 0 mg/l in extremely poor water conditions to as high as 15 mg/l in \( 0^\circ C \) water. Levels below 5 mg/l are however stressful to most aquatic organisms (Ida, 1997).

(v) **pH** is a general measure of the acidity or alkalinity of a water sample (Ida, 1997) and is indicated on a scale of 0 to 14 (acidic \( \rightarrow \) neutral (7) \( \rightarrow \) alkaline). The pH of natural waters is determined largely by geological and atmospheric influences.

### 3.2.2.2 - Chemical water quality monitoring

Water from all breeding tanks was sampled and prepared for Inductively Coupled Plasma Mass Spectroscopy (ICP-MS) analysis. ICP-MS is a type of mass spectrometry that is highly sensitive and capable of analyses of a range of metals and several non-metals at below 1 ppb. Selected samples were also prepared for Atomic Absorption Spectroscopy (AAS) for selected metal analysis.

For this purpose, water samples (14 ml) for these two procedures were collected in 15 ml plastic conical tubes (Figure 3.1 (8)), acidified with 1 ml 65% Nitric acid (Merck) immediately after collection, and stored at 4°C until further analysis at an independent certified laboratory. Representative water samples were also collected from the breeding setup for analysis including total hardness, ammonia, nitrates and nitrites.
According to Barnhoorn et al. (2003) and Fatoki (2003), environmental health researchers reported that endocrine-disrupting chemicals (EDC’s) are present in South African waters with some sources showing estrogenic activity (Aneck-Hahn, 2002; De Jager et al., 2002; Timmerman, 2003). To confirm EDC free baseline conditions, additional water samples were also collected for selected EDC analyses. These samples (2.5l), were collected in glass bottles (Figure 3.1 (8)), pre-rinsed in 100% methanol (Merck) and stored at 4°C until further analysis at an independent certified laboratory using Gas Chromatography Mass Spectroscopy (GC-MS).

3.2.3 Specimen monitoring

Throughout the breeding process, specimens were monitored regarding their behaviour, mortality and growth rate. These three aspects were monitored to identify and remove any possible sick specimens from the system. During the breeding process, it was observed that C. gariepinus fry and juveniles displayed cannibalistic behaviour. It was therefore necessary to transfer stronger, bigger specimens to separate tanks. This was done during the first few months to ensure that the desired sample size from the same brood would be obtained without losing specimens due to this behaviour.
3.3 PHASE 2: SPECIMEN SAMPLING AND NECROPSY

The objective of the second phase of the project was to: (1) collect sexually mature specimens bred during phase 1 for histological assessment (preferably 50:50 sex ratios); (2) perform a necropsy on all individuals; (3) and calculate somatic indices and the condition factor for each specimen. The execution of a detailed necropsy, along with a record of the index values as mentioned above, provide the researcher with supporting data which might correlate with and/or support the diagnosis of possible pathological conditions during the histological assessment, and collectively indicate general fish health.

3.3.1 Sample size and gender ratio

As stated by Bernet et al. (1999), there is no absolute recommendation for an optimal sample size because it varies according to the objective of the study. For this project, a sample size of 20 specimens (± 50:50 sex ratio) per species was chosen. Additionally (as mentioned in section 3.2.1.3 (iii)), five, four month old second generation *O. mossambicus* specimens (F2 group) were selected for an overview of the general histological structure and orientation of selected target organs.

The sex of each specimen, was classified either according to secondary sexually characteristics (the presence of a distinct uro-genital papilla) in *C. gariepinus*, or according to the breeding colours and pronounced enlarged upper mandible of male *O. mossambicus* specimens (Figure 3.5 (2, 3 & 7)).

The sexually mature specimens of both species were randomly selected for histological analyses and assigned a three digit reference number (Figure 3.5 (4)), for example **O01** or **C15**. The first letter indicates the species: **O** = *Oreochromis* or **C** = *Clarias* and the last two digits indicate the order of sampling: **01** = first specimen or **15** = fifteenth specimen. These reference numbers are specifically important in...
histological studies where all organs are processed separately. For the F2 group, reference numbers OF201 - OF205 were assigned.

