Thermal induction of tetraploidy and assessment of selected DNA quantification methods in African sharptooth catfish, *Clarias gariepinus*

by

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Declaration

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Date: April 2022

Dedication

This work is dedicated to my family

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Abstract

Producing viable African sharptooth catfish (*C. gariepinus*) tetraploids has become imperative and potentially beneficial in aquaculture for mass production of sterile triploids, which can be generated by mating natural diploids with tetraploids. The objectives of the current study were to assess the correct shock initiation time between egg fertilisation and heat shock induction of tetraploidy and to validate three methods for DNA quantification namely flow cytometry (Sysmex® PI method), chromosome preparations (Giemsa staining) and silver nitrate staining to quantify Nucleolar organising regions (NORs).

Properly conditioned broodstock with good physical condition (CF 3.1 ± 0.3%), pre-fertilised egg quality (> 1000µ egg diameter) and fecundity (RSI >11%) were employed for spawning, since low quality fertilised eggs (< 1000 µ egg diameter) could not survive ploidy manipulation procedures. To successfully induce tetraploidy, fertilised eggs incubated at 25 °C ± 0.1 were immersed in a water bath (40.5 °C ± 0.1) for 2 minutes at shock initiation times (IT) from 40 – 50 minutes post fertilisation. The IT treatments within this range were spaced one minute apart i.e. 40 – 45 minutes and the remaining IT treatments were 30 seconds apart i.e. 45.5 – 50 minutes. Fertilisation completion was set at 15 seconds after the addition of activated sperm to eggs. The thermal shock led to a decrease in hatching and larval survival percentage, and with a progressive trend from about 41% (40 minutes post-fertilisation treatment) to 1% (50 minutes post fertilisation treatment). Ploidy status determination of African sharptooth catfish was evaluated by testing procedures respectively developed for chromosome preparations, silver nitrate-NOR staining and flow cytometry. Flow cytometry was selected as the preferred DNA quantification method when excluding erythrocyte karyotyping (thrombosis issues), since the other procedures proved to be inconsistent, tedious and unreliable. The slightly modified *CyStain® PI Absolute P* protocol used for flow cytometry

analysis in the current investigation proved to be a rapid and efficient method for processing and determining the karyotype of minced 2 DPH larvae or finclips (non-destructive option). Production of tetraploid offspring (60 – 90+%) was predominant when heat shocked from the 45 minute to 47 minute setpoint initiation times post-fertilisation, with peak frequency of occurrence (>90%) at 47th minutes post fertilisation induction setpoint. Microscopic determined nuclear sizing (erythrocytes) and nuclear sizing electronically by Coulter counter^{TM} efficacies should be tested for comparison in future studies to flow cytometry DNA quantification in terms of ploidy verification accuracy, test rapidity, and cost effectiveness. The outcome of the current work unlocks the opportunity to produce all triploid *C. gariepinus* offspring by crossing female tetraploid with male diploids, and could potentially facilitate in the future commercial production of triploid African sharptooth catfish in regulated eco-sensitive regions.

Notes

This thesis is compiled as five chapters. The layout covers the general introduction, literature review, methods, results and discussion and conclusion of the study. The opinions expressed, together with the conclusions arrived at in this thesis are those of the author and are not necessarily attributed by the Department of Forestry, Fisheries and Enviromental Affairs, the Department of Animal Sciences and Department of Genetics, Stellenbosch University.

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List of abbreviations

ACU	Animal Care Unit
CAF	Central Analytical Facilities
CL	Caudal Length
CPD	Citrate Phosphate Dextrose
CSIR	Council for Scientific and Industrial Research
CV	Coefficient of Variation
CB	Cytochalasin B
°C	Degree Celsius
DAFF	Department Agriculture, Forestry and Fisheries
DAPI	4,6-diamidine-2-phenylindole
DNA	Deoxyribonucleic acid
Dph	Day post hatch
ECA	Economic Commission for Africa
EDTA	Ethylene-diamine-tetra-acetic acid
FAO	Food and Agriculture Organisation of the United Nations
FCF	Fulton Condition Factor
FSC	Forward Scatter
G	Gram
GFP	Green Fluorescent Protein
GIFT	Genetically Improved Farmed Tilapia
GTH	Gonadotropic hormone
HCL	Hydrochloric acid
IBWT	Inter-Basin Water Transfer
IUCN	International Union for Conservation of Nature
Kg	Kilogram
KPa	Kilopascal
LC	Least concern
LHRHa	Luteinising hormone-releasing hormone (LHRH) agonist
Min	Minute/s
MFI	Multi fluorescent Intensity
ml l^{-1}	millilitre per litre
Ml	Millilitre
Mm	Millimetre
Nm	Nano meter
NAOH	Sodium hydroxide
NORs	Nucleolar Organiser Regions
PI	Propidium Iodide
Ppm	Parts per million
Psi	Pounds per square inch
RAS	Recirculating Aquaculture system
RSI	Roe Somatic index
rDNA	Ribosomal Deoxyribonucleic acid
rRNA	Ribosomal Ribonucleic acid

RNase A	Ribonuclease A
SANS	South African National Standards
AgNORs	Silver-stained Nucleolar Organising Regions
SSC	Side Scatter
TAN	Total Ammonia Nitrogen
TSI	Testes Somatic Index
TL	Total Length
TPF	Treatment post fertilisation
TW	Total weight
µg kg^{-1}	microgram per kilogram
µl	Micro litre

Chapter 1 General introduction

1.1 Background

Hunger is a global concern, with the Food and Agriculture Organisation (FAO) of the United Nations reporting that there were 821 million malnourished people worldwide in 2017, of which 257 million were in Africa (FAO, 2018). Furthermore, the FAO approximated that about 59 million children in Africa were stunted and malnourished in the same year and South Africa formed part of this projection (FAO & ECA, 2018).

Aquaculture was introduced as means of sustainable food production by the colonial governments throughout Africa between the 1940s and 1950s, with the primary goals to improve nutrition in rural areas, supplementary income generation, diversification to reduce crop failure risks and employment creation in rural areas (Brummet *et al*, 2008; Babatunde *et al*, 2020). The top producing African countries in aquaculture are Egypt, Nigeria, Uganda, Ghana, Tunisia, Kenya, Zambia,Madagascar, Malawi and South Africa (Satia, 2017a). However, their contribution towards the global aquaculture production is still insignificant (about 2.7%) (Halwart, 2020). In Africa, the aquaculture sector employs approximately 6.2 million people, with a large share of the employees being women that are engaged in large scale commercial farms (Satia, 2016). Therefore, development of aquaculture has been identified as a complementary solution in reducing the food crisis, also leading to improved nutrition and income in poorer communities in these African countries including South Africa (FAO, 2018).

Fish consumption per capita has doubled worldwide in the past five decades (FAO, 2018). The majority of this increase in demand has been supplied by the growing aquaculture industry,

as capture fisheries have struggled to sufficiently increase their catch rates (FAO, 2018). In 2016, the production from both marine and inland capture fisheries amounted to approximately 91 million tonnes and has since plateaued at about this level over the past twenty years (FAO, 2019). Marine fisheries demonstrated a general decrease in production, standing at 79.3 million tonnes in 2016 from 86.4 million tonnes in 1996 (FAO, 2019). Unlike marine fisheries, the global production for inland fisheries has increased gradually since 1988, and was nearly 12 million tonnes in 2016 (FAO, 2019). According to the FAO, the figures reported to them are believed to be underestimated as FAO does not have a status tracking system in place like it does for marine fisheries (FAO, 2019).

Fish is an important food source for the health of billions of consumers in both developed and developing countries due to its special nutritional properties. It provides for essential micronutrients, such as vitamins A, B and D, as well as minerals, which makes it particularly attractive in the current fight against malnutrition in low income and food deficient countries (FAO, 2017a). Fish is highly recommended by human nutritionists as a good source of protein and healthy fatty acid due to its elevated ratio of Omega 3: Omega 6 fatty acids, and as a cheaper source of animal protein (FAO, 2017a).

The aquaculture industry involves culturing of mollusc, crustacean and fish species of either marine, freshwater, euryhaline or stenohaline origin (FAO, 2017a). Among the important freshwater fish species is the African sharptooth catfish, *Clarias gariepinus*. The freshwater species of the genus *Clarias* and their hybrids are predominantly cultured for their fast growth rate, disease resistance and tolerance to high-density culture (Huisman & Richter, 1987; Haylor, 1993). Clariid catfishes have a distinctive attribute of being able to breathe in atmospheric air

and can endure in waters with low levels of dissolved oxygen. This character makes them particularly attractive to aquaculture (Bruton, 1979).

1.2 Rationale

Problem statement

Technology deficient and extensive traditional aquaculture production methods such as the use traditional extensive or intensive aquaculture systems (fish ponds, pens and cages) with little or no genetic interventions; have become less reliable as they hinder sustainability and are associated with slow growth of fish, inefficient harvest, and poor fecundity as well as reproduction (Dunham, 2004). The development of improved aquaculture products can contribute to increased fish production, which is one of the fundamental solutions in meeting the future food demands of an expanding human world population (Anderson *et al*., 2017). Aquaculture is a technology-driven sector and requires diverse scientific input from a number of disciplines (reproduction, nutrition, physiology, genetics, animal health and disease, etc.) to improve growth and production in the industry (DAFF, 2016). Major improvements have been achieved through enhanced husbandry procedures, improved nutrition, enhanced disease diagnosis and therapies, and the application of genetics to production traits. However, more genetic research and application can greatly contribute to production efficiency and increasing sustainability (Dunham, 2004). Genetic improvement studies employed conventional methods such as selective breeding, hybridisation and crossbreeding (Gjedrem & Robinson, 2014). A considerable amount of work has also been carried out where modern biotechnological techniques such as polyploidisation, monosex, transgenesis and genetic markers are applied (Gjedrem & Robinson, 2014). Some of these technologies are applied to continuously improve

farmed species, in order to achieve genetic gains in successive generations, while others are used for instant short-term benefits (FAO, 2019). Genetic engineering has improved a variety of traits, including growth rate, feed conversion efficiency, disease resistance, tolerance of low water quality, thermal tolerance, body shape, dressing percentage, carcass quality, fish quality, fertility, reproduction and marketability (Dunham, 2004, FAO, 2019). Although several aquaculture species have been greatly improved through the application of genetics, the majority remains underdeveloped (Dunham *et al.*, 2001).

In South Africa, the aquaculture of African sharptooth catfish is well developed, and most of the farmers use relatively high population density ponds, raceway and recirculation systems (DAFF, 2017). In 2015, the catfish sub-sector had 13 operating farms (DAFF, 2017) in South Africa. From 2006-2011, the sub-sector contributed a total production of 1060 tons, however, no records of commercial production were officially reported since 2012. This is attributed to most farmers producing fingerlings for the export market rather than growing the fish to market size on the farm (DAFF, 2017). Although the artificially manipulated breeding of African sharptooth catfish is a well-understood exercise, fingerling supply is still below fish farmers' demand. One of the major causes is the low survival rates of hatchery-produced fingerlings, associated with lack of proper genetic management practices or genetic interventions (FAO, 2008). Mass catfish production requires fast-growing and uniformly-sized fingerlings for fish farmers (Eyo *et al*., 2003).

Although catfish aquaculture production shows promise in improving food security and job creation in South Africa, the fish is suitable for culture in all provinces except in the Western and Eastern Cape provinces due to legislative restrictions. The culture of the species is limited in these

provinces due to its predatory-omnivore nature, reproductive and invasive potential (Impson et al., 2017). These attributes can cause African sharptooth catfish to compete for food with local biota and, thus, result in ecological imbalance in suitable habitats where it does not occur naturally (Kadye & Booth, 2012). The legislative restrictions in these geographic regions could, however, be amended or lifted if there is a possibility of producing sterile triploids that have minimal ecological impact in non-native environments such as the Western Cape or Eastern Cape in South Africa. Therefore, biotechnological interventions are required for successful aquaculture of this species in these potential areas in order to meet consumer demand while not affecting ecosystems or biodiversity conservation. The current study focuses on polyploidy induction in the African sharptooth catfish. The selected technique is important in fish reproduction strategies and provides a rapid approach for neonate gonadal sterilisation, sex control, improvement of hybrid viability, and clonation (Lakran & Ayyappan, 2003). Ploidy manipulations can produce sterile, unisex or highly homozygous groups of animals (Piferrer *et al.*, 2009). Worldwide expansion of aquaculture requires species or stocks more adapted to localised conditions and less likely adversely to affect the genetic diversity of natural populations in the event of escape, as a result of prolific reproduction (Hulata, 2001). Therefore, the induction of ploidy, specifically triploidy, can produce sterility in animals. Sterility induced in triploid individuals not only result in improved growth rates and organoleptic quality of their flesh (Nell, 2002), but has a significant potential use in the genetic containment of aquaculture species (Piferrer *et al.*, 2009).

Tetraploid broodstock production can be used to facilitate the induction of triploid fish (Piferrer *et al.*, 2009). Tetraploid fish are important to aquaculture as they can be utilised to produce large numbers of sterile triploid fish through simple crosses between tetraploids and

diploids (Guo *et al.*, 1996). This method has shown to be viable in other aquaculture species and is likely to represent a more commercially-viable and reliable means of mass production of triploids. The only chromosome-set manipulations reported in African sharptooth catfish have been induced triploidy and meiogynogenesis (Henken *et al*., 1987; Volckaert *et al.*, 1994). Váradi *et al.* (1999) produced diploid gynogens by employing interspecific sperm and induced tetraploidy in African sharptooth catfish (*Clarias gariepinus*). However, the study focused more on the production of homozygous gynogens over heterozygous tetraploidy induction. Tetraploid induction is an intermediate step in producing triploid individuals. The success of treatments to induce polyploidy depends on the time of initiation of the shock, magnitude of the shock, duration of the shock, genetics and quality of the gamete (Piferrer *et al.*, 2009).

The current work will open the opportunity for production of triploid offspring by crossing tetraploids with diploids. Successful production tetraploids could help address the shortages in fingerling supply experienced by farmers in the catfish industry. The information obtained from the current investigation on the optimal or correct protocol for inducing tetraploidy and how to detect ploidy in African sharptooth catfish would be beneficial to farmers and the development of the catfish industry.

1.3 Aim and objectives

The aim of the study was to validate the success of a tetraploidy induction protocol in the African sharptooth catfish, using heat shock as a method of induction and chromosome preparations, silver nitrate NORs staining and flow cytometry as tetraploidy verification methods.

This aim was achieved through the following objectives:

1. To assess the correct shock initiation time interval between egg fertilisation and thermal induction of zygotic tetraploidy in African sharptooth catfish*, Clarias gariepinus*.

2. To validate flow cytometry (Sysmex® PI method) and selected staining techniques, namely Giemsa–chromosome preparations and silver nitrate staining for identifying nucleolar organising regions (NORs) as methods for tetraploidy verification in *C. gariepinus*.

Chapter 2 Literature review

2.1 Aquaculture as global food sector contributor

The world population is estimated to increase to above 9 billion by 2050, while the current projection of the population is approximated to be over 7 billion people (FAO & ECA, 2018). Due to these projections, food insecurity and nutrition have become global concerns as human societies are faced with the challenge of having to provide food and livelihoods to this population (FAO & ECA, 2018). According to the United Nations Food Agriculture Organisation (FAO), fisheries and aquaculture have demonstrated an essential role in fighting hunger, while ensuring food security and nutrition, as well as sustainable economic independence, especially in the developing world (FAO, 2018).

Generally, capture fisheries were the main supply of fish protein worldwide; however, many of these fisheries have been fully exploited or overfished (FAO, 2018). As a result, many of the world capture fisheries have plateaued or collapsed over the past three decades, leaving aquaculture as an increasingly important source of fish (), including in developing countries (Belton *et al*., 2016). Fish is generally known and highly recommended by the health promoting industry (human health nutritionists) as a healthy source of high-quality protein, essential amino- and fatty-acids, minerals and vitamins in human nutrition; and is an affordable source of animal protein (FAO, 2017a). The FAO has defined aquaculture as the farming of aquatic organisms, which are harvested and owned by a particular individual or corporate body (FAO, 2014). The farming involves a level of intervention in the rearing process, such as feeding, regular stocking and protection from predators, in order to enhance production (FAO, 2014).

To date, China is the leading aquaculture producer, contributing the highest exports of fish and fish products worldwide (FAO, 2018). Whilst global capture fisheries are at a plateau, aquaculture excelled as the fastest growing sector in food production, as opposed to other major production sectors, and contributes for 50% of the world's fish that is used for food (FAO, 2018). In 2018, the total global fish production increased to about 179 million tonnes, with aquaculture representing 46% of the total production and 52% for human consumption (FAO, 2020). Of the total tonnes of fish produced in 2018, over 156 million tonnes (about 88%) were used for direct human consumption and this proportion has demonstrated a significant increase in recent decades (FAO, 2020). The global food fish consumption per capita increased by about 1.5% on average per year, from 9.0 kg in 1961 to 20.5 kg in 2018 (FAO, 2020). Figure 2.1 illustrates the ebbing trend in world capture fisheries, as opposed to the growth trend of aquaculture from 1950-2018.