3.3.2 Necropsy

A necropsy was performed to identify and examine any macroscopic abnormalities regarding external features, blood and internal organs. The aspects examined in the necropsy are similar to those included in the Quantitative Health Assessment Index for Rapid Evaluation of Fish Condition in the Field by Adams et al. (1993).

Each specimen was removed from the tank and placed on a clean plastic dissection board (Figure 3.5 (6)). The eyes were covered with a paper towel to minimise stress. Blood was drawn from the dorsal caudal artery at the posterior region of the lateral line (Figure 3.5 (5 & 9)), using a new sterile disposable syringe (1 ml) and a 26G needle (NPC) for each individual. For total plasma protein concentration (TP) determination, blood was collected in 4 ml heparin vacutainers for O. mossambicus and 4.5 ml EDTA vacutainers for C. gariepinus. The samples were centrifuged at 30 000 rpm for 15 minutes. Plasma was transferred to 2.0 ml Eppendorf Safe-Lock tubes and stored at -40°C. Samples were prepared using a Total Protein Kit (Roche), and analysed in triplicate using a Universal Microplate reader. In addition, a second blood sample was collected per specimen for haematocrit determination using standard techniques. Blood was collected in micro-haematocrit capillary tubes, centrifuged at 30 000 rpm for 10 minutes and analysed using a Hawksley micro – haematocrit reader.

Following the blood collection, a macroscopic examination of the external features of each specimen (opercula, eyes, skin and fins were executed to identify any aberrations, erosion or abnormalities as well as the presence of any possible external parasites. Each specimen was weighed and the standard and total lengths were recorded (Figure 3.5 (8)). These measurements were then used to calculate the condition factor for each specimen.
The condition factor quantifies fish condition and is an indicator of general fish health (Adams et al., 1993). Although the condition factor is usually more applicable to field specimens, it was included in this study to establish a baseline value for specimens of which the nutritional information and history were known. Fulton’s condition factor (CF) was calculated for each specimen in this study. A value of (1) indicates a good health status. It was calculated as described by Carlander (1969):

\[
CF = \frac{\text{Total body mass (g)} \times 10^5}{\text{Total body length}^3 \text{ (mm)}}
\]

After recording the body measurements, each specimen was sacrificed by severing the spinal cord just anterior to the dorsal fin. Selected organs were dissected out using new scalpel blades and clean dissection tools. A macroscopic examination of major visceral organs (liver and bile colour, spleen, hindgut, kidney, gills, heart, gonads and pseudobranch) (Figures 3.6 and 3.7) was performed by identifying any possible nodules, cysts, growths, inflammation or discoularation of any of the organs. The percentage mesenteric fat present was also determined (section 3.3.3) and any internal parasites were identified if present.

The selected target organs for the histological assessment as well as those examined and measured as part of the necropsy were removed and sampled in the following order: gills → heart → liver (and gall bladder for bile colour determination) → spleen → gonads → kidney. Once dissected out, the liver, spleen, gonad and mesenteric fat mass were recorded for the calculation of somatic indices. In addition, the testis length was recorded. The liver, gills, gonads, kidney and heart were then sampled for histological analyses as will be further discussed in section 3.4.

### 3.3.3 Somatic indices

Somatic indices indicate the organ mass relevant to the body mass and can indicate possible hypertrophy or atrophy after contaminant exposure. For this project, the somatic indices for the liver (Hepato-somatic index), spleen (Spleno-somatic index) and gonads (Gonado-somatic index) were determined as well as the mesenteric fat
The hepato-somatic index (HSI) is calculated by dividing the total liver mass by the total body mass and is expressed as a percentage.

\[
\text{HSI} \, (\%) = \left( \frac{\text{liver mass (g)}}{\text{body mass (g)}} \right) \times 100
\]

The spleno-somatic index (SSI) is calculated by dividing the total spleen mass by the total body mass and is expressed as a percentage.

\[
\text{SSI} \, (\%) = \left( \frac{\text{spleen mass (g)}}{\text{body mass (g)}} \right) \times 100
\]

The gonado-somatic index (GSI) was calculated by dividing the total gonad mass by the total body mass and is expressed as a percentage.

\[
\text{GSI} \, (\%) = \left( \frac{\text{combined gonad mass (g)}}{\text{body mass (g)}} \right) \times 100
\]

The fat index (FI) was calculated by dividing the total mesenteric fat mass by the total body mass and is expressed as a percentage. However, mesenteric fat deposits in fish can vary widely depending not only on food availability and feeding regimes, but on other interacting factors such as fish size, sex, time of year and stress level. Because of these interacting variables, the fat index can not be assigned normal or abnormal values (Adams et al., 1993) but merely an index value associated with the condition of the specific specimen.

\[
\text{FI} \, (\text{Fat}\%) = \left( \frac{\text{mesenteric fat mass (g)}}{\text{body mass (g)}} \right) \times 100
\]
3.4 PHASE 3: TISSUE SAMPLING AND HISTOLOGICAL PROCESSING

Following the necropsy, the objective of the third phase of the project was to collect histological samples from the selected target organs from both species, and to prepare these samples for the histological assessment. Tissue sampling and histological processing includes all procedures executed from the initial removal of the organ from the test specimen, to the final microscope slide used for light microscopy analysis.

3.4.1 Tissue sampling

Tissue sampling included the following successive steps: (1) fixation of tissue samples; (2) decalcification (if applicable or necessary); (3) tissue processing (washing, dehydration, clearing, infiltration and embedding); (4) sectioning; (5) staining; (6) and mounting. The execution of proper histological processing will avoid or minimise the occurrence of any artifactual changes to the target organ’s histological structure. It will also subsequently ensure an accurate microscopic analysis.

Tissue samples from each of the selected target organs of both species were sampled at specific, predetermined morphological regions, or, included the sampling of the entire organ (Figure 3.8). These morphological regions (usually midsections of an area) were chosen according to the structure and/or function of each organ. As the aim of this study is to provide baseline histological data as reference for exposed specimens, these sample regions also correspond with those used in histopathological assessments in toxicity studies. The liver, gills, gonads and heart are usually easily sampled. However, due to the anatomical position of fish kidney, a specific sampling method was used. To avoid damaging the kidney of the smaller *O. mossambicus* specimens, the entire kidney was removed and fixed together with the dorsally located spinal cord and skeletal muscle. Consequently, it was easier to remove proper kidney samples after fixation.
The larger size and specific shape of *C. gariepinus* kidney allowed for relatively easy sampling without having to remove the entire organ. The time period between dissection and fixation was kept to a minimum to avoid any possible anoxic changes to occur. Each organ was then sampled (± 4mm x 4mm x 4mm) at the specified morphological regions (Figure 3.8). For the F2 group, it was decided to randomly select five specimens from the group. These fish were weighed (total body mass) and measured (total length), and sacrificed by severing the spinal cord and were subsequently fixed as whole specimens.

### 3.4.2 Histological processing

#### 3.4.2.1 – Fixation

Samples of the liver, heart, gills and kidney were fixed for 72 hours in 10% neutrally buffered formalin (10%NBF) (Humason, 1979). The testes, ovaries and whole specimens (F2 group) were fixed for 24 hours in Bouin’s solution. For the F2 group, the tail end and head (just behind the opercula opening) of each specimen was excised to allow proper infiltration of the fixative. Labelled 50mℓ screw top glass bottles were used for the fixation process (Figure 3.8 (7)). The amount of fixative was predetermined to ensure a 10:1 ratio of fixative to tissue volume, to allow optimal and proper penetration and to prevent tissue autolysis to occur. Bouin’s solution was the choice of fixative for the whole specimens because two of its components, Acetic acid and Picric acid, decalcify bone adequately and therefore minimise sectioning problems. Tissue fixed with Bouin’s solution also seems to stain more vividly with routine histological stains such as H&E (Yasutake and Wales, 1983).