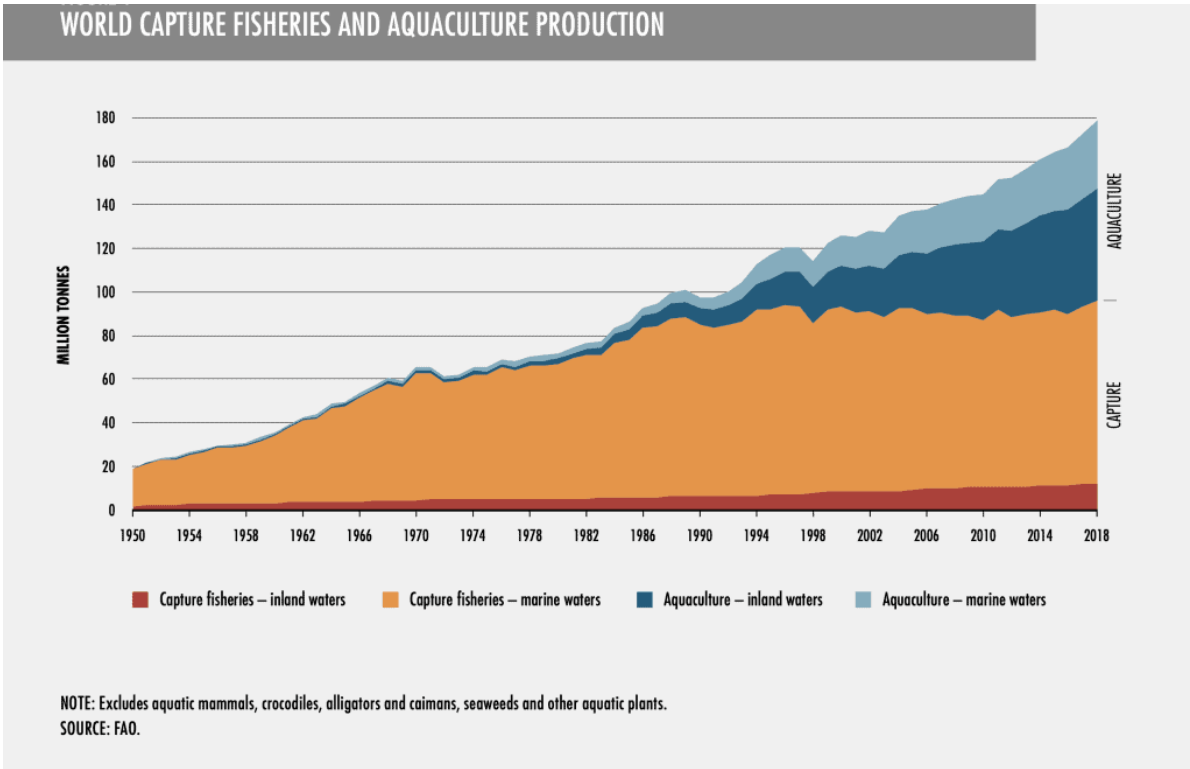


Figure 2.1 World capture fisheries and aquaculture production (10^6 tonnes) trends from 1950-2018 (FAO, 2020)**.**

The finfish sector is the highest contributor to food fish globally, with 54.3 million tonnes of the total production (which contributed a value of USD 139.7 billion). Following the finfish sector are molluscs at a total production of 17.7 million tonnes, valued at USD 34.6 billion, and crustaceans, producing 9.4 million tonnes valued at USD 69.3 billion, while the other production came from marine invertebrates (435 400 tonnes, USD 2 billion), aquatic turtles (370 000 tonnes, USD 3.5 billion) and frogs (131 300 tonnes, USD 997 million) (FAO, 2020). Freshwater aquaculture has a production of 47 million tonnes that contributes a value of USD 104.3 billion and presently surpasses marine and coastal aquaculture, which produced 7.3 million tonnes with a contribution of USD 35.4 billion to the economy (FAO, 2020). This is probably due to the higher costs related with marine aquaculture, which requires building complex land-based aquaculture facilities for sensitive and high-value species or farming in rough waters (e.g., South Africa). Freshwater

aquaculture, on the other hand, can be practiced in simple ponds with more hardy or tolerant fish species that require far less capital input (Brummett *et al.*, 2008).

2.2 Aquaculture in South Africa

Aquaculture production operations occur in freshwater (inland), brackish water and marine (coastal) environments, though most production activities take place in freshwater (FAO, 2019). These operations, especially freshwater, are either subsistence, small-scale market-driven or large industrial scale (Satia, 2017b). In Africa, large industrial-scale fish farming occurred in 1924 with tilapia (*Oreochromis niloticus*) in Kenya and, to date, approximately 170 species have been farmed in 26 African regions (FAO, 2019). According to country reports by FAO, this number of species could be underestimated as many countries reported producing more species or species items than they report through the regular FAO statistic (FAO, 2019). In 2014, freshwater aquaculture production contributed a value of USD 1.6 billion, which was obtained from farm gate price with no value addition. The majority of the production, about 98%, was from inland aquaculture, mainly of indigenous and omnipresent species of tilapia and the African sharptooth catfish. About 93% of the total production was contributed by African countries, which included Nigeria, Uganda, Ghana, Kenya, Zambia, Madagascar and South Africa (Satia, 2017b).

Although the aquaculture industry has shown rapid development in South Africa, it still is a minor contributor towards the country's economy (DAFF, 2015; DAFF, 2018). South Africa's per capita fish consumption is relatively low, with only 6.1 kg reported in 2016; however, South Africa is generally a net exporter of aquaculture products. The annual value of exports surpassed imports by USD 174 million during 2017, with imports valued at USD 424 million and exports at USD 598 million (FAO, 2018). In 2016, marine aquaculture, which is the fastest-growing sector in

South Africa, contributed 3660 tonnes towards the total production, accounting for 67% of the production. Freshwater aquaculture on the other hand was responsible for 1840 tonnes, which accounted for 33% of all the aquaculture production in the same year (FAO, 2018). Marine aquaculture can only realistically be practiced in the coastal provinces such as the Eastern, Western and Northern Cape as well as KwaZulu-Natal. Freshwater aquaculture on the other hand, holds great potential as it can be practiced in all nine provinces of the country (DAFF, 2017). The main limitations for freshwater fish culture have been government policies and suitable water supply of both the right quality and quantity (FAO, 2018).

Freshwater species currently farmed in South Africa include finfish species such as Rainbow trout and brown trout (*Onchorynchus mykiss and Salmo trutta*), Mozambique and Nile tilapia (*Oreochromis mossambicus* and *Oreochromis niloticus*), African sharptooth catfish (*Clarias gariepinus*), common carp (*Cyprinus Carpio and Ctenopharyngodon idella*), ornamental fish (*e.g.,* koi carp), crocodiles (*Crocodylus niloticus*) and invertebrates, such as Marron crayfish (*Cherax tenuimanus*) (DAFF, 2017). In 2015, trout aquaculture remained the most valuable freshwater aquaculture subsector in South Africa, with total production at 1497.30 tonnes (82.43 percent); following this were tilapia and marron crayfish aquaculture at 325.29 tonnes (17.90 percent) and 4.00 tonnes (0.22 percent), respectively. The catfish subsector did not produce in 2015, since production costs were higher than selling price (DAFF, 2018).

Although African sharptooth catfish aquaculture recorded zero production in 2015, it has demonstrated great production potential over the past years, producing 1060 tonnes during 2006-2011. In 2014, 53.3 tonnes of African sharptooth catfish were exported to foreign markets, at a value of approximately R 0.8 million (DAFF, 2016). African sharptooth catfish was exported

to countries such as Democratic Republic of Congo (highest importer at 99.4 percent) and Cote D'Ivoire (at 0.6 percent) (DAFF, 2016). In 2015, however, the African sharptooth catfish exports market decreased significantly by 97% when compared to 2014. South Africa exported 3.4 tonnes of African sharptooth catfish, at a value of approximately R 202 564. Most of the exports went to Zambia, Nigeria and Botswana (DAFF, 2017). At the same time, 11 tonnes of catfish were imported into South Africa, with majority of it coming from Vietnam (90%), while Thailand contributed 10% (DAFF, 2017). The total value of African sharptooth catfish imports was approximately R 0.2 million. The simultaneous export and import of catfish in South Africa is mostly due to the production of African sharptooth catfish declining below the local demand, and therefore, catfish from foreign countries were imported to satisfy the market (DAFF, 2017). The trends of catfish import and export have demonstrated the growing demand of catfish and the need to sustainably produce catfish locally in order to meet this demand.

2.3 Global culture of African sharptooth catfish

Clarias gariepinus is a good candidate for aquaculture owing to its ability to feed on a variety of food items, its rapid growth, a high degree of hardiness and survival in poorly oxygenated waters (Britz & Hecht, 1988). Due to its economic importance, this species is widely cultured in various regions in the world (FAO, 2016). Nigeria is the leading producer, with the highest annual production of African sharptooth catfish, followed by the Netherlands, Brazil, Hungary, Kenya, Syrian Arab Republic, South Africa, Cameroon and Mali (FAO, 2016). The FAO reported a total global production of 246 476 tonnes of *C. gariepinus* during 2015.

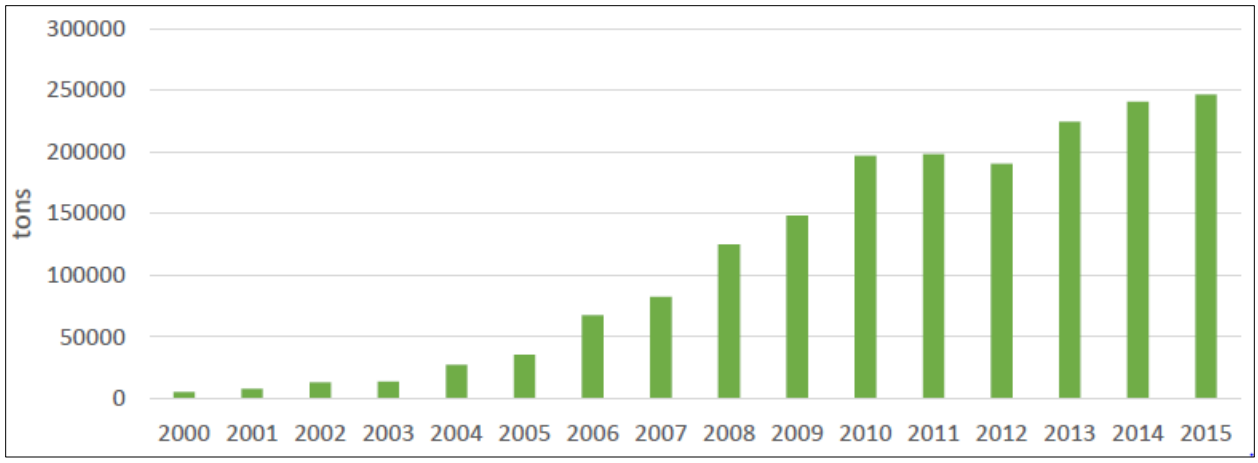


Figure 2.2 The global aquaculture production of African sharptooth catfish (FAO, 2016).

The African sharptooth catfish is an indigenous species to Africa, the Middle East (Skelton, 2001) and high-fish producing Asian countries such as China, Indonesia, Thailand and Malaysia (FAO, 2018). However, the production statistics for these regions are currently unavailable to the FAO (FAO, 2018). Therefore, the total African sharptooth catfish production could be significantly underestimated. As an example, the production data reported by the Nigerian Federal Department of Fisheries from 2001 to 2012 were much higher compared to the official FAO statistics (Dauda *et al.*, 2018). This inconsistency was said to be attributed to the farming of African sharptooth catfish hybrids that occur not only in Africa, but also in most Asian countries. Therefore, it was difficult to separate the data for pure African sharptooth catfish and that of hybrids and the FAO did not capture the output under the name African sharptooth catfish but reported this as *Clarias* sp. (FAO, 2017b; Dauda *et al.*, 2018). Table 2.1 shows the production statistics for catfish in Nigeria.

Table 2.1 African sharptooth production in Nigeria from 2001-2012 (Anetekhai, 2018).

Species	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012
Clarias gariepinus	19518	24530	24542	35160	45084	67662	68100	115000	137516	180482	199015	228508
Clarias hybrid	390	491	491	703	902	1353	1362	2300	9168	12032	13268	15234
Heterobranchus sp	1464	1840	1841	2637	3381	5075	5108	8625	6112	8021	8845	10156
Total	21372	26861	26874	38500	49367	74090	74570	125925	152796	200535	221128	253898
Total job creation-production and marketing	21372	26861	26874	38500	49367	74090	74570	125925	152796	200535	221128	253898

2.4 African sharptooth catfish culture in South Africa

Freshwater aquaculture in South Africa has progressively increased for the past three decades (DAFF, 2017). However, it is still at a developing stage and its contribution towards the country's economy is minor in terms of production (DAFF, 2017). South African sharptooth catfish aquaculture is developing slowly; however, it is technologically developed in terms of the farming strategies employed. These technologies includes extensive to intensive production models (DAFF, 2017). Most of the farmers use relatively high population density ponds, raceway or recirculating aquaculture systems. Other potential production systems include aquaponics, cage culture and flow-through systems (DAFF, 2017). According to the DAFF African sharptooth catfish feasibility study report (DAFF, 2018), all of these production systems have proven to be economically viable, depending on farming strategies and product price. South African sharptooth catfish production is largely carried out by smallholder producers, with a few key government supported initiatives (DAFF, 2018). A total of two farms were reported in South Africa in 2008, which accounted for permanent employment of eight individuals while temporarily employing three other individuals. According to Britz *et al.* (2009), catfish aquaculture during this year contributed R 3.6 million. Currently, there are 13 catfish farms in

South Africa (DAFF, 2017). These farms are typically located in the Free State, Limpopo, North West, Gauteng, and the Eastern Cape (DAFF, 2017). In recent years, the catfish industry has seen an increase in production, which can be attributed to the development of farming technology, improved farm management and changing consumer behaviour (DAFF, 2018). A total annual production of 1060 tonnes of catfish recorded since 2006-2011, however, the industry has recorded zero production since 2012 to 2015 as shown in figure 2.3 (DAFF, 2017). This absence of information and data on production is attributed to the fact that the majority of South African sharptooth catfish farmers do not present their farming data to the relevant governmental departments for reporting purposes (DAFF, 2018). Furthermore, a report produced by the DAFF (2017) indicated that there was a shift of focus for producers, with most farmers concentrating on producing catfish fingerlings for the export market rather than growing the fish to market prize. This has also contributed to the unavailability of production data to the DAFF for reporting purposes, during the period 2012-2015 (DAFF, 2017; 2018).

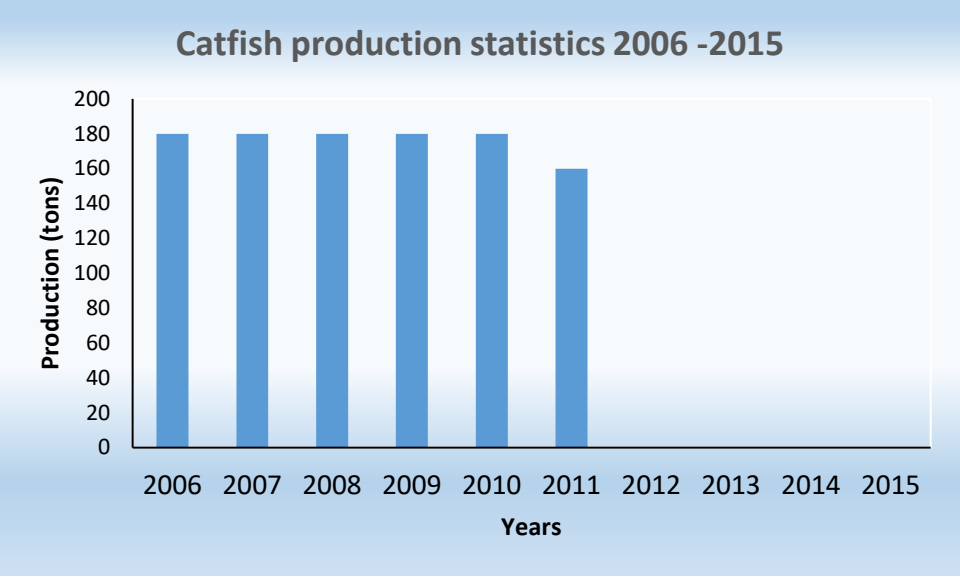


Figure 2.3 Production data for African sharptooth catfish from 2006-2015 in South Africa (DAFF, 2017).

Catfish aquaculture was forecast to result in significant positive socio-economic improvements; however, in South Africa, the high costs involved with feed are a major constraint in keeping pricing attractive (Cambray, 2003). Recently, the shutting down of the South African sharptooth catfish industry is associated with the farmers closing their operations as a result of production costs exceeding the selling prices, which was directly due to the high-feeds prices (FAO, 2017; DAFF,2018). The feed cost reducing incentives are driven by the DAFF research in collaboration with Universities such as the University of Limpopo. Therefore, cheaper fish meal alternatives such as acid fermented biowaste can replace fishmeal completely and facilitate the profitable commercial production of African sharptooth catfish (DAFF Aquaculture Research and Development personal communication).

The South African sharptooth catfish market is relatively small with production estimated to range from about 100 tonnes per annum (DAFF, 2018). Farm gate sales are a major component in small-scale enterprise farms (DAFF, 2018). The products which are mainly consumed by local market are fresh and drained whole fish, and are mostly driven by the exports from Western and Eastern Africa (DAFF, 2018). It has been reported that there is an existing demand within the South African sharptooth catfish market for value added products such a sausages and burger patties (DAFF,2018). The local market holds a great pontetial and has the ability to grow faster considering the large foreign population which resides in South Africa. There are about 3 million foreigners in South Africa, of which 100 000 are Western and East Africans (DAFF, 2018). However, the great pontential of African sharptooth catfish aquaculture could be unleashed if most value-added products were consumed by the South African middle class. Therefore, creative marketing and education have become important tools for improving consumer's

knowledge and acceptance of consumers and also for influencing their buying preferences (DAFF, 2018).

2.5 The taxonomy, biology and distribution of *Clarias gariepinus*

2.5.1 Species classification

Clarias is the third most diverse catfish genus in Africa, which consists of 32 species (Teugels, 1984; Skelton, 2001). Six subgenera in this genus identified by Teugels (1982a; 1982b; 1986) include *Anguilloclarias*, *Brevicephaloides*, *Clarias*, *Clariodes*, *Dinotopteroides* and *Platycephaloides*. The only two validated species under the subgenus *Clarias* are *C. gariepinus* and *C. anguillaris*. *Clarias gariepinus* is an economically valuable species, as it is the most cultured catfish in Africa and is rated third among the most cultured catfishes worldwide (Garibaldi, 1996). In the 1980s, the genus *Clarias* was re-evaluated, which resulted in several widespread species being synonymised, whereby *Clarias capensis* of southern Africa, *Clarias mossambicus* of central Africa and *Clarias lazera* of West and the North African came to be known as *Clarias gariepinus* (Teugels, 1986).

The taxonomic classification of African sharptooth catfish is as follows:

Order: Siluriformes

Family: Clariidae

Genus: *Clarias*

Species: *Clarias gariepinus* (Burchell, 1822)

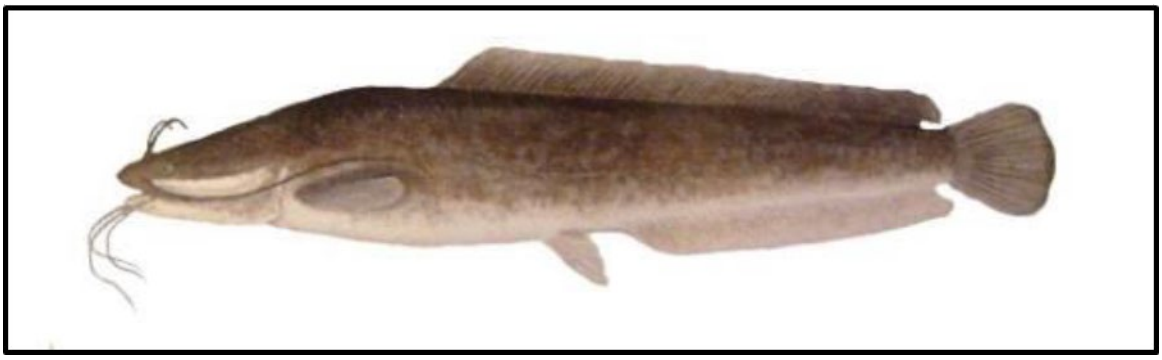


Figure 2.4 *Clarias gariepinus* is readily recognised by its cylindrical body with scaleless skin, flattened bony head, small eyes, elongated spineless dorsal fin and four pairs of barbels around a broad mouth. The upper surface of the head is coarsely granulated in adult fishes but smooth in young fish (Skelton, 2001).

2.5.2 Distribution and potential environmental impact

African sharptooth catfish, *Clarias gariepinus*, is widely distributed in Africa, and with relative geographical longitude occurrence ranging from the Orange River in South Africa to the Nile River in North Africa. It is native in parts of Asia (Israel, Syria and southern Turkey) (Skelton & Teugels, 1992). In South Africa, it is non-endemic to the coastal Western Cape and Eastern Cape provinces (Impson *et al*., 2017). The distribution covers as far south as the Orange River system in the west and the Umtamvuna river in the east of South Africa. The native range of African sharptooth catfish covers most of the African continent with the exception of Northwest Africa, Upper and Lower Guinea, and the Cape provinces of South Africa (**Figures 2.5 and 2.6**). Skelton (2001) stated that South Africa possibly has the most extensive distribution of African sharptooth catfish. This species has been widely introduced in many parts of the world through aquaculture (Radhakrishnan et al., 2011). The geographical distribution of the species is attributed to several aspects of its biology and ecology, as well as to its ability to tolerate physiologically adverse environmental conditions (Hecht & Appelbaum, 1988). Biologically, *C. gariepinus* is an excellent aquaculture species, as amongst other qualities, it can be reared and grown at ultra high densities

(>200 kg m^{-3}) (DAFF, 2018). The species is hardy and extremely adaptable under high density and suitable rearing conditions in a RAS production system (DAFF, 2018). This is largely due to the species' air-breathing ability (Hecht *et al*., 1988) owing to a multi-branched accessory air-breathing organ composed of modified gill arches, which enables it to breathe air in drying pools or poorly oxygenated water (Teugels, 1986, 1996; Skelton, 1993). Its air-breathing ability also allows the species to spread easily, as it can translocate terrestrially to other water bodies, since it is able to tolerate extreme dry condions for short periods (Skelton, 1994). The fish's widespread distribution includes temperate, subtropical and tropical environments (Hecht *et al*., 1988). *Clarias gariepinus* is an omnivore, feeding on a wide variety of natural prey under diverse conditions. The species is also highly fecund and is easily spawned under captive conditions (Haylor, 1992; Hecht *et al*., 1996).

Although their impact in introduced areas is unknown, concern has been raised over its predation and competition impact on local biota (Khan & Panikkar, 2009; Kadye & Booth, 2012). In the Eastern Cape Province, South Africa, the fish was translocated primarily through the Orange/Fish inter-basin water transfer (IBWT) scheme, which was completed in 1976 (Cambray & Jubb, 1977). The African sharptooth catfish has since spread into several other river and impoundments through secondary IBWT schemes, angler introductions and, to a lesser extent, aquaculture (Cambray, 2005). Translocated catfish from the Orange River system have been established in many Eastern Cape rivers, including the Great Fish, Sundays, Kouga, Swartkops, Keiskamma, Buffalo, Nahoon and Mtata rivers, and in many reservoirs (de Moor & Bruton, 1988; Kadye & Booth, 2012).

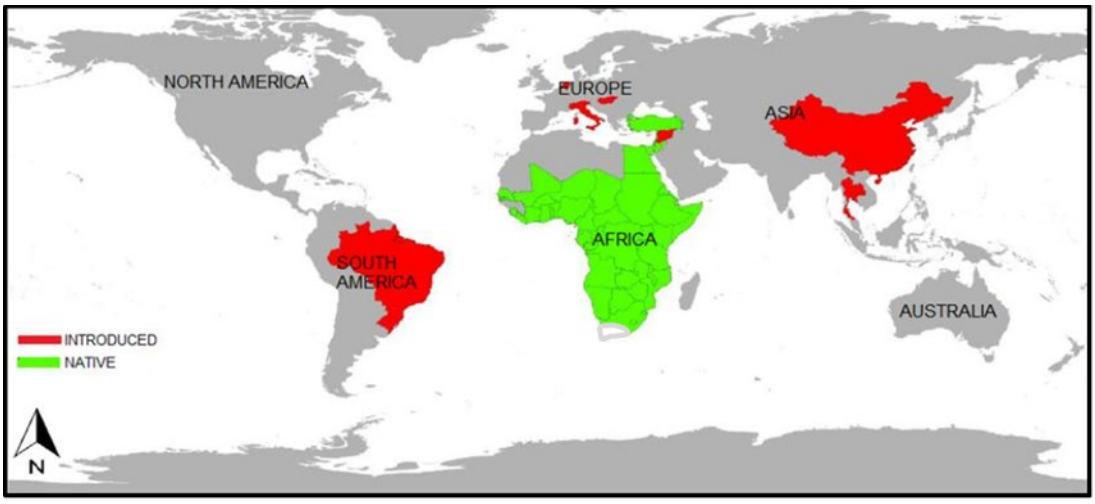


Figure 2.5 The spread of *Clarias gariepinus*, in regions where it is native (green) and introduced (red) globally (GISD, 2012).

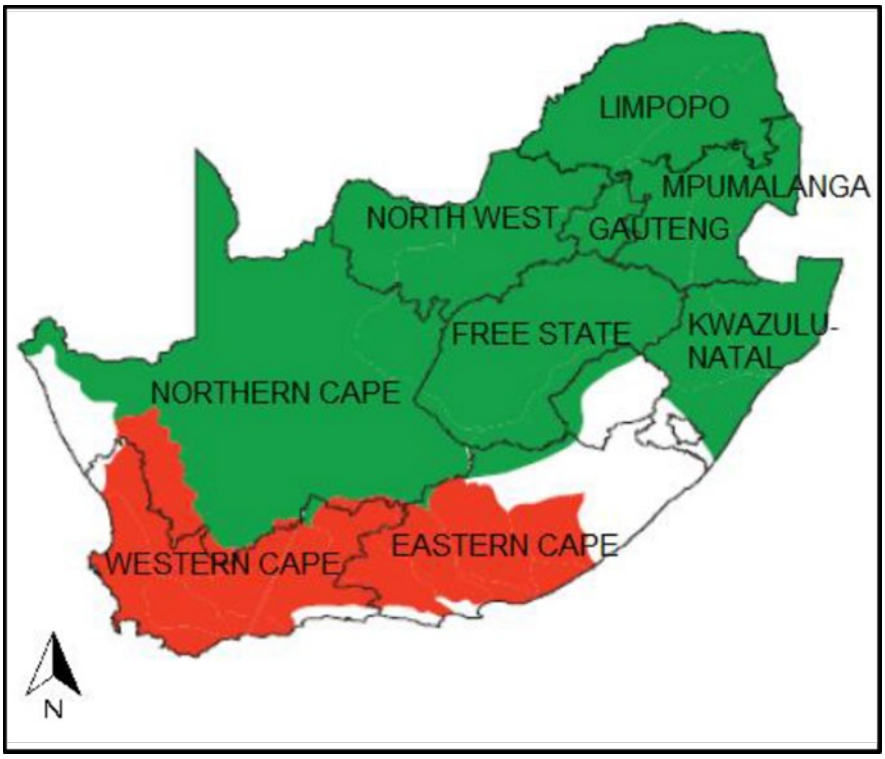


Figure 2.6 Geological regions where *Clarias gariepinus* naturally occurs and its introductions within South Africa (Picker & Griffiths, 2011).

2.5.3 Reproduction

In the wild, *C. gariepinus* undergoes a seasonal trend in gonadal maturation, which begins in winter and is usually associated with the rainy season. The annual reproduction cycle of *C. gariepinus* is predominantly influenced by abiotic factors, such as changes in water temperature and photoperiodicity, with the final triggering of spawning caused by a raise in water level due to rainfall (de Graaf *et al*., 1995). In South Africa, spawning in the wild takes place at water temperatures above 18 °C, usually at 22 °C (Hecht *et al*., 1988). The size at first maturity varies greatly, from 200 to 300 g upwards (with 150 to 800 mm total length) and is usually at an age of one to three years (Bruton, 1979; Hecht *et al*., 1988). Shoals of fish relocate or move up the river or towards edges of still water bodies to prepare for breeding (de Moor & Bruton, 1988). Sexual mature catfish begin their courting, breeding and the laying of eggs at night often after rain (Bruton, 1979). Eggs normally attach to either aquatic or terrestrial plants which were immersed in the water body by flooding induced by the rain (Bruton, 1979). The eggs hatch as soon as 24 to 36 hours after breeding (Bruton, 1979) and the offspring do not receive any parental care from their parents (Hecht *et al*., 1988). Bruton (1979) found that the standard fecundity of African sharptooth catfish was approximately 45 000 eggs for a 2 kg fish.

Another attribute making *C. gariepinus* an attractive aquaculture species is the ease to manipulate reproduction thereof in captive aquaculture conditions. In captivity, African sharptooth catfish cannot spawn voluntarily due to the absence of environmental cues, such as the rise in the water level and overflow of shallow areas. Van der Waal (1972, 1978) and Hogendoorn (1977) were the first authors to attempt to artificially spawn, strip and incubate the eggs of *Clarias gariepinus*. Their efforts allowed the possibility for the intensive cultivation of

African sharptooth catfish larvae. Artificial propagation in the African sharptooth catfish industry was introduced because the semi-natural methods of propagation used in early production trials were found to be insufficient for the mass production of fry on a commercial scale (Kelleher & Vincke, 1976; Richter, 1976; Van der Waal, 1978). Artificially-induced spawning eliminates environmental variables, such as spawning area, temperature, light and other climatic factors, thus resulting in the development of fry production into a highly controlled hatchery-based operation. Artificial propagation requires the selection and conditioning of broodstock, hormone-induced spawning, hand stripping of eggs, and fertilisation using sperm from sacrificed males (Britz & Hecht, 1988). The adhesive fertilised eggs hatch within less than one day (at 24°C), and larvae are reared under controlled environmental conditions using formulated artificial feed supplemented with live feed (Britz & Hecht, 1988).

According to Britz and Hecht (1988), fish of between one and five kilograms are the most practical size for artificial spawning. However, smaller or bigger fish may also be used. The relatively high fecundity of the catfish is an advantage with respect to its culture. According to the observations by Van der Waal (1972) and Bruton (1979), females in the one to five kilogram range may yield between 30 000 and 500 000 eggs.

Broodstock can be wild caught in a gravid condition, or manipulatively brought to seasonal maturity in captivity. The brood fish rapidly reach gonadal maturity and spawning condition within a period of one month if the water temperature is elevated and kept at 28 °C and the fish are fed a high protein diet (42-48%) (Richter *et al.*, 1987). Ripe females are screened by the size of their abdomen, redness of cloaca and the condition of the eggs (cannula method). It is generally not possible to judge the ripeness of male fish by means of any external examination.

Males are sacrificed and their testes are dissected to reveal their condition and development state. The testes lie dorsally and to the rear of the abdominal cavity along with its associated seminal vesicles (Hogendoorn & Vismans, 1980). The presence of ripe sperm in the testes is indicated by a white, opaque, milky colour spreading from the distal margin into median body of the testis. Unripe testes are usually smaller and transparent. The testes of dissected males need not go to waste as the sperm will remain viable for at least 24 hours if the whole testis is stored at approximately five degrees Centigrade (Hogendoorn & Vismans, 1980).

In southern Africa, artificially-induced spawning was previously mediated by injecting African sharptooth catfish or common carp homoplastic pituitary extracts, which require the injection of fish pituitary gland homogenate into the female, to stimulate final egg maturation and ovulation. The pituitary synthesises and stores gonadotrophic hormone (GTH), which modulates gonad maturation and spawning in teleost fish (Britz & Hecht, 1988). Gonadotrophic hormone is released into the bloodstream in response to certain natural cues, such as rising water and temperature levels, which induce spawning activity of African sharptooth catfish. Intramuscularly- or intraperitoneally-injected homoplastic pituitary extract facilitates the direct introduction of GTH into the bloodstream, thereby circumventing the natural cues required for ovulation (Britz & Hecht, 1988).

Male and female African sharptooth catfish are induced to spawn simultaneously with administered hormone [pituitary extract or nowadays synthetic luteinising hormone-releasing hormone agonist (LHRHa)] Hawarry *et al*.,2016. The body mass of pituitary donor and receiver fish are approximately equal. Luteinising hormone-releasing hormone agonist is administered according to the analog available and the supplier's prescription dose, which normally varies

between 1 and 20 µg.kg^{-1}. Prior to the stripping of females (spontaneous gravitational egg release), one or two males (fertility risk reduction) are sacrificed (1 ml.L^{-1} 2-phenoxy ethanol and decapitation) before the testes are removed. Each testis is dissected along the distal margin using a sharp blade and the semen squeezed over the eggs. Eggs and semen are then gently mixed using a soft rubber spatula. A small quantity of water from the incubation trough is added, and gently stirred for another minute. The addition of water activates the sperm and causes the eggs to swell and become adhesive. The swelling of the eggs causes the egg micropyle to close (Britz & Hecht, 1988).

Fertilised egg incubation, hatching and larval rearing are preferably maintained under low light intensity or even darkness. The optimum temperature range for incubation is 27 to 30 °C. Eggs are, however, very tolerant of temperature fluctuations and will hatch successfully from 17 to 33 °C. Hatching is temperature dependent and takes between 20 and 24 hours within the optimum temperature range. At temperatures below 23 °C, fertilised egg mortalities are often high due to a longer delay (metabolism reduction) before egg hatching, which increases the likelihood of fungus (*Saprolegnia* spp.) spreading from dead eggs to live eggs, eventually destroying the latter (Britz & Hecht, 1988).

2.6 Genetic interventions in aquaculture

Due to the increasing global demand for aquaculture products, a number of improvements have been made in the technologies employed in the aquaculture industry to meet this demand. The advancement of biotechnological principles for worldwide aquaculture became crucial for supporting the significant expansion in the farming of aquatic animals and also for preventing ecological disruptions in natural aquatic ecosystems (De Beer, 2004; FAO, 2019). Over the past

years, genetic biotechnology has played an innovative role through the improvement of important aquaculture species (Hafsa *et al*., 2010). The main goal in most biotechnological advances has been to target traits, such as fast growth, feed utilisation and disease resistance, while minimising the production time and cost for market size individuals (De Beer, 2004; Dunham, 2004). However, very few species have been genetically improved such as Nile tilapia (*Oreochromis niloticus*) and Atlantic salmon (*Salmo salar*) (Khan *et al*., 2008; Hafsa *et al*., 2016).