#### 3.4.2.2 – Decalcification

The F2 group was further decalcified after fixation to ensure ease during sectioning. For this purpose, specimens were placed in an EDTA solution for 168 hours post-fixation, before routine tissue processing continued.
3.4.2.3 – Tissue processing

After fixation, samples fixed in 10% NBF were washed in running tap water for ± 12 hours and then dehydrated in rising concentrations of ethanol (30% → 50% → 70% → 80% → 90% → 95% → 100% → 100%) for 30 – 60 minutes per ethanol concentration (van Dyk, 2003). Samples fixed in Bouin’s solution were rinsed in water and then washed in several replacements of 70% ethanol preceding further dehydration as mentioned above. All samples were then cleared (5 - 10 min) in Xylene until transparent or clear. Clearing time was not specific for all samples and was influenced by the tissue type and the size of the sample. Once cleared, the samples were transferred to a series of rising concentrations of liquid paraffin wax in an oven (60 °C). Proper infiltration with paraffin wax allow for easy and proper sectioning. The samples were then embedded in paraffin wax blocks (± 2cm x 2cm x 2cm).

3.4.2.4 – Sectioning

The wax blocks were allowed to cool properly and stored at 4°C for 24 hours before sectioning. Sections, 5µm thick, were cut for each sample to produce three slides per organ. Two slides were reserved for H&E staining and the third were stored as a backup, or, if required for other staining techniques. Disposable microtome blades were used and replaced after each organ. The sections were stretched in a distilled water bath (45°C) and then positioned on glass microscope slides using a distilled water and albumin solution. Once dried, this solution prevents sections washing off during the staining process. The microscope slides were air dried and stored in a dry oven (45 °C) for 12 hours.

3.4.2.5 – Staining and mounting

Two histological stains were employed and included a routine Haematoxylin and Eosin (H&E) stain as well as the Periodic Acid Schiff (PAS) stain. The procedure followed for each of these stains was according to the adapted methods as listed by Van Dyk (2003). Once stained, all slides were mounted with Entellan.
3.5 PHASE 4: QUALITATIVE HISTOLOGICAL ASSESSMENT

The objective of the fourth phase of the project was to qualitatively assess, and describe the histology of the selected target organs, and thereby complying with the first main objective of the study (refer to Chapter 1, section 1.2).

3.5.1 Light microscopy analysis and histological description

All samples prepared for histological assessment were analysed using light microscopy (Leica DMLS – ICCA). Depending on the histological detail of the different structures within organs, slides were examined using four objective levels (4X, 20X 40X and 100X -oil). Digital images were taken to show the histological structure of selected target organs and were analysed using IM50 Image Manager Software. All histological sections were carefully examined and incorporated in the final microscopic description of the normal histology of the selected target organs.

3.5.2 Gonadal developmental staging

As part of the qualitative histological assessment, the developmental stage of the gonads were determined by identifying certain histological features as defined by McDonald et al. (2000) in the Biomonitoring of Environmental Status and Trends Program (BEST) (Schmitt and Dethloff, 2000) (Table 3.2), adapted from Treasurer and Holiday (1981), Nagahama, (1983), Rodriquez et al. (1995) and Goodbred et al. (1997). Subsequently the sexual maturity of the sample groups could be confirmed. Oocyte diameter was measured using IM50 Image Manager Software.
### Table 3.2 Gonadal reproductive stage determination (McDonald et al., 2000).
(Adapted from Treasurer and Holiday (1981), Nagahama (1983), Rodríguez et al. (1995) and Goodbred et al. (1997)).