Traditionally, selective breeding, crossbreeding and hybridisation have been the most commonly used genetic technologies to improve aquaculture species for many years (FAO, 1995). Selective breeding programmes have been used to improve the breeding value of a fish population by selecting and mating only fish with desired traits such as faster growth rates, flesh quality or colour (FAO,1995). This long-term technology allows brood fish to transmit their superiority to their offspring, therefore, producing genetic gains in each generation. It is often the most profitable strategy for strain enhancement as well as domestication (Gjedrem & Robinson, 2014). For example, selective breeding has been successfully used to improve growth performance and disease resistance in Atlantic salmon (*Salmo salar*) (Gjedrem, 2004). Selective breeding programmes were also applied to produce the Genetically Improved Farmed Tilapia (GIFT) strain of *Oreochromis niloticus*, which has shown exceptional genetic gains in growth rate and has thus outperformed other strains (Bentsen *et al*., 2017) . Crossbreeding and hybridisation can be used to combine favourable qualities from two genetically different groups and have shown an advantage of hybrid vigour (heterosis) in the offspring (Bartley *et al*., 2001). Crossbreeding programmes usually involve different strains within a species (intraspecific hybridisation), but different species can also be hybridised (interspecific hybridisation) (Bartley

et al., 2001). Interspecific hybridisation has resulted in fish with improved growth rates, manipulated sex ratios, sterile animals, improved flesh quality, increased disease resistance, improved tolerance to environmental extremes and other altered traits (Bartley *et al*., 2001). These are short-term genetic improvement strategies that do not require the same level of record keeping nor management as long-term projects. In addition, they can result in significant gains in short period of time. Hybrid fishes commonly used in aquaculture include cyprinids (e.g. Grass carp x bighead carp; silver carp x bighead carp; crosses between common carp, rohu and catla catla), salmonids (e.g. Brown trout x brook trout; Atlantic salmon x brown trout) and ciclids (eg. Tilapia such Mossambique tilapia x Nile tilapia; Nile tilapia x blue tilapia) (Bartley *et al*., 2001). Another genetic improvement method which can produce significant one-time gains in the short term is polyploidisation (FAO, 2019).

Genetic manipulation, particularly polyploidy induction, has been used to enhance growth rates in some commercially important fish including Atlantic salmon, rainbow trout, Nile tilapia and channel catfish (Wolters *et al.*, 1982; Chourrout *et al.*, 1986; O'Flynn, 1997; Pechsiri & Yakupitiyage, 2005) and shellfish species (common mussel, Pacific oyster and abalone) (Boudry *et al.*, 1998; De Beer, 2004; Prins, 2011). The most common and normal chromosome complement is two sets referred to as diploid (Dunham, 2004). Polyploidy is the possession of more than two sets of chromosomes and the common forms applied in aquaculture include triploidy (having three sets of chromosomes) and tetraploidy (with four sets of chromosomes) (Piferrer *et al.*, 2009).

In African sharptooth catfish, the only chromosome set manipulations reported have been induced triploids, meiogynogenesis and mitotic gynogenesis (Henken *et al.*, 1987; Volckaert *et al.*, 1994) and tetraploids (auto-inactivated sperm) (Váradi *et al.*, 1999). Meiogynogens are produced if activation occurs without fertilisation or through fertilisation by sperm that has been irradiated to inactivate the DNA, and the resumption of Meiosis II is disrupted, with a resulting diploid zygote receiving both sets of chromosomes from the female parent. Mitotic gynogens can occur from disruptions of the first mitotic division (Volckaert *et al.*, 1994). Váradi *et al.* (1999) produced diploid gynogens and tetraploids in African sharptooth catfish. However, the focus of their work was mostly on producing homozygous gynogens over heterozygous tetraploidy induction. Therefore, there is still a need to standardise a protocol for inducing tetraploidy in African sharptooth catfish.

2.7 Triploidy in aquaculture

The interest in the induction of triploidy in aquaculture has been stimulated since it results in functional sterility. Triploidy has been studied considerably in teleost fish by numerous researchers (Purdom, 1983; Thorgaard, 1983; Hussain *et al.*, 1991; Pandian & Koteeswaran, 1998; Arai, 2001; Felip *et al.*, 2001; Tiwary & Ray, 2004). Examples include Rainbow trout, (*Oncorhynchus mykiss*); Atlantic salmon (*Salmo salar*), Grass carp (*Ctenopharyngodon idella*), Common carp (*Cyprinus carpio*), tench (*Tinca tinca*), tilapias (*Oreochromis* spp.), Pacific salmon (*Oncorhynchus kisutch*) and African sharptooth catfish (*Clarias gariepinus*).

The occurrence of polyploidy in natural populations is mainly attributed to numerical mutations of chromosomes (Guo & Allen, 1994; Comai, 2005; Zhou & Gui, 2017). Spontaneous polyploids were observed in several phylogenetically distant orders, including both wild and farmed fish

species (Schultz, 1969; Thorgaard & Gall, 1979). In vertebrates, polyploid species were reported for different groups, from amphibians (Stöck *et al.*, 2002) to, occasionally and to a rare extent, mammals (Gallardo *et al.*, 1999). Polyploidy is lethal in mammals and birds (Chourrout *et al.*, 1986). Polyploids can originate either from alterations of meiotic or mitotic processes in specimens within a species (autopolyploidy) or by reproductive contact among species (allopolyploidy) (Piferrer *et al.*, 2009).

Current interest in polyploidy induction is essentially due to its potential application in aquaculture, for the production of triploid and tetraploid fish (Purdom, 1993; Pandian & Koteeswaran, 1998; Piferrer *et al.*, 2009; Dunham, 2011; Zhou & Gui, 2017). Artificial induction of triploidy, the most popular ploidy manipulation in fish and shellfish, has been used to create sterile individuals and this resolves the problems caused by sexual maturation, such as decreased body growth rates, higher incidence of diseases, or organoleptic deterioration in taste of the edible parts (Piferrer *et al.*, 2009).

2.7.1 Effects of triploidy in aquaculture

Artificially produced triploids differ from conspecific diploids in three fundamental ways: they are generally more heterozygous (Allendorf & Leary, 1984; Leary *et al.*, 1985), they have larger but fewer cells in a variety of tissues, and their gonadal development is disrupted to some extent. Benfey (1999) reviewed the effect of these aspects on the physiology and behaviour of triploid fishes. Beaumont and Fairbrother (1991) supported Benfey (1999) and the previously mentioned authors by providing three main reasons why sterile aquaculture organisms are beneficial for commercial aquaculture:

a) Due to the redirection of energy normally used to produce gametes, which now becomes available for somatic growth, the adult triploids should grow faster (Beaumont & Fairbrother, 1991).

b) In breeding seasons, mature animals produce ripe gonads which often make them less attractive and therefore difficult to market; on the other hand, their flavour is negatively affected by depletion in the glycogen levels during this period. This disadvantage is eliminated in sterile animals (Beaumont and Fairbrother, 1991; Chao *et al.*, 1993; Boudry *et al*., 1998).

c) Sterility reduces the risk of accidental introduction of non-native species from aquaculture facilities into the environment, since such intruders will not be able to reproduce (Beaumont & Fairbrother, 1991; Sheehan *et al.*, 1999).

There are a number of hypotheses that explain the mechanism for faster growth rate in triploids, particularly in fish, but there are also contradictions to the general assumption that triploids grow faster than diploids (Tabarini, 1984; Ruiz-Verdugo *et al.*, 2000; Piferrer *et al.*, 2009). Because of increased heterozygosity, some triploid populations may demonstrate heterosis or hybrid vigour, which occur due to the higher possibility of having more than two different alleles at each gene (Beaumont & Fairbrother, 1991; Hawkins *et al.*, 2000; Magoulas *et al.*, 2000; Garnier-Gere *et al.*, 2002). However, diploids might show reduced growth compared to triploid genotypes because of higher homozygosity. The increased levels of homozygosity can result in a higher level of expression of deleterious mutations in the diploid state (Zouros *et al*., 1996). Based on the gene-dose hypothesis, it was postulated that for a triploid genotype, there are three homozygous alleles at each gene, therefore, three times the gene product might be available.

Furthermore, due to the presence of three gene templates in triploids rather than the two found in diploids, the gene products affecting or increasing growth transcribe faster (Magoulas *et al.*, 2000).

Triploid genotypes also demonstrate a generally increased cell size, as the cells have to accommodate more DNA due to the presence of three sets of chromosomes in the nuclei (Guo and Allen , 1994). Because body size correlates with cell size in some organisms, (Ihssen *et al.*, 1990), the probability is high that triploid organisms would grow faster and reach larger ultimate sizes than diploids.

However, studies have shown that during the juvenile and immature adult phase, triploid fishes normally grow equal to or less than diploids, depending on the species and environmental conditions (Cotter *et al*., 2002). Studies have shown that, in species such as the Atlantic salmon, triploids versus all female diploids do not present significant growth differences, at least from 0 - 9 months of age, in freshwater when the two genotypes are grown in separate tanks (Benfey & Sutterlin, 1984; Quillet & Gaignon, 1990; Galbreath *et al.*, 1994; O'Flynn, 1997; Cotter *et al.*, 2002). This support the results achieved with rainbow trout reared under appropriate environmental conditions (Sheehan *et al.*, 1999; Leggatt *et al.*, 2006; Wagner *et al.*, 2006). In overall, triploids in shellfish normally accumulate the same growth as diploids during their juvenile stage. This information suggests that triploids do not obtain an inherent higher growth rate than diploids until they reach a maturity stage (Sheehan *et al.*, 1999).

Adult triploid freshwater fishes demostrated faster growth than diploids in salmonids (except coho salmon), Nile tilapia (*Oreochromis niloticus*), tench, perch, cyprinid loach and in

catfishes, such as channel catfish (*Ictalurus punctatus*) (Wolters *et al*., 1982), but not in African sharptooth catfish (Zanuy *et al.*, 2001). Faster growth in these species is attributed to increased anabolic opportunity created, since nutrients are not channelled to sexual organs but to somatic growth (Wolters *et al*., 1982). The faster growth in triploids has shown to be species specific since in some studies of fish showed that triploid cells are bigger, but triploid individuals neither reach larger ultimate sizes, nor grow faster than diploids (Ihssen *et al.*, 1990; Pandian & Koteeswaran, 1998). Benfey (1999) reviewed the findings on bigger cell size in triploids, where they demonstrated and concluded that, increased cell sizes led to decreased cell numbers in different fish organs, such as the brain, retina, etc., and, therefore, did not demonstrate any growth advantage to triploids.

In reviews by various authors, shock-induced triploids were indicated to have lower early survival in terms of reduced viability of eggs, developing embryos and hatched larvae up to the first feeding stage, when compared to diploids (Chourrout, 1988; Ihssen *et al.*, 1990; Thorgaard *et al*., 1992; Pandian, and Koteeswaran, 1998; Benfey, 1999; Felip *et al.*, 2001; Hulata, 2001; Gomelsky, 2003; Tiwary *et al*., 2004; Maxime, 2008). In case of treatment not being 100% effective, some alleged triploids groups may contain many diploids that have failed to respond to the shock method. Cherfas *et al.* (1994) demonstrated that shocked diploids and triploids presented the same survival during early stages, but had lower survival than the unshocked diploid controls. The outcome of their study suggests that the induction shock is the main factor responsible for early-minimal survival, whereas the triploidy status itself may be the cause of reduced survival later on.

In a study on rainbow trout by Weber *et al.* (2014), shock-induced triploids obtained by direct induction were compared with interploid or intercross triploids, produced by crossing tetraploids with diploids, in terms of their performance. The study demonstrated that triploids produced by crossing tetraploid with diploid individuals attained better growth rates than their diploid and induced-triploid counterparts. The enhanced survival of interploids compared well to that of diploids, whilst shock-induced triploids failed to compare (Chourrout *et al.*, 1986; Blanc *et al.*, 1987; Myers & Hershberger, 1991).

2.7.2 The application of triploidy for biological containment (biosecurity)

There is need for the biological containment of fish and shellfish from commercial aquaculture activities to prevent genetic contamination of surrounding wild populations. Sterility as a result of triploidy can be a tool exploited to ensure biological containment. This confinement may be necessary to restrict the excessive reproduction of overly fecund species or act as a safeguard against the threats of competition or predation imposed on natural populations by escapees of exotic species (Piferrer *et al.*, 2009; Prins, 2011). Recently, containment of domestic stock has become of great significance as a result of the development of aquaculture activities in regions or habitats that are not natural or native to these species. Therefore, reproductive sterility resulting from triploidy induction is a potential strategy to achieve such containment (Piferrer *et al.*, 2009; Prins, 2011)

Diploid grass carp, for example, has been used in water bodies in the United States of America as a biological control for aquatic plants, which has resulted in reduced application of herbicides. Other benefits include improvement of recreational quality, and flood-control linked to the flow of water bodies influenced by excessive aquatic vegetation (Zajicek *et al.*, 2011).

Regardless of these benefits, there is a possibility for escaped grass carp to reproduce and affect the ecosystem or fish communities in natural environments. Therefore, to reduce the possible negative ecological effects, functionally sterile triploid grass carp is now used in many states for controlling aquatic plants (Zajicek *et al.*, 2011). Triploid grass carp provides an advantage as a biological control agent for aquatic plant management, while removing the risk of having increasing numbers of non-native fish populations due to reproduction. There have been concerns over whether triploid carp acquired complete sterility during induction and whether the protocols used for producing triploid grass carp are sufficient to stop introductions of prolific diploid carp into the environment (Zajicek *et al.*, 2011). Based on studies by Fuller (2003) on various species of carp, including grass carp, it was confirmed that triploids are able to revert to a diploid state and are capable of reproducing. Therefore, triploid induction does not totally remove the potential of fish populations establishing in non-native habitats. In shellfish, trials on triploid oyster, *Crassostrea gigas*, have shown that the alleged sterile triploids had mature gonads and were able to reproduce in some cases (Allen *et al.*, 1996). The results of these trials also showed the presence of mosaics in the triploid stocks of *Crassostrea gigas*. These outcomes validated that triploidy is unstable; however, they could not prove that the mosaics became sexually active.

2.7.3 Methods for triploidy induction

Cell division suppression can be achieved by several methods that include physical or chemical treatments. Physical treatments applied during ploidy induction include pressure shock (Chourrout, 1984; Lou & Purdom, 1984; Benfey *et al.*, 1988; Peruzzi & Chatain, 2000; Xu *et al.*, 2008) and temperature shocks (Chourrout, 1980; Thorgaard *et al.*, 1981). Temperature shock

treatment can either be cold (Felip *et al.*, 1997; Piferrer *et al.*, 2003) or heat shock (Garrido-Ramos *et al.*, 1996; Rougeot, 2003). The effectiveness of these treatments is mainly dependent on the intensity of the shock in physical treatments and concentrations of chemicals in chemical treatments.

In fish, eggs are arrested on their release, at the metaphase stage of Meiosis II as shown in , while in mollusc, eggs are restricted at the prophase or metaphase of Meiosis I during release (Colas and Dubé, 1998; Piferrer *et al.*, 2009). Application of physical or chemical shock during meiosis I or meiosis II can suppress cell division and prevent the extrusion of either the first or second polar body in case of shellfish but only the second in case of fish. The processes occur while allowing chromosomal division and hence results in the production of triploids (Piferrer *et al.*, 2009). Chemical treatments use agents that interfere with the microtubules during cell division, hence preventing the extrusion of a polar body. According to Teskeredžić *et al.* (1993), Johnson *et al.* (2004) and Haffray *et al.* (2007), physical treatment is predominantly successful and widely used to induce triploidy in fish. Chemical treatments were found to be more successful in inducing triploidy in shellfish than physical treatment (Beaumont & Fairbrother, 1991). Triploids can also be obtained by indirect methods based on interploid crossing, where normal eggs are fertilised with the diploid sperm from a tetraploid male (Wang et al., 2002; Francescon *et al.*, 2004; Nam and Kim, 2004).

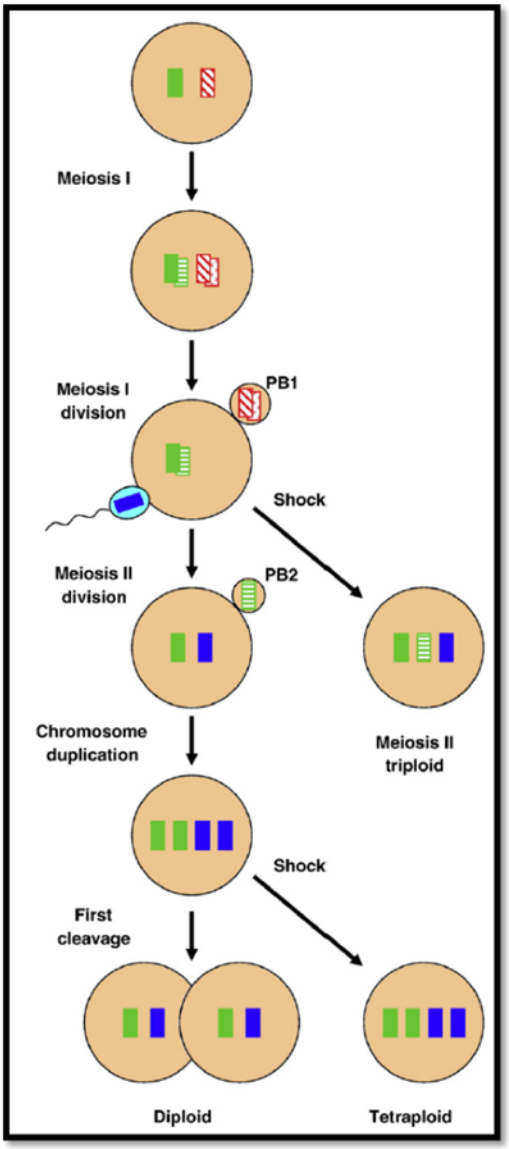


Figure 2.7 An illustration of ploidy or chromosome manipulation in fish. Eggs are released at meiosis II and fertilisation occurs to continue with meiosis. Physical or chemical shock is appllied during meiosis II or first cleavage to suppress cell division while allowing chromosomal division producing triploids (meiosis II suppression) or tetraploids (first cleavage suppression). Each bar inside the cell represent one chromosome while overlapping bars show the sister chromatids following DNA replication during meiosis I (Piferrer *et al.*, 2009).

2.7.3.1 Direct procedures for triploidy induction

Chemical treatment

Amongst the most frequently used methods of inducing triploidy is chemical shock by means of Cytochalasin B (CB) treatment. Cytochalasin B is a fungal metabolite (produced by the fungus *Helminthosporium dematioideum*) that is used to inhibit the formation of micro-filament in cells by inhibiting actin polymerization (Beaumont & Fairbrother, 1991; Gérard *et al*., 1999). Cells treated with CB do not display distinguished cytoplasmic cleavage furrows or filaments of the contractile ring required for a successful cytokinesis 24 (division of cytoplasm during meiosis) (Beaumont & Fairbrother, 1991). According to Beaumont and Fairbrother (1991), induction using CB as a treatment method requires reduced critical timing of treatment and generally results in maximum percentages of triploidy than other treatments in shellfish.

Cytochalasin B has been used successfully in the South African abalone (*H. midae*) (Stepto & Cook, 1998), the Australian blacklip abalone (*H. rubra*) (Liu *et al.*, 2004), the Pacific abalone (*H. discus hannai*) (Zhang *et al.*, 1998) , the small abalone (*H. diversicolor supertaxa*) (Yang *et al*., 1998) and numerous oyster species (Barber *et al*., 1992; Gérard *et al.*, 1999). However, this treatment method is costly and often results in undesirable levels of ploidy, due to higher polyspermy and increased percentages of abnormalities, as well as higher mortality rates during early larval development when compared to other methods (Beaumont & Fairbrother, 1991). Additional concerns have been expressed with regard to the health hazard to humans associated with high cytotoxicity and carcinogenic activity of CB, even though its use on a commercial basis has been approved in the United States America (Beaumont & Fairbrother, 1991; Liu *et al.*, 2004). Allen and Stanley (1979) produced few triploid cells and polyploid-diploid mosaics, which

comprised diploid, triploid, as well as tetraploid cells, after exposure of fertilised eggs of Atlantic salmon (*Salmo salar*) to Cytochalasin B. Another potential chemical to induce polyploidy is an alkaloid colchicine, which inhibits the formation of microtubules during cell division, therefore, preventing the chromosomes from segregating. Exposing fertilised egg of brook trout (*Salvenlinus fontinalis*) to colchicine, similar mosaic polyploid-diploids were produced by Smith & Lemoine (1979).

Chemical shock treatments such as calcium and caffeine treatment were reported to have little success in inducing triploidy and result in poor larval development and survival (Scarpa *et al.*, 1994). Thorgaard (1983) suggested that the use of chemicals should be discontinued in finfish since they are less adaptable to mass production of triploids.

Hydrostatic pressure treatment

Hydrostatic pressure treatment is a successful application for selected finfish and abalone species. Johnson *et al.* (2004) used hydrostatic pressure shock to induce 96% triploidy in Chinook salmon. They applied hydrostatic pressure for 5.0 min at 6.89 x 104 kilopascal (kPa) (10,000 psi) of pressure, at 30 minutes after fertilisation. Kerby *et al.* (2002) induced triploidy in Sunshine bass by employing hydrostatic pressure shock treatments to ova at 4 to 5 minutes intervals after fertilisation at a pressure of 8000 pounds per square inch (PSI) or 1.5 to 2 minutes. In a study by Chourrout (1984), retention of the second polar body in rainbow trout eggs was induced by 7000 psi early pressure shocks (applied 40 minutes after insemination) lasting 4 minutes. This resulted in all-triploid progenies after fertilisation with functional sperm, and in high yields of heterozygous diploid gynogenetic fry after insemination with gamma-irradiated sperm. All-

tetraploid progenies were produced by 7000 psi late pressure shocks (applied 5 h 50 min after insemination) lasting 4 minutes, when a normal sperm had been used. Hydrostatic pressure has demonstrated to produce more consistent results, survival of treated eggs and per cent triploidy than temperature shocks and other treatments in various species (Cassani & Caton, 1986; Bury, 1989).

Temperature treatment

The application of species-specfic heat shock settings, shortly after fertilisation has been the most successful technique used to induce polyploidy in salmonids (Chourrout, 1980, 1982; Thorgaard *et al*.,1981; Chourrout & Quillet, 1982; Lincoln & Scott, 1983). Other studies have used cold shocks only, with varying success (Lincoln *et al*.,1974; Chourrout, 1980; Lemoine and Smith, 1980; Refstie *et al*., 1982). Cold shocks are suitable to use with fish that have small eggs but fish with large eggs show higher intrinsic variation with respect to inductions using temperature shock (Piferrer *et al.*, 2009).

Temperature shocks may prevent second polar body extrusion by altering development rates, disrupting the microtubules of the meiotic spindle or through changes in the density of cytoplasm. Heat shocks cause depolymerisation of tubulin polymers that form microtubules (Rieder & Bajer, 1978), which are essential for the formation of the spindle apparatus. Heat shocks may also inhibit spindle formation and aster movement (Gaillard & Jaylet, 1975). When applied shortly before first cleavage division, heat shocks inhibit cytokinesis and cause zygotes to undergo two genomic replications with only one cytoplasmic division.

2.7.3.2 Indirect procedures for triploidy manipulation

Interploidy crosses between tetraploids and diploids

Another method that can be employed to produce triploid fish is by mating a tetraploid individual with a diploid individual to produce a brood of triploid fish called intercross triploids (Weber *et al.*, 2014). This can be achieved by first producing viable tetraploid animals. Artificial induction of tetraploidy of a diploid species is theoretically feasible through the suppression of the first cleavage division (), using chemical or physical (heat/cold shock or pressure shock) methods. In previous studies, these methods produced viable tetraploids in some fish species (Piferrer *et al.*, 2009). Yoshikawa *et al*. (2008) reviewed that only viable mature and fertile tetraploids could be obtained for rainbow trout (*Oncorhynchus mykiss*), blunt snout bream (*Megalobrama amblycephala*) and mud loach (*Misgurnus mizolepis*) but not in some other tested species of aquaculture importance. For example, tetraploid fish were also produced in channel catfish, *Ictalurus punctatus* (Bidwell *et a*l.,1985), tilapias of the genus *Oreochromis* (Mair, 1993), grass carp (*Ctenopharyngodon idella*) (Cassani *et al.*, 1990; Zhang *et al*., 1993) common carp [review by Gomelsky (2003)], tench (*Tinca tinca*) (Flajšhans *et al*., 1993), Indian carps, *Labeo rofita* and *Catla catla* (Sarangi & Mandal, 1994), and yellow perch (*Perca flavescens*) (Malison *et al.*, 1993). However, in a number cases, low yields of larvae produced were reported, which either did not survive to the fingerling stage or died later on. Váradi *et al.* (1999) also produced tetraploids, also at a very low percentage, using radiation-inactivated sperm. Thorgaard *et al*. (1981); Chourrout (1982, 1984) and Chourrout *et al.* (1986) initially used hydrostatic pressure treatment to successfully produce the tetraploids in rainbow trout. At the adult stage, the tetraploids were crossed with diploid females to produce triploid progenies.

2.7.4 Tetraploidy in aquaculture

Tetraploid induction has been achieved by pressure, temperature, or chemical shocking of zygotes in many species, including tilapias (*Oreochromis* sp.) channel catfish (*Ictalurus punctatus*) yellow perch (*Perca flavescens*) and masu salmon (*Oncorhynchus masou*) (Bidwell *et al*., 1985; Myers, 1986; Malison *et al*., 1993; Goudie *et al*., 1995; Malison & Garcia-Abiado, 1996; El Gamal *et al*., 1999; Sakao *et al*., 2006). Tetraploid broodstocks are used for mass production of triploids through crossing tetraploids with diploids (Weber & Hostuttler, 2012; Weber *et al*., 2014).

Tetraploidy has two potential advantages, which are overall increased heterozygosity, leading to heterosis (Diter *et al*., 1988), and gene redundancy, which masks recessive alleles (in gametes as well as zygotes) and provides evolutionary potential for diversification of gene function. Disadvantages of tetraploidy include changes in cell architecture that drive a decrease in cell numbers to maintain a similar body size to diploids (especially in shellfish). Diploid spermatozoa from tetraploid fish may exhibit reduced fertility, as their enlarged heads have more difficulty in passing through the oocyte micropyle (Chourrout *et al.*, 1986; Blanc *et al.*, 1993).

According to findings of Nam & Kim (2004), the mud loach tolerated polyploidy better. About 6% of tetraploid males permanently produced diploid sperm, while the rest released haploid or aneuploid sperm. By crossing normal diploid females with tetraploid males releasing diploid sperm, they achieved 100% triploids. These findings indicated that the sperm ploidy determination is of great importance and, hence, must be taken into consideration for successful production of triploids (Piferrer *et al.*, 2009).

In rainbow trout, the production of unreduced eggs and enlarged spermatozoa in tetraploids limits their value as a tool to produce triploids (Chourrout *et al.*, 1986; Blanc *et al.*, 1987; Weber & Hostuttler, 2012). Studies have focused on developing procedures with more precision to establish the appropriate period for applying pressure shock when inducing tetraploidy and such an intervention has improved production (Hershberger & Hostuttler, 2007; Weber & Hostuttler, 2012). Furthermore, it was established that the eggs from tetraploids obtained in the second generation had improved quality compared to eggs from the first generation tetraploids (Weber & Hostuttler, 2012). The improvements achieved in these studies, combined with work from previous studies demonstrating that sperm size of tetraploids is moderately heritable (Blanc *et al.*, 1993), confirm the feasibility of producing tetraploids if production traits of intercross-triploid fish suggest that efforts are validated.

Weber *et al.* (2014) conducted a study to compare growth rates of nine sets of rainbow trout families, each composed of triploids produced from pressure shock (directly induced triploids, 3Ns), triploids from tetraploid and diploid crosses (intercross triploids, 3NCs), and diploid siblings through to two years of age. Diploid and intercross triploid fish demonstrated greater body weight when compared with pressure-induced triploid fish for the most duration of the study. However, in the final stage, intercross triploid fish acquired body weight exceeding that of the diploid and pressure-induced triploid fish, which accumulated similar body weight.

2.8 Ploidy determination

Ploidy level identification plays a significant role in ensuring the success of chromosome set manipulation programmes, particularly ploidy induction (Mukti *et al.*, 2016). An efficient ploidy analysis is prerequisite in breeding activities as larger numbers of individuals need to be tested

to avoid wasting unnecessary space and time rearing groups of larvae with low levels or undesired ploidy (Jankun *et al*., 2007). Therefore, it is necessary to utilise a fast and an accurate method for ploidy level identification to assess the success of ploidy induction during the early developmental life stages. Knowledge of ploidy levels during these stages is useful since the induction treatment does not always result in 100% desired ploidy population (Mukti *et al.*, 2016). It is also of utmost importance to monitor the changes in the percentage of ploidy in question (e.g., triploidy or tetraploidy) over a period of time. This is a common occurrence possibly because of differential mortality between diploids and tetraploids or triploids (Cassani *et al.*, 1990; Malison *et al.*, 1993; Nam & Kim, 2004). Various methods can be utilised to identify levels or percentages of ploidy in treated groups (Jankun *et al.*, 2007; Pradeep *et al.*, 2011).

2.8.1 Flow cytometry

Flow cytometry has been described as the quantification of cells in a flow system, which delivers particles in single file past a point of measurement (Ormerod, 1998). One of the initial applications of flow cytometry was the measurement of the DNA content in cells for the rapid identification of phases of the cell cycle, apart from mitosis. The technique is a powerful tool that has been applied in multiple disciplines, such as immunology, virology, molecular biology, cancer biology and infectious disease monitoring (McKinnon, 2018). Flow cytometry has been effectively used for the study of the immune system and its response to infectious diseases and cancer (Fernando *et al.*, 1994; McKinnon, 2018). It allows for the simultaneous characterisation of mixed populations of cells from blood and bone marrow, as well as solid tissues that can be dissociated into single cells, such as lymph nodes, spleen, mucosal tissues, solid tumours, etc. In addition to the analysis of populations of cells, other major applications of flow cytometry include

chromosome analysis, measurement of the viability of cells, as well as cell sorting (Ormerod, 1998; McKinnon, 2018). Using ploidy measurements, the ploidy of tumours can be confirmed by an estimation of the S phase fraction so as to indicate the growth state of a tumour (Fernando *et al.*, 1994). Flow cytometry has been used successfully to detect ploidy in a number fish species (Thorgaard *et al.*, 1982; Allen, 1983; Lamatsch *et al.*, 2000) and shellfish (Chaiton & Allen, 1985; De Beer, 2004). The cell cycle produces different elements that can be detected by flow cytometry (Fairbanks & Anderson, 1999).

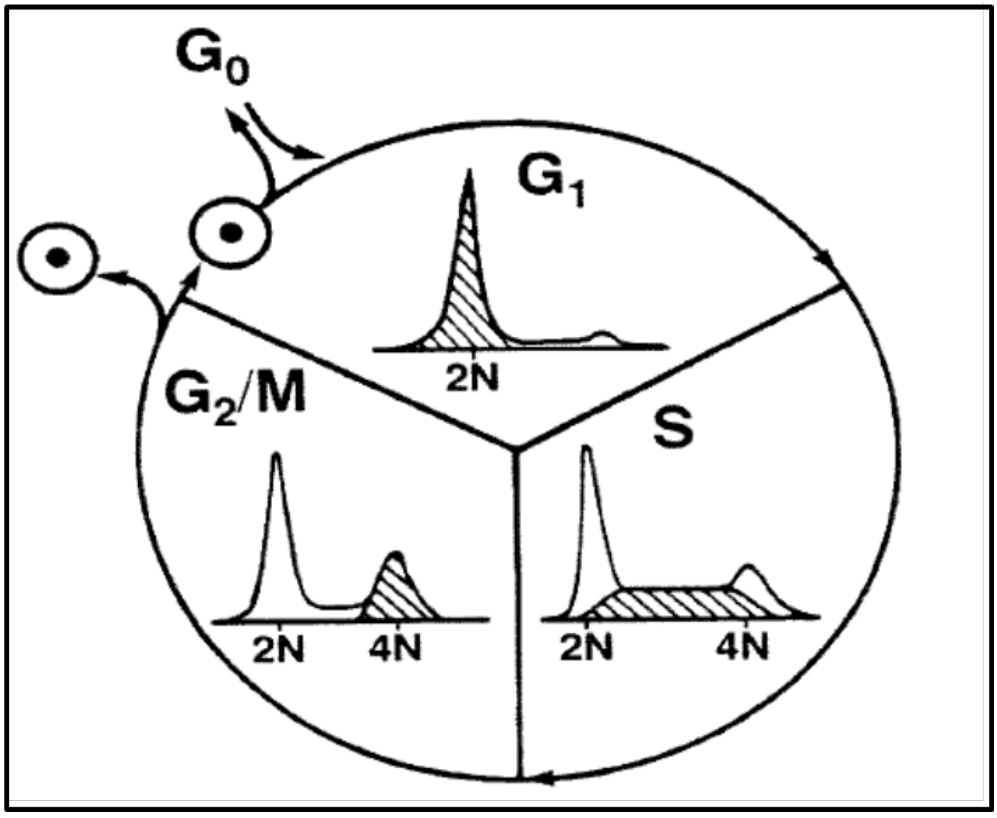


Figure 2.8 Schematic representation of the cell cycle illustrating the flow cytometric elements at each phase (Rabinovitch *et al.*, 1981).

The cells in the G_0 state are very quiescent with little cellular functions, and therefore, no growth or division (). In preparation for division, somatic cells enter the G_1 phase of the cell cycle, which is the synthetic growth phase, during which there is an increase in RNA and proteins

essential for DNA replication are produced. The S-phase or synthesis phase starts after the G_1 phase as DNA replication begins. During this period, the DNA content of the cell increases until it has doubled. At the end of DNA synthesis, the cell enters the G_2 phase of the cell cycle, where DNA repair and reorganisation of the DNA structure takes place prior to division. The DNA is divided equally during the final stage of the cell cycle, mitosis, as the cell divides to form two new daughter cells. Most cells in our body are arrested at the G_1 phase of the cell cycle (Fairbanks & Anderson, 1999).