<table>
<thead>
<tr>
<th>Stage</th>
<th>Testis characteristics (Male)</th>
<th>Ovary characteristics (Female)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Undeveloped (immature):</td>
<td>Undeveloped (immature):</td>
</tr>
<tr>
<td></td>
<td>Little to no activity in germinal epithelium; immature stages of spermatogenesis (largely spermatocytes); no spermatozoa observed.</td>
<td>Pre-vitellogenic oocytes observed exclusively; oocyte diameter &lt; 250 µm: cytoplasm stains basophilic with H&amp;E.</td>
</tr>
<tr>
<td>1</td>
<td>Early spermatocytes:</td>
<td>Early development:</td>
</tr>
<tr>
<td></td>
<td>Mostly thin germinal epithelium with scattered spermatogenic activity; spermatocytes to spermatids predominate; few spermatozoa observed.</td>
<td>&gt; 90% pre-vitellogenic, remaining oocytes early to mid-vitellogenic; oocytes slightly larger (up to 300 µm); late peri-nucleolus through cortical alveolar stages.</td>
</tr>
<tr>
<td>2</td>
<td>Mid-spermatogenic:</td>
<td>Mid-development:</td>
</tr>
<tr>
<td></td>
<td>Germinal epithelia are of moderate proliferation and maturation of spermatozoa and equal mix of spermatocytes, spermatids and spermatozoa, present.</td>
<td>Majority of observed follicles are early and mid-vitellogenic; oocytes larger, 300-600 µm diameter, and containing peripheral yolk vesicles; globular and uniformly thick chorion (5-10 µm in black basses, 10-20 µm in common carp); cytoplasm is basophilic, yolk globules eosinophilic.</td>
</tr>
<tr>
<td>3</td>
<td>Late spermatogenic:</td>
<td>Late development:</td>
</tr>
<tr>
<td></td>
<td>Thick germinal epithelium; diffuse regions of proliferation and maturation of spermatozoa; all stages of development are represented, but spermatozoa predominate.</td>
<td>Majority of developing follicles are late vitellogenic; oocyte diameter is 600-1000 µm; eosinophilic yolk globules distributed throughout the cytoplasm; chorion thickness is 10-30 µm in black basses, 40-50 µm in common carp.</td>
</tr>
<tr>
<td>4</td>
<td>N/A</td>
<td>Late development/hydrated:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Majority of developing follicles are late vitellogenic; follicles are much larger (&gt;1000 µm).</td>
</tr>
<tr>
<td>5</td>
<td>N/A</td>
<td>Post-ovulatory:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Spent follicles, remnants of the theca externa and granulose.</td>
</tr>
</tbody>
</table>
3.6 PHASE 5: QUANTITATIVE HISTOLOGICAL ASSESSMENT

The protocol used for the quantitative histological assessment was published by Bernet et al. (1999). This assessment tool has since formed part of various published papers (Schmidt et al., 1999; Schmidt-Posthaus et al., 2001; Chun-Yao et al., 2004; Bernet et al., 2004; Nero et al., 2005) and is also used in histological assessments in toxicity studies within the department. This protocol is appropriate for these studies because as stated by the authors it is: (1) applicable to any given organ; (2) leads to a standardised quantification method, because, by using specific criteria for the classification of histological alterations, variation among investigators is reduced; (3) it allows the possibility of legitimate comparison between different studies; (4) and with restrictions, between different organs (Bernet et al., 1999).

It is important to note that this assessment tool was developed to objectively compare the histological integrity of specimens from polluted and unpolluted systems, and to better understand the significance of histopathological findings after contaminant exposure. However, no reference intervals or values have been included by the authors that categorise normal and pathological conditions, as this may vary between different species. In other words, no range of normal histological characteristics has been stipulated. It is only stated that the higher the index value, the more severe the organs are affected (Bernet et al., 2004). Thus, in most studies, index values calculated for unpolluted sites (control groups) serves as reference for those calculated for polluted sites.

Hence, the objective of the fifth phase of the project was to quantitatively assess the histological integrity of the selected target organs and thereby, together with the somatic indices, condition factor and blood parameters, establish associated quantitative baseline values and reference intervals for *C. gariepinus* and *O. mossambicus*. These results therefore complied with the second main objective of this study (refer to Chapter 1, section 1.2). For use in our laboratory, the index assessment forms, stipulating the assessment criteria, were slightly modified in terms
of abbreviations used and the description of the histological alterations listed. However, these modifications do not affect the index’s applications or calculations in any way. The quantitative histological assessment was executed by three histology researchers by means of a multi-headed microscope to ensure an accurate and unbiased assessment. In the following section, the protocol for the quantitative assessment will be described as stated by Bernet et al. (1999).

3.6.1 Quantitative assessment protocol

3.6.1.1 – Assessment structure
For each target organ chosen for this study, the respective pathological changes (from here on referred to as alterations), are classified into five reaction patterns (rp). According to Bernet et al. (1999), these patterns represents a slight modification of the classification of Takashima and Hibiya (1995), and are also in accordance with the recommendations of Sindermann (1979), who proposed this classification for the histopathological assessment of experimental studies. Each reaction pattern includes several alterations (alt) which concern either functional units of the organ or an entire organ. The five reaction patterns and the subsequent alteration under each pattern are cumulatively referred to as the histological description of that specific organ in terms of the assessment protocol.