During a flow cytometry analysis, measurements and information about the biological attributes of cells, such as their physical and/or chemical properties, are collected. These measurements are carried out as the cells or particles pass, preferably singly or as an individual file, through the measuring apparatus in a fluid stream (). The point of measurement is light focused on particles, recording their fluorescence and the light scattered by these particles. The procedure allows simultaneous analyses of multiple cells thus enabling ease and rapid data

collection on these cells. The generated data is computerised and thereafter transformed into histograms (Ormerod, 1998; De Beer, 2004).

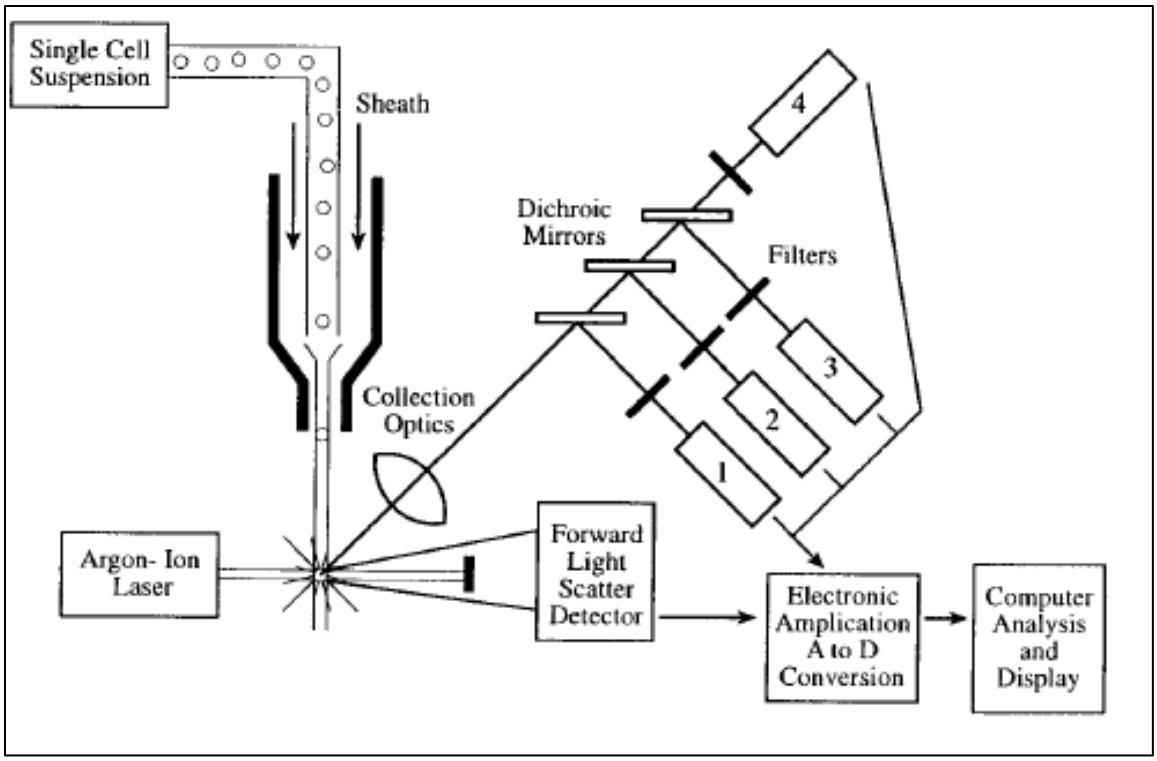


Figure 2.9 Illustration of a flow cytometer(Brown & Wittwer, 2000).

A flow cytometer rapidly analyses single cells or particles as they flow past single or multiple lasers while suspended in a buffered salt-based solution. Each particle is analysed for visible light scatter and one or multiple fluorescence parameters. Visible light scatter is measured in two different directions, the forward direction (Forward Scatter or FSC), which can indicate the relative size of the cell, and at 90° (Side Scatter or SSC), which indicates the internal complexity or granularity of the cell. Signals are collected by the forward angle light scatter detector, the side scatter detector (identified as 1 of), and multiple fluorescence emission detectors (2–4 as shown in). The signals are magnified and translated into a digital format for analysis and display

on a computer screen (Brown & Wittwer, 2000). Light scatter is independent of fluorescence (McKinnon, 2018).

In a flow cytometer, the optical system was devised to measure illuminated cells (Mandy *et al*., 1995). For the detectors to measure fluorescence, the particles must be stained with a fluorescent dye. When detecting ploidy for example, various fluorescent dyes are obtained that bind stoichiometrically to DNA. These dyes include DAPI (4,6-diamidine-2-phenylindole), Ethidium Bromide, Acridine Orange and Hoechst 33342 (Vindeløv & Christensen, 1994; Ormerod, 1998; Yang *et al.*, 2000). Because of the hydrophobicity of the environment, these dyes fluoresce strongly when bound to DNA (Ormerod, 1998). A commonly used dye for ploidy analysis, is the red fluorochrome called Propidium Iodide (PI). Propidium Iodide binds specifically to double-stranded nucleic acids by intercalating between base pairs, without base specificity. It absorbs in blue-green with a wavelength of 493 nm, while fluorescing a red wavelength of 630 nm and has an absorption spectrum with maxima at 287 and 488 nm. The Propidium Iodide readily enters and stains non-viable cells, but it is not permeant to the membrane of viable cells. Therefore, to stain the nuclei, viable cells are first treated to permeabilise their membranes or extract their nuclei. Since it can also bind to double stranded RNA, the enzyme ribonuclease A (RNase A) is used for the removal of the RNA molecules prior to staining (Vindeløv & Christensen, 1994; Ormerod, 1998).

The amount of dye absorbed by the cell is directly proportional to the amount of DNA in the nucleus. Stained nuclei of triploid cells will therefore emit 1.5 times the fluorescence of diploid nuclei (Nell, 2002) while tetraploid cells will emit double the fluorescence of diploid nuclei. Flow cytometry allows the collection of data from tens of thousands of cells/nuclei within a few

minutes (Ormerod, 1998). Although flow cytometry has proven to be a powerful and fast method for ploidy determination, it can, however, be very expensive (Johnson *et al*.,1984).

2.8.2 Chromosome counting/Karyotyping

The chromosome counting technique analyses the number and morphology of chromosomes in the nucleus of a cell. The technique is amongst the direct methods which allow direct ploidy level determination since the number of chromosomes reveal the amount of DNA molecules (Jankun *et al.*, 2007). The success of chromosome counting for ploidy level determination relies on a good chromosome preparation protocol. It is normally challenging to apply the chromosome spread method to a larger number of small sized fish. To carry out chromosome preparation analysis in fish, tissue samples may be obtained from various parts of the fish body, such as the fin and gill epithelia, blood (Jankun *et al.*, 2007) and whole body (Shao *et al.*, 2010).

In standard protocols for chromosome preparation, the application of a spindle toxin such as colchicine is common in order to inhibit cells at their metaphase stage (Kligerman & Bloom, 1977). The selection of the right concentration and duration of incubation of the chemical is important for obtaining clear and distinguishable metaphase chromosome spreads (Rieder & Palazzo, 1992). Exposing cells to the spindle toxins in inadequate concentration and/or for an insufficient period may be unsuccessful in arresting the cells at the metaphase stage, whereas excessively high concentrations and/or prolonged periods of exposure may lead to chromosomal condensation (Wood *et al*., 2001). According to Moore & Best (2001), it is necessary to incubate the cells or larvae in a hypotonic solution when using the technique so the nuclei can swell and disperse the chromosomes on the slides. Poor choice of a hypotonic solution and a suitable

period of incubation could either result in entangled and overlapping chromosomes or loss of chromosomes (Baksi & Means, 1988).

Following the hypotonic solution step are the fixation of the samples using Carnoy's fixative solution, and preparation of a cell suspension. Furthermore, it is required to take careful consideration of factors such as the evaporation rate of the fixative and preheating of slides while preparing the slides, as they could have a negative effect on the quality and quantity of chromosomal spread (Moore & Best, 2001). It is essential to apply proper spreading methods during the preparation of the slides to prevent the chromosomes from washing out during the staining process. Different staining methods are applied to stain chromosomes for various purposes following slide preparation. This includes classic staining techniques such as aceto-orcein, haematoxylin, Giemsa, Wright and Leishman stains. Prolonged incubation and highly concentrated staining solutions could yield a darkened background covering the gap between chromatids, whereas diluting the concentration of staining solutions and/or a short duration for incubation results in unclear chromosomal spreads (Moore & Best, 2001; Wang *et al.*, 2010; Calado *et al.*, 2013).

The staining procedure can be done on both histological and cytological specimens. Karami *et al.* (2015) determined the efficiency of the protocol by investigating the dependency of the chromosome preparation parameters at different developmental stages of two species, African sharptooth catfish (*Clarias gariepinus*) and the Zebra fish (*Danio rerio*). It was found that taking into consideration the parameters such as the concentration of colchicine and/or incubation time, the type of hypotonic solution and age of larvae used, which differed from one fish species to the other, the protocol proved efficient, especially in African sharptooth catfish.

2.8.3 Nucleoli counting (Silver nitrate-NORs staining)

The method of counting nucleoli is a simple low-cost option for identifying ploidy in different fish species. For most fish species, the nucleoli in interphase cells represent the level of ploidy in the animal. Therefore, this method may be applied to numerous fish species since most of those analysed were demonstrated to have just one chromosome with a nucleolar organiser region for each haploid genome (Phillips *et al*., 1986).

The nucleolus organiser regions (NORs) consist of repetitive gene segments of ribosomal DNA (rDNA) composed of unit clusters of 18S, 5.8S and 28S, which code for ribosomal RNA. The rDNA regions or NORs form the nucleolus, which is responsible for synthesising rRNA to produce ribosome subunits and, hence, is important in producing proteins. The silver (Ag-NOR) staining method can be used identify the NORs due to the argyrophilic nature of their non-histone acidic proteins (Thippeswamy *et al.*, 2015). During the staining, silver attaches to NOR proteins such as the RNA polymerase I subunit linked to rRNA. This allows all NORs and nucleoli with activity during an existing interphase to be visualised under the microscope. In fish, specimens are collected from fins and gills epithelia or blood specimens to detect and count nucleoli in interphase cells. The nucleoli and NOR regions in chromosomes have distinct brown-black dots in a nucleus (Howell & Black, 1980). These dots are Ag-NOR centres and are considered as “ribosomal factories”. The silver staining technique identifies neither rRNA nor rDNA but the acidic protein (Thippeswamy *et al.*, 2015). For experimental ploidy determinations, diploid fish will have at most two nucleoli, a maximum of three nucleoli per cell in triploids, whereas a total of four nucleoli per cell can be identified in tetraploidy induced fish (Jankun *et al.*, 2007).

2.8.4 Nuclear and cell size measurement of erythrocyte

Nuclear sizing involves comparison of the nuclear size/volume of cells of diploids with that of triploids and tetraploids (Nell, 2002) . The degree of ploidy is reflected by size of the nucleus since it contains the chromosomal material. The nuclei of triploid cells are 1.5 times, while tetraploids cells are double, the volume of diploids cells nuclei and will therefore have a greater diameter (Benfey *et al.*, 1984; Beaumont & Fairbrother, 1991). Red blood cells (erythrocytes) are normally used for ploidy detection in fish (Benfey *et al*., 1984; Rottmann *et al*., 1991). The minor and major axes of the erythrocyte nucleus in blood smears are measured using a micro spectrophotometer (Benfey *et al*., 1984). Studies by Child and Watkins (1994) on the Manila clam, which were in agreement with studies performed by Nell (2002) on oysters, showed that measuring of the diameter of cell nuclei from gill tissue and haemolymph also successfully distinguished diploids and triploids. An improved method for estimating ploidy electronically, using nuclear sizing, is the Coulter Counter™, calibrated to read both diploid and triploid red blood cell nuclei volumes (Benfey *et al*., 1984; Rottmann *et al*., 1991). Nuclear sizing is a relatively simple technique with the advantages that the method requires only a high-power microscope, basic microbiological and haematological equipment. It is also a cheap and easy method of determining ploidy (Child & Watkins, 1994). In a comparison of triploid induction validation techniques, Harrell *et al*. (1995) concluded that nuclear sizing (particle size analysis) is the simplest and quickest method for evaluating ploidy in fish.

Conclusion

The African sharptooth catfish is ideally suited for extensive, semi-intensive and intensive production in South Africa provided that an acceptable production temperature range of 25-28

°C can be maintained. South Africa has seen trends of simultaneous export and import of catfish and these are mostly due to the production of African sharptooth catfish declining below the local demand. As a result, catfish from foreign countries were imported to satisfy the market. This has shown the growing demand of catfish and the need to sustainably produce catfish locally in order to meet this demand. Economic production of this species in South Africa is still a challenge but promising research outcomes will reduce feed cost and secure business profitability. Commercial exploitation of the species in the Eastern and Western Cape provinces is prohibited due to its invasive potential. Producing sterile or triploid seedstocks for growing fish in the mentioned prohibited provinces will open the door for prospective catfish farming in these areas, therefore, extending the collective production of this species in South Africa. Triploid stocks can be hatchery produced by crossing ploidy manipulated female broodstock (tetraploids) with non-manipulated diploid male breeders and then testing 100% triploid status in the offspring. Tetrapoidy induction and verification methods are available but will require research determined procedures for the African sharptooth catfish.

Chapter 3 Materials and methods

3.1 Study location

The experiment was conducted between August 2018 and November 2019 at the Aquaculture research division within the Welgevallen Experimental Farm, Stellenbosch, South Africa (33°56'33"S 18°51'56"E). The farm is used by Stellenbosch University for research and training and is within walking distance from the campus. Stellenbosch has a Mediterranean climate characterised by dry summers and mild winters, with a mean annual temperature of 16.4°C. Stellenbosch experiences low to moderate rainfall, with an average of 802 mm, mainly occurring during the winter months (June, July and August) (Conradie *et al*., 2002). Experiments were conducted in an indoor, temperature controlled recirculating aquaculture system (RAS) in which broodstock and hatchery were respectively kept.

3.2 Experimental design, animals and system

Before commencing with this trial, this research study applied and obtained ethical clearance from the Stellenbosch University Research Ethics Committee: Animal Care and Use (Protocol# ACU-2018-7285, attached in Appendix A). All experimental procedures have been approved by the forementioned committee, following the guidelines of the South African National Standards (SANS10386:2008) regarding the care and use of animals for experimental and scientific purposes.

The parameters used in the experiment to determine the optimum conditions for induction of tetraploidy were heat shock initiation times post-fertilisation, as well as a consistent incubation

temperature (25˚C) prior to heat shock initiation. The experimental system consisted of an indoor temperature controlled recirculating system located at Welgevallen Experimental Farm, Stellenbosch University. The system consisted of 2 x 3 kL circular high density polyethylene (HDPE) tanks placed in a closed building equipped with an air-conditioning fan to regulate the tempature both in the water and atmosphere. One of the tanks was dedicated for housing the broodstook fish, while the other tank was compartmentalised with 34 net bags and used as the hatchery for keeping eggs or larvae. The water temparature was maintained at 26 – 28 °C, operating through a thermostat. Brood stock was kept in a 22% shade cloth net bag (Alnet®) tank liner at a density of about 14kg m^3 in a single 3 kiloliter circular high density polyethylene (HDPE) tank with 2m diameter and 1m functional water depth. Circulating water exit the tank via centre bottom drain fitted with a 110mm T-piece connected to two horizontal and multiple vertical slotted (4mm) PVC extension pipes (100mm) that was closed terminally with 110mm PVC end plugs. The tank exited water then entered a Speck 450w Badu Porpoise® pump on the suction side via a PVC saddle (110 x 50mm) saddle connection. The main 110mm exit pipe was then reduced by a 110mm x 50mm reducing bush, and with finally connected 50mm extension pipe that can drain the broodstock tank by valve control to the effluent reticulation system of the facility. The mentioned circulation pump with delivery pipe (50mm) connected to the multiport valve of a 3 bag sand filter (sand diameter 2-3mm). The latter was equipped with a pressure meter (1-5 bar resolution). Sand filter was back washed when filter resistance reading exceeded 1bar. Backwashed water was directed to the mentioned effluent system of the facility. Make up water (dechlorinated municipal water) was automatically added by hosepipe connected to compensate for backwash water loss. Pre-filtered water was then proportionally split by

respective valve controls to supply water to the biofilter (~1kl/h), inline Ultrazap® UV sterilizer (55w/h) and Ultrazap® thermostat controlled element heater (2kw) (2kl/h). Filters were cleaned and a third of the water in the system was replaced every two weeks. Water entered the biofilter centre area vertically and was injected through a 50mm venturi jet prior to its forced release into the centre surface in order to create a moving fluidized bed biofilter. The biofilter housing was a plastic drum (100L), mounted higher than the broodstock keeping tank and positioned on an elevated brick platform. The biofilter drum contained 30 litres of Kaldnes® biomedia (semi-buoyant), also allowing for 50% expansion volume. The media quantity was biometrically determined, using empirically determined FCR-, waste production and potential ammonia production figures related to the maximum allowed biomass of breeder fish and the daily percentage (<2 %) of body weight feed mass offered to breeders. Additionally, the mentioned net bag lining in the breeder tank was also calculated as a surplus surface addition for microbial biofilter establishment. Water exited the biofilter via a centre (inner drum) vertically mounted 63mm pipe with upper part turning 90^{o} towards the exit flange of the filter side wall - by connecting it to a centrally mounted 63mm T-piece. The latter levelled at about 80% of the depth of the drum. The T-piece connected so that it has only one horizontal pipe connection. The upper vertical opening of the T-piece functioned as an emergency overflow (unlikely blocking of slots by biomedia). The bottom of the vertical pipe connected to an end plug, above which horizontal slots (6mm), spaced 4mm apart, and were cut halfway around the pipe for a distance of about 300mm, in order to prevent biomedia escape during filter operation. The exit pipe of the biofilter released water above surface in the breeder tank, enabling additional aeration treatment to the broodstock tank water. The mentioned additional water line via the UV sterilizer and heater

finally entered the breeder tank with similar supply configuration and additional water aeration as for the biofilter exit pipe. Overhead lighting was only used when broodstock were handled, or when the system was checked for any required maintenance. The broodstock tank was also covered with 22% shade net in order to prevent fish that may try to escape by jumping.

The hatchery tank, recirculation and filtration system outlay was similar in comparison to the discussed broodstock keeping system except for the following differences:

1. The sand filter was replaced with a zeolite and filter mat unit (100L plastic drum filter) also mounted on a brick platform as discussed for the biofilter. The filter was packed top to bottom with four different density filter mat layers graded from coarse to fine as a top to bottom series. The mat filter was suspended by slotted inverted pack crate suspended by four corner pillars of bricks. The compartment below the crate was packed with zeolite coarse gravel. The centre abstraction pipe and outflow/overflow from filter similar than discussed above for the biofilter. Zeolite was used as ammonia absorber since the hatchery was intermittently used and therefore no biofilter could be established.
2. A 6mm stainless steel frame was designed and constructed to suspend 16 net (Teralyne fabric) compartments each measuring 65cm (L) x 70cm (outer width) x 50cm (d). The net compartments stitched and with corner weights attached. The latter consisted of 3 marbles in net pocket –attached to each of four outer net corners of bag (Figure 3.1).
3. A separate valve controlled supply line from the pump delivery manifold was connected to a 50mm PVC pipe ring (made with 8 x 45^{o} elbows and pipe spacers) - that supplied a jet of water to each of the 34 net bags. The jet of water was facilitated by drilling a 5mm hole in the ring pipe above each net bag. The ring was suspended by lines and hooks from

the ceiling and were adjusted to be horizontal and 40cm above the surface of the water in the net bags. The flow strength of all jets could be adjusted by the mentioned control valve (Figure 3.1).

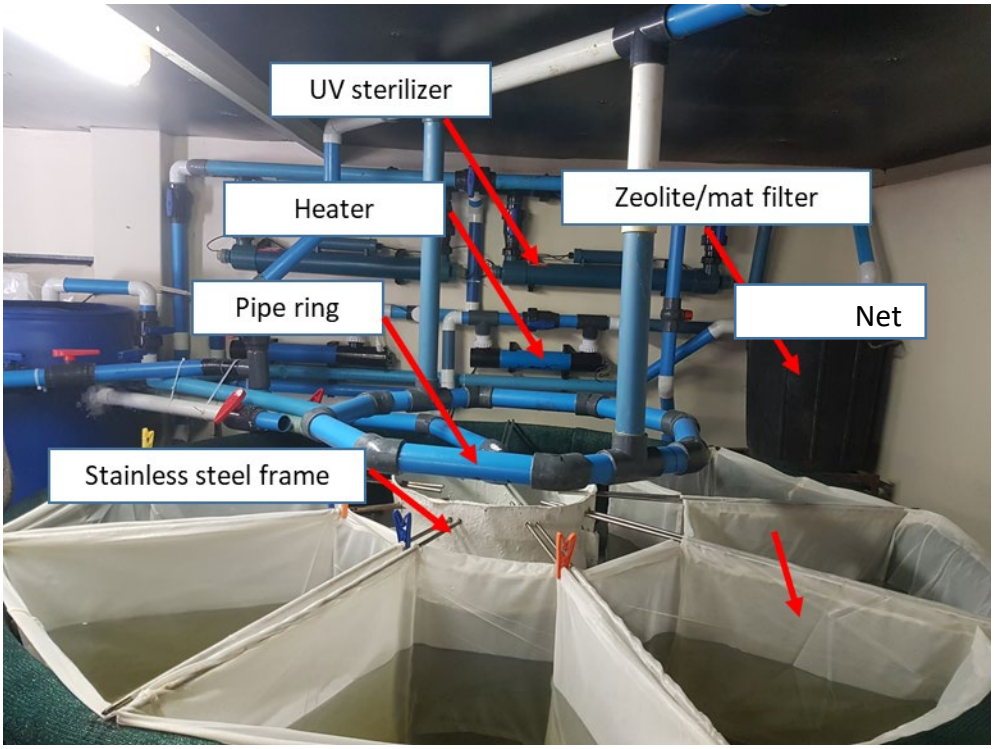


Figure 3.1 The hatchery tank with small net bag compartments for keeping fish eggs and larvae as per treatment. Illustration of different components that contribute to the functionality of the hatchery system.

Ten males and ten female African sharptooth catfish obtained from the Welgevallen Experimental farm, were randomly selected and allocated to the 3kL tank, where they were held to acclimatise to the RAS system for three weeks before commencement of trial. During these three weeks, all fish were fed commercial trout pellets, which during the trial, were supplemented with raw chicken livers fed to fish to satiation. This feeding regimen conditioned and prepared the broodstock fish for breeding and spawning. The material used for the heatshock induction consisted of newly spawned eggs and sperm obtained from the broodstock. The experimental design consisted of 16 treament groups (16 shock initiation time treatments

applied after fertilisation) and untreated control group. Shock initiation times (IT) of 40, 41, 42, 43, 44, 45, 45.5, 46, 46.5, 47, 47.5, 48, 48.5, 49, 49.5 and 50 minutes post-fertilisation were used. Eggs adhesiveness was used to attach fertilised eggs to trial tagged bottle brushes. The eggs were incubated at 25 °C ± 0.1 in a separate temperature controlled bath until heat shock initiation time, when it was transferred to heat shock water bath at a temperature of 40.5 °C ± 0.1 for a duration of 2 minutes. Control eggs were not heat treated. Six of the treatments were spaced one minute apart (40 – 45 minutes) while the other ten (45.5 – 50 minutes) were separated by 30 seconds. All treatments were replicated two times, therefore two net bag compartments were randomly assigned to each of the treatments (32 samples). For ploidy testing, three methods were employed namely flow cytometry, chromosome preparations and silver nitrates staining for identification of nucleolar organiser regions (NORs). Fish produced from one artificial breeding session were grown to 20 weeks of age before obtaining blood and fin tissue samples for flow cytometry and silver nitrate NORs staining ploidy verification respectively. Samples were sourced from 20 fish per treatment tanks (sampling was done in duplicate for each treatment). Ploidy analysis was also performed using flow cytometry and chromosome preparations in two-days post hatch (2DPH) larvae. For flow cytometry two samples sizes were used, therefore n=5 and n=20 larvae per treatment, while for chromosome preparations only 20 larvae per treatment. All sampling were done in duplicate for each treatment.

Executable tasks were sequentially strategised as follows:

a) Programmed induced spawning of conditioned broodstock and recorded timed interval settings between egg fertilisation and heat shock at a consistent incubation temperature.

b) Application of heat shock (40.5°C) to fertilised eggs that completed incubation time as per interval setting from fertilisation for 2 minutes.

c) Preparation of tissue (blood, fins or two-day post-hatch (DPH) larvae) for DNA quantification

d) DNA quantification using chromosome staining, NORs staining (nucleolar organizing regions) or flow cytometry methods

e) Comparing selected DNA quantification methods in terms of efficacy, repeatability, rapidity

f) Determining which treatment intervention (post-fertilisation time interval to heat shock) yielded predominant populations of tetraploid larvae

3.2.1 Brood stock conditioning and artificial spawning

African sharptooth catfish broodstock were reared at 8.3 $kg.kL^{-1}$ stocking density in a single 3 kL circular tank high density polyethylene (HDPE) tank and conditioned by feeding them rotationally with trout pellets and raw chicken livers to satiation twice daily (8:00 and 18:00). Fish were also fed on alternate days with formulated Aquaplus® trout pellets (6 mm) soaked in a mixture of fish oil, chicken liver and egg yolk. The prepared mixture contained 800 g livers, nine chicken egg yolks, and 100 ml fish oil per 400 ml water which were blended (kitchen blender at high speed) for one minute. Two-kilograms of pellets were soaked with this mixture, portioned into equal sizes of 400 g and frozen for required use. This feeding programme was selected as it proved superior to fast track *Clarias gariepinus* broodstock conditioning based on observations and empirical results from breeding trials (Fouché, 17 May, 2018, pers. comm.).

Water quality was kept optimal using recirculating aquaculture system (RAS) technology, which maintained the following parameters continuously: Temperature = 26 °C – 28 °C; pH = 6.9 – 7.3; total ammonia nitrogen (TAN) < 0.02 mg/l; Oxygen > 5 mg/l; NO_3 < 40 mg/l; Alkalinity > 60 ppm; Calcium carbonate hardness > 40 ppm.

Two females with conspicuously swollen bellies and with light-red to red cloacae were selected for spawning. Two males were selected when having prominent urogenital papillae, preferably with reddish tips. Both the male and female breeders selected for spawning were injected with Aquaspawn® at 0.5 $ml.kg^{-1}$ and kept in separate four water containers to prevent hormone-induced aggressive behaviour and harmful attacks on fellow breeders (Fouché, 2 August 2018, pers. comm.).

Latency time to spawning was approximately 12 – 13 hours at 25°C. Spawning-prepared breeders were then sedated in a bath (40 L) with 2-phenoxyethanol at 1 ml 3.3 L^{-1} water before the males were killed by pithing and then decapitation. The testes were then surgically removed, weighed on a digital balance (0.1 g resolution) and these data were recorded (). Milt was extracted from testes by severing it with a blade and pressing the contents into a clean 250 ml glass beaker with 50 ml 0.9% saline, and the severed testes were finally added. The mixture was gently stirred and the remaining testes tissue finally removed.

Both sedated males and females were measured in centimetres (cm) (caudal length), weighed [in grams (g)] with a digital scale (0.1 g resolution) and the morphometric data were recorded in Table 4.1. Females which released eggs gravitationally when held with head upright were ready for manual egg stripping. Eggs were stripped gently by abdominal massage into a dry

4 L bowl and then weighed. Mean data values were generated from the morphometric data calculated by using the following formulas:

- Testes-somatic index: TSI (%) = WT/WB X 100, where WT represents the weight of the testes and WB the total weight of the breeder fish.
- Roe somatic index: RSI (%) = WR/WB X 100, where WR represents the weight of the stripped eggs and WB for the total weight of the breeder fish.
- Fulton condition factor (FCF): FCF (%) = TW/CL^3 x 100, where TW = Total weight (g) and CL = Caudal length (cm).

3.2.2 Fertilisation and heat-shock treatment

The prepared saline-sperm mixture was then added to the eggs with gentle mixing. Chlorine-free tap water (24 hour aged aerated water) was added to the egg-milt-saline mix at approximately twice the volume of the eggs stripped. A period of 15 seconds was timed and estimated as the window period for completion of the fertilisation process. A timer was activated to measure the interval between egg fertilisation and heat shock. Bottle brushes were used as the egg adherence substrates. The brushes were respectively marked with different shock initiation times (IT) post-fertilisation intervals to the heat shock point from 40 – 50 minutes. These brushes were soaked in the egg bowl while mixing gently to constantly suspend eggs in the bowl, allowing easy and even attachment. They were then positioned vertically in a temperature-controlled bath (25 °C) and made to float (with a polystyrene float attached below the brush handle). The brushes with the eggs were exposed to heat shock at 40.5 °C for 2 minutes between 40 and 50 minutes after fertilisation and 16 different treatment (initiation times (IT) post-fertilisation intervals) were

generated within this range (40 – 50 minutes). The intervals were a mixture of two treatment batches, one batch spaced by one minute (40, 41, 42, 43, 44, and 45) and the another separated by 30 seconds (45.5, 46, 46.5, 47, 47.5, 48, 48.5, 49, 49.5 and 50) respectively, making a total of 16 treatment groups. After the eggs were heat-shocked as per the selected IT treatment group (marked brush), the brushes were transferred to respective hatchery net bag tanks marked with similar specified IT measurements as indicated on the brush. The treatments were replicated two times, therefore each treatment was allocated to two net bags tanks. Control egg brushes did not receive any heat shock treatment and were also replicated twice.

3.2.3 Estimation of survival

The temperature in hatchery tanks was raised to 28 °C to accelerate hatching time (20 hours at 28 °C) to prevent a lethal invasion of dead egg fungus hyphae into live eggs. The survival percentage of embryos at the time of hatching (eyed embryos) was determined by using a square area (2 cm^2) of bottle brush hair to count dead and eyed eggs respectively. The mean of three squares was used to determine approximate % survival of eyed eggs as a qualitative measure of hatching success. Sample preparation for flow cytometry blood analysis, and nucleolar organiser regions (NORs) determination in fin tissue required raising hatched larvae to juveniles (20 weeks and approximately 200 g each). All other ploidy determination methods were employed using 2 days post hatch (DPH) larvae. The latter stage was preferred since it is not known if tetraploid larvae can only exist, with growout offspring only having diploid survivors.

3.3 Ploidy status determination using DNA staining techniques

3.3.1 a.) Chromosome staining (Giemsa) and metaphase spread counts (vinblastine Reanal)

Two days post hatch (DPH) fry of African sharptooth catfish were treated with vinblastine Reanal 10 mg ml^{-1} for 2 hours to arrest somatic cells in the metaphase mitotic stage. Control and treated samples were prepared in duplicates per treatment (40 – 50 minutes, IT heat shock treatment 1-minute interval from 40 – 45 minutes, and 30 seconds interval from 45.5 – 50 minutes). Samples of 20 larvae (n=20) were obtained from each repetition in both control and treatment net bag tanks; a total of 34 samples. These samples were incubated in a hypotonic solution (distilled water) for 20 minutes and fixed overnight in a 3:1 mixture of methanol and acetic acid (Carnoy solution). Air-dried slides were prepared according to Yamazaki *et al.* (1981) and stained with 5% Giemsa. Photomicrographs were taken with an Axiocam ERc5s camera mounted to a Zeiss Axio light microscope (400 x magnification). The ploidy status of each individual larva to be determined based on the presence of least 10 countable metaphase spreads.

b.) Chromosome preparation (colchicine)

Live 2 days post hatch (dph) (20 larvae per tank) were kept in 0.05 % colchicine for 3 hours at room temperature (at 25 °C) (Pradeep *et al.*, 2011; Karami *et al.*, 2015). Samples of 20 larvae were obtained from each repetition (n=20, replicated twice for each treatment) in both control and treatment net bag tanks. The sampled larvae were anaesthetised in a chilled isotonic solution (0.9% saline) and transferred into distilled water (hypotonic solution) at room temperature for 30 minutes (Shao *et al.*, 2010; Pradeep *et al.*, 2011; Karami *et al.*, 2015). Following their removal from hypotonic solution, the specimens were placed in wells with freshly prepared Carnoy's fixative (methanol: glacial acetic acid, 3:1) at 4°C for 40 minutes. This step was repeated again by

placing larvae in a new solution of Carnoy's fixative for another 20 minutes at 4°C (Pradeep *et al*., 2011; Karami *et al*., 2015). To obtain a cell suspension, the larvae were transferred onto a cleaned and dried microscope slide and a drop of distilled water was poured onto larvae (to promote dissociation of embryonic cells). A sharp scalpel blade was used to chop the larvae until a whitish solution or homogenate was achieved.

The homogenate was spread onto the slide by covering it with a cover slip. The slides were passed through a burner flame. The slides were then rinsed in acetone and air dried for 10 minutes to dry oil droplets. A freshly prepared 11% Giemsa solution was used to stain the slides for 45 minutes (Pradeep *et al*., 2011; Karami *et al*., 2015). After removal from the staining solution, the slides were dipped in xylene for 10 minutes and air dried. To check for metaphase spreads, the slides were observed under Zeiss Axio light microscope at 1000 x magnification with oil immersion.

3.3.2 Silver nitrate staining nucleolar organiser regions (NORs) determination procedure

Fin clips of African sharptooth catfish (control and treated groups) were sliced on a clean slide and few drops of 50% acetic acid were applied to the samples. The samples were left dry in air at room temperature. Treatment and control samples were prepared in duplicate per treatment (40 - 50 minutes, IT heat shock treatment one minute interval from 40 – 45 minutes, and 30 seconds (from 45.5 – 50 minutes). Samples from 20 juvenile fish were obtained for each repetition in both control and treatment net bag tanks. A total of 34 samples ere kept separately in smaller net bags). Sample preparations were carried out according to a method used by Howell & Black (1980) and Kim *et al.* (2017), with samples stained with silver nitrate by using two solutions. Solution A was prepared with 0.5 g of gelatine, 25 ml of double-distilled water and 0.25

ml of formic acid, containing formaldehyde (2% final concentration). In preparation of an aqueous solution, solution B, silver nitrate (5 g) was mixed together with double-distilled water (10 ml). Aluminium foil was used for covering the two solutions (A and B), which were then placed in the dark and kept away from reacting with any source of light. To stain the slide, 50 µl drops of solution A and 100 µl drops of solution B were applied to the slide and a clean 3ml disposable pipette was used to mix gently. The following step was to place the slide on a hot plate at 60 °C, protected to shield out any light for the staining to occur. The slide was removed from the hot plate when the staining solution turned golden brown, washed gently fro beneath and left to dry in air. Nucleolar organiser region identification and counts were attempted, using a Zeiss Axio light microscope (20 x magnification).