Reaction pattern 1 (rp₁): Circulatory disturbances (CD)
Circulatory disturbances result from a pathological condition regarding the flow of blood and tissue fluid. Fluid content alterations in tissues related to inflammatory processes are considered in reaction pattern 4. The alterations included in reaction pattern 1 are:

1. **Haemorrhage** (Blood leaking from blood vessels).
2. **Hyphaema** (Congestion of blood caused by venous or arterial processes).
3. **Aneurysm** (Dilation of arterial blood vessels).
(2) **Intercellular oedema** (Stagnant tissue fluid which has leaked from capillaries into tissue).

**Reaction pattern 2 (rp$_2$): Regressive changes (RC) ▼**

Regressive changes are processes which terminate in a functional reduction or loss of an organ. The alterations included in reaction pattern 2 are:

1. **Architectural and structural alterations** (Changes in tissue structure as well as in the shape and arrangement of cells).
2. **Plasma alterations** (Changes in cellular plasma caused by hyaline droplets (granular degeneration), colloidal droplets (colloid degeneration), degenerative fatty vacuolation or hydropic glycogen droplets (glycogen degeneration), calcareous degeneration, and thickening of the fine fibres of connective tissue (hyaline degeneration)).
3. **Deposits** (Intercellular accumulations of substances primarily caused by degenerative processes).
4. **Nuclear alterations** (Changes in the nuclear shape and structure of chromatin: pyknosis and karyorrhexis).
5. **Atrophy** (Reduction in volume of cells and/or decreasing amount of intercellular substances).
6. **Necrosis** (Morphological state of a cell or a tissue which appears after irreversible loss of cell function).

**Reaction pattern 3 (rp$_3$): Progressive changes (PC) ▼**

Progressive changes are processes which lead to an increased activity of cells or tissues. The alterations included in reaction pattern 3 are:

1. **Hypertrophy** (Enlargement of cell volume or tissue without increase in cell number).
(2) **Hyperplasia** (Enlargement of tissue or organ by a greater number of cells without change in the volume of the cell).

**Reaction pattern 4 (rp₄): Inflammation (I) ▼**

Inflammatory changes are often associated with processes belonging to other reaction patterns. Therefore it is often difficult to attribute inflammatory changes to one single reaction pattern. Hence, the authors use the term “inflammation” in a very strict sense. The alterations included in reaction pattern 4 are:

1. **Exudate** (Fluid containing a high protein concentration and a large amount of cellular debris exuded from blood and lymph vessels).
2. **Activation of the reticulo-endothelial system (RES)** (Hypertrophy of the RES, which consist of endothelial cells and macrophages that line the small blood vessels).
3. **Infiltration** (Leucocytes penetrating the walls of the blood vessels and infiltrating the surrounding tissue).

**Reaction pattern 5 (rp₅): Tumour (T) ▼**

A tumour is an uncontrolled cell and tissue proliferation (autonomous proliferation). Tumours are divided into two classes:

1. **Benign tumours** (Differentiated cells which replace or displace the original tissue – these tumour cells resemble the cells of the normal tissue).
2. **Malignant tumours** (Poorly differentiated, rapidly multiplying cells which invade and destroy resident tissues; metastasis may be observed).

**3.6.1.2 - Importance factor (w)**

According to Bernet *et al.* (1999), the relevance of a histological alteration depends on its pathological importance, i.e. how the specific alteration affects organ function.
and the ability of a fish to survive. The pathological importance is therefore taken into account in the assessment protocol by assigning an importance factor ($w$) to every alteration listed in the histological description. The alterations are classified into three importance factors (1, 2 & 3) depending on the reversibility of specific alteration:

<table>
<thead>
<tr>
<th>Value</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Minimal pathological importance. The alteration is easily reversible as the exposure to irritants (toxicants etc.) ends.</td>
</tr>
<tr>
<td>2</td>
<td>Moderate pathological importance. The alteration is reversible in most cases if the irritant is neutralised.</td>
</tr>
<tr>
<td>3</td>
<td>Marked pathological importance. The lesion is generally irreversible, leading to partial or total loss of the organ function.</td>
</tr>
</tbody>
</table>

3.6.1.3 - Score value ($a$)
In the assessment protocol, every alteration is assessed by assigning a score value ranging from zero to six (0, 2, 4 & 6) to each alteration depending on the degree and extent of the specific alteration:

<table>
<thead>
<tr>
<th>Value</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No occurrence of the alteration</td>
</tr>
<tr>
<td>2</td>
<td>Mild occurrence of the alteration</td>
</tr>
<tr>
<td>4</td>
<td>Moderate occurrence of the alteration</td>
</tr>
<tr>
<td>6</td>
<td>Severe occurrence of the alteration</td>
</tr>
</tbody>
</table>

3.6.1.4 - Mathematical calculation of indices
By assigning an importance factor ($w$) and a score value ($a$) to each alteration identified in a target organ, four different indices can be calculated: (1) Organ index
(2) Reaction index

(3) Total organ index

(4) Total reaction index. If the lesions within one organ are studied only, the first two indices are applicable. If the several organs of a fish are examined, the latter two indices will be applicable.

(1) Organ index \((I_{org})\)

**Goal:** Determine the degree of damage to a single organ. The index value is calculated by the sum \((\sum)\) of the multiplied score values \((a)\) and importance factors \((w)\) of all alterations \((alt)\) identified in all the reaction patterns \((rp)\) of a specific organ \((org)\). A high index value indicates a high degree of damage. Calculating the organ index allows a comparison between the extent of damage in the same organ in different individuals.

\[
(I_{org}) = \sum_{rp} \sum_{alt} (a_{org all rp all alt} \times w_{org all rp all alt})
\]

(2) Reaction index \((I_{rp})\)

**Goal:** Determine the degree of damage within a single reaction pattern to a single organ. The index value is calculated by the sum of the multiplied score values \((a)\) and importance values \((w)\) of all alterations \((alt)\) identified in a single reaction pattern \((rp)\) of a specific organ. The quality of the lesions in an organ is expressed by the reaction index.

\[
(I_{rp}) = \sum_{alt} (a_{1 org 1 rp all alt} \times w_{1 org 1 rp all alt})
\]

(3) Total organ index \((TotI_{org})\)

**Goal:** Determine the cumulative degree of damage to all target organs examined. The index value is calculated by the sum of the multiplied score values \((a)\) and importance factors \((w)\) of all alterations \((alt)\) identified in all the reaction patterns \((rp)\) of all organs \((org)\). This index represents a measure of the overall health status based on histological alterations.

\[
(TotI_{org}) = \sum_{org} \sum_{rp} \sum_{alt} (a_{all org all rp all alt} \times w_{all org all rp all alt})
\]
(4) Total reaction index (TotI\textsubscript{rp})

**Goal:** Determine the degree of damage within a single reaction pattern to all organs. The index value is calculated by the sum of the multiplied score values \((a)\) and importance factors \((w)\) of all alterations \((alt)\) identified in a single reaction pattern \((rp)\) of all organs \((org)\). This index represents the quality of the histological alterations in all examined organs of an individual fish.

\[
(TotI_{rp}) = \sum_{rp} \sum_{alt} (a_{all\ org\ rp\ all\ alt} \times w_{all\ org\ rp\ all\ alt})
\]

**3.6.2 Statistical analyses**

All applicable data were statistically analysed at the statistical consultation services (STATKON) of the University of Johannesburg. All specimen data (body mass, total length, standard length and age) and somatic indices, condition factor and necropsy data are presented as frequencies and percentages with subsequent means ± standard deviation (SD). Statistical differences between males and females of selected variables were determined using analysis of variance (Independent T-test) and Levene’s test for equality of variance. Pearson’s correlation coefficients were applied to examine relationships between body mass, total length, HSI, GSI, SSI, FI, CF, haematocrit and total protein for each species. Differences were considered to be significant at \(p \leq 0.05\). All water quality data are presented as means ± standard deviation (SD). For the Quantitative Histological Assessment the organ index, total organ index, reaction index and total reaction index were calculated for each specimen. The means for all organ indices and reaction indices were calculated for both species as well as for the total organ and reaction indices.
3.7 REFERENCES


MATERIAL & METHODS