3.3.3 Chromosome preparation (colchicine)

Live 2 days post hatch (dph) (20 larvae per tank) were kept in 0.05 % colchicine for 3 hours at room temperature (at 25 °C) (Pradeep *et al.*, 2011; Karami *et al.*, 2015). Samples of 20 larvae were obtained from each repetition (n=20, replicated twice for each treatment) in both control and treatment net bag tanks. The sampled larvae were anaesthetised in a chilled isotonic solution (0.9% saline) and transferred into distilled water (hypotonic solution) at room temperature for 30 minutes (Shao *et al.*, 2010; Pradeep *et al.*, 2011; Karami *et al.*, 2015). Following their removal from hypotonic solution, the specimens were placed in wells with freshly prepared Carnoy's fixative (methanol: glacial acetic acid, 3:1) at 4°C for 40 minutes. This step was repeated again by placing larvae in a new solution of Carnoy's fixative for another 20 minutes at 4°C (Pradeep *et al*., 2011; Karami *et al*., 2015). To obtain a cell suspension, the larvae were transferred onto a cleaned and dried microscope slide and a drop of distilled water was poured onto larvae (to promote

dissociation of embryonic cells). A sharp scalpel blade was used to chop the larvae until a whitish solution or homogenate was achieved.

The homogenate was spread onto the slide by covering it with a cover slip. The slides were passed through a burner flame. The slides were then rinsed in acetone and air dried for 10 minutes to dry oil droplets. A freshly prepared 11% Giemsa solution was used to stain the slides for 45 minutes (Pradeep *et al*., 2011; Karami *et al*., 2015). After removal from the staining solution, the slides were dipped in xylene for 10 minutes and air dried. To check for metaphase spreads, the slides were observed under Zeiss Axio light microscope at 1000 x magnification with oil immersion.

3.3.4 Flow cytometry method

A pre-calibrated flow cytometer (BD FACS Melody, BD Biosciences, San Jose, USA) was used for determining ploidy. The prepared control diploid larvae samples of African sharptooth catfish were used as standards during the analysis. Samples were analysed in duplicate (a total of 34 samples; two samples per treatment, n=5 and n=20 larvae per tube). Propidium Iodide (PI) was the flouroscent dye that was used in this experiment. Propidium Iodide is excited at 488 nm and, with a relatively large Stokes shift, emits at a maximum wavelength of 617 nm. The optimal excitation laser was set at 488 nm for experiments. The software used for data acquisition was BD FACS Chorus software v1.1 (BD Biosciences, San Jose, USA), but further analysis was conducted using the FlowJo™ 10.6.1 program (BD Biosciences, San Jose, USA).

The fluorescence intensity of stained nucleus is recorded and digitally converted from the flow cytometer to the computer. Before running the flow cytometer, samples were vortexed to

obtain a suspension of nuclei, which was then transferred to a 5 ml Becton Dickinson flow cytometry tube. In order to avoid debris while reducing electronic noise a threshold of 200 was set. DNA fluorescence was used as the thresholding parameter. For each sample, a minimum of 20 000 nuclei were obtained at a rate of 100 to 250 nuclei per second to generate histograms for analysis. A diploid control group was used as internal standard for each group of treatments and its DNA content was compared with that of the treated groups. Flow cytometry histograms were plotted using the curve-fitting program ModFit LT (Verity Software House, Topsham, ME, USA).

3.3.4.1 Blood and larval sample preparation for flow cytometry analysis

Method 1 (Blood + heparin +EDTA)

About 1 ml blood samples obtained through caudal vein puncture were collected from juvenile fish, which weighed approximately 200 g. Blood was collected into heparinised EDTA-containing vacutainer tubes, which were inverted 8 to 10 times before they were stored in a freezer at -20 °C. The samples were transported in ice from the Welgevallen experimental farm to the Central of Analytical Facilities (CAF) laboratory 24 hours later for further preparation and flow cytometry analysis.

Nuclear extraction was done using the Sysmex® method (Sysmex Partec GMBH, Germany). Blood samples were thawed at 37 °C in a temperature-controlled water bath. Thirty four (34) samples of 200 µL were taken and transferred with an auto-pipette into the flow cytometer tubes. An amount of 500 µL nuclear extraction buffer was added to one sample tube at a time and vortexed for 2 seconds a minute later, after addition of extraction buffer. The samples were then incubated for 30 seconds at room temperature before they were filtered through a 50 µm

polyamide screen filter into the sample tubes. Following the sample filtration step, 2 ml staining solution (which is comprised of Staining Buffer, Propidium Iodide and RNAse A stock solution) was added. The samples were vortexed and incubated for 30 to 60 minutes at room temperature in a dark room, covered with a protective foil. After 40 minutes of incubation, the samples were analysed using a standard flow cytometry procedure as described in section 3.3.4.

Method 2 (Blood +EDTA+ CPD)

Similar protocol steps were followed as in Method 1, but, with modification. Teruflex® CPD (citrate-phopshate-dextrose mixture) red blood cell preservative/anticlotting solution was additionally used in an effort to prevent blood clotting.

Table 3.1 The Citrate-Phosphate Dextrose (CPD) solution consisted of the following components per 100ml water**.**

Citric acid monohydrate - 0.327g
Sodium citrate (dehydrate) - 2.630g
Monobasic sodium phosphate (monohydrate) - 0.222g
Dextrose anhydrous - 2.320g

Approximately 0.14 ml of CDP solution was drawn in a sterile syringe needle prior to blood withdrawal *via* caudal vein puncture. The blood was transferred by vacuum pressure into a heparinised vacutainer. The samples were filtered three times (50 µ screen) to remove any possible blood clots before the staining procedure (see Method 1). In this method, 100 µl of distilled water was also added to the samples following the last step of filtration. The samples were incubated for 40 minutes just as in the first method, prior to flow cytometer analysis.

Method 3 (2DPH larvae)

To determine the ploidy status of each treatment group, control and heat shock treated (40 – 50 minutes IT post fertilisation) 2 DPH larvae (rearing temperature days post hatch was set at 25 °C) were prepared for flow cytometry karyotyping using the Sysmex® method (Sysmex Partec GMBH, Germany) as indicated in Method 1. To increase cell numbers for analyses, cells from either five larvae or 20 larvae were respectively pooled and the ploidy type frequency distribution finally compared (flow cytometry results). Cells were prepared using a modified Sysmex®sample preparation protocol. Briefly, the respective larval (2DPH) samples were put in marked tubes and 500 µl of Nuclei Extraction Buffer was added. A slow rotation (approx. 1500 rotations per minute) tissue homogeniser (LabX Janke & Kunkel Ultra-Turrax TP 18/10S1 Homogeniser 18/10 S1) was used for three seconds to homogenise the sample before the homogenate was incubated for 60 seconds. The sample was then filtered through a 50 µm polyamide screen filter, using micropipette dispenser injection pressure to transfer the liquidised product through the screen into a flow cytometry test tube. Following sample filtration, 2 ml of the staining solution was added, the sample was mixed using a vortex and then incubated for 30-60 minutes at room temperature until required for flow cytometry analysis. This step was performed in the dark since Propidium Iodide is light sensitive. Flow cytometry analysis followed as indicated in section 3.3.4.

3.3.4.2 Software analysis of raw data

The curve-fitting program ModFit LT (Verity Software House, Topsham, ME, USA) was used for analysis of raw data. Flow cytometry histograms were produced using this software program. These histograms describe the distribution of fluorescence signals from the nuclei (Yang *et al.*, 2000). The peak position of the histogram is measured by channel numbers on the horizontal axis

and reflects the relative DNA content per nucleus, while the number of nuclei recorded is shown on the vertical axis (Peruzzi & Chatain, 2000; Yang *et al.*, 2000). The area under the curve represents the relative contribution of a certain ploidy (Allen, 1983).

3.4 Statistical analysis

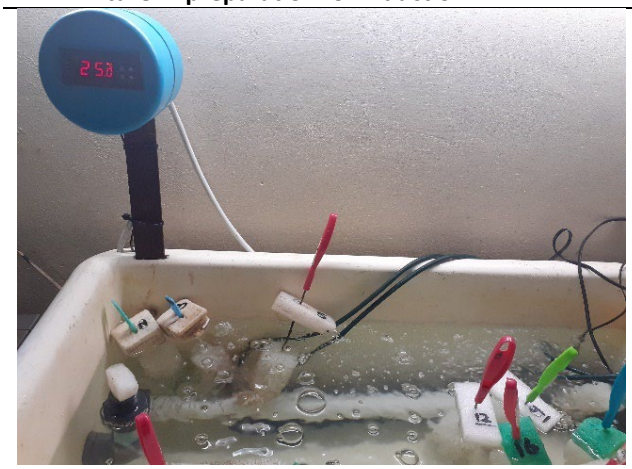
Microsoft Excel and Statistical Analysis Systems (SAS) were used to analyse morphometric data. Microsoft excel was used to graph the percentages of ploidy produced per treatment. The karyotype data were not subjected to statistical calculations since the percentage frequency of occurrence of represented nuclei quantity per karyotype were detected as either diploid or tetraploid. The ratio aspect between the occurrence frequencies of the two karyotypes were deductively used to determine tetraploidy induction success per time interval setting from fertilisation to heat shock intervention.

a. **Conditioned brookstock male and female being anesthetised in a holding tank in preparation for artificial spawning.**

b. **Mixture of stripped eggs (dark) and milt (foamy) before fertilisation.**

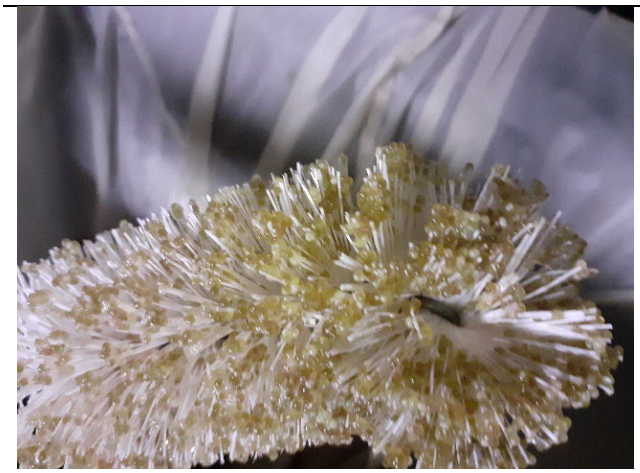
c. **Fertilised eggs before the brushes are dipped in the egg-mixture in preparation for induction.**

d. **The warm-water bath set up at 25°C for incubation of embryos prior to the induction procedures.**



e. **Fertilised eggs attached to brushes during the incubation period at 25°C in the aerated water bath.**

f. **Heat-shock inducing warm water bath at 40.5°C in which treatment brushes are transferred the incubation tank.**



g. **Close up look at the embyros in the hatchery tank.**

h. **The RAS for keeping broodstock and larvae.**

i. **The hatchery tank with small bag compartments for keeping fish eggs and larvae as per treatment.**

j. **Becton Dickinson FACSMelody flow cytometer at the Central Analytical Facilities Fluorescence Microscopy Unit Main campus, Department of Physiology Stellenbosch University used for analysis of samples.**

Figure 3.2 An illustration highlighting the main processes and events during the tetraploid induction procedure.

Chapter 4 Results and discussion

4.1 Morphometric data, hatching success and egg distribution on brushes

Table 4.1 indicates mean values (± SD) of morphometric parameters for 20 *C. gariepinus* broodstock (n = 10 per sex), which were used in the study. The mean testes somatic indices (TSI) for hydrated testes was 0.3 ±0.1%, while the mean roe somatic Indices (RSI) of females averaged 11.2 ±1.1%. The mean body mass of males was roughly equal to those of females (male group = 1142.0 ± 300.8 g; female group = 1123.1 ± 137.4 g), whereas the mean Fulton condition factor for females was higher (male group: 2.9 ±0.3%; female group: 3.3 ±0.3%).

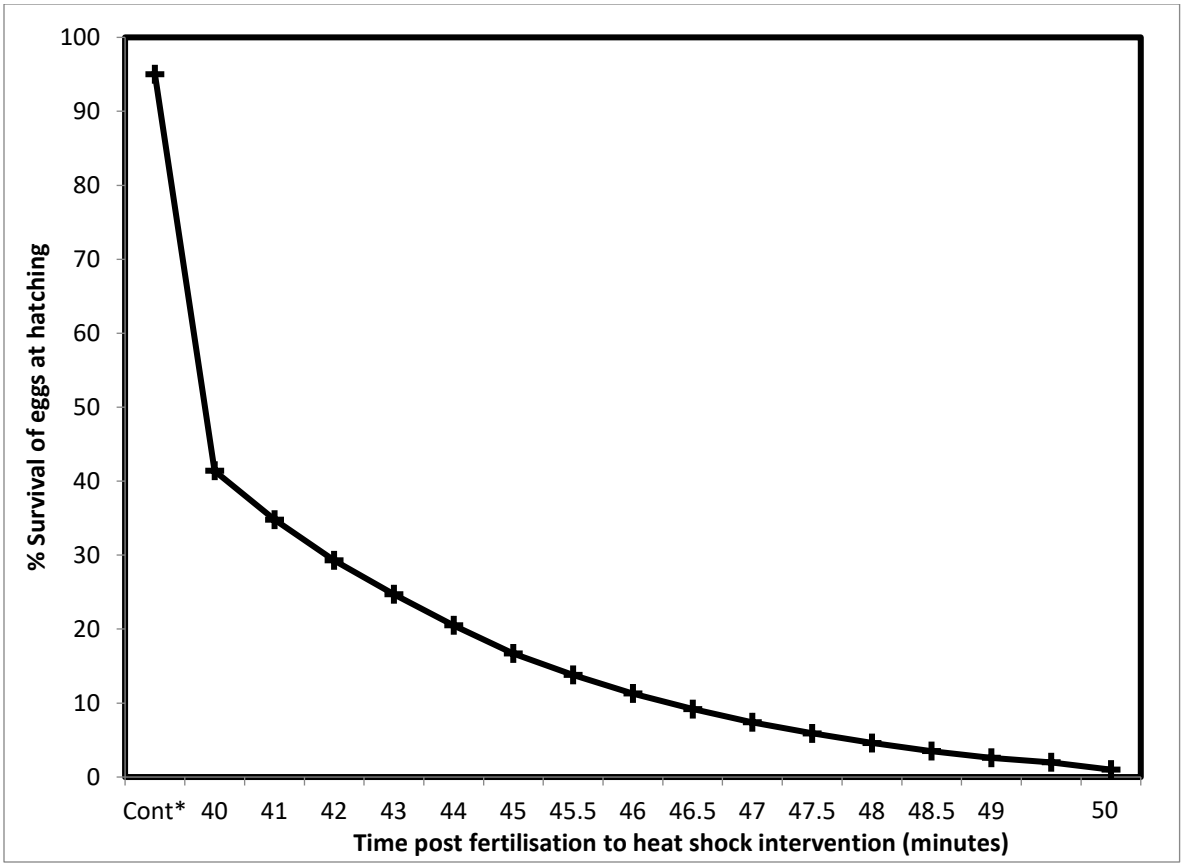
In fish, the condition factor reflects the physical and biological state of the fish, as well as changes related to feeding conditions, infections and physiological factors such as stress, maturity and spawning (Le Cren, 1951). It indicates the general wellbeing of a fish, where the fish is physically fit and healthy when the condition factor is 1% or greater, while a fish is in poor growth condition when the condition factor is less than 1% (Jisr *et al.*, 2018). In the current study, the condition factor was an indication that the resilient breeders were in a good health and sexual maturity state and were properly conditioned. The approximate threefold higher values for both the sexes of African sharptooth catfish brood stock are indicative of complete nourishment and proper husbandry conditions. Also, the endocrine intervention causes hydration of the gonads and a concomitant increase in body mass (Saka, 2015). Ovaries hydrated proportionally much more than testes (morphometric data) and, therefore, also achieved the highest FCF percentage value.

Table 4.1 Average morphometric measurements of *C. gariepinus* male and female (n=10 per sex) breeders.

Sex	**BM±SD** (g) (n=10)	**BL±SD** (cm) (n=10)	**RM±SD** (g) (n=10)	**RSI±SD** (%) (n=10)	**TM±SD** (g) (n=10)	**TSI±SD** (%) (n=10)	**FCF±SD** (%) (n=10)
Female	1123.1±137.4	32.6±1.9	123.8±13.0	11.2±1.1			3.3±0.3
Males	1142.0±300.8	34.2±4.1			5.4±3.2	0.3±0.1	2.9±0.3

SD=standard deviation; BM = body mass; BL=body length; RM= Roe mass; RSI (%) = roe somatic index; TM= Testes mass; TSI (%) = testes somatic index and FCF (%) = Fulton condition factor.

The line graph in Figure 4.1Figure 4.1 indicates the survival percentage of embryos as determined from three randomly selected 2 cm² brush areas at the time of hatching for each treatment group marked by a respective IT post-fertilisation window length. The egg distribution was not uniform between brushes but eggs were in high enough numbers to provide surplus surviving larvae for flow cytometry tests (n=5 or 20) per treatment group. The fertilised eggs were strongly adhesive as compared to previous attempts where experimental non-adhesive eggs displayed poor hatching success and larval survival (heat-shock intervention). The survival of heat-shocked embryos was substantially lower than the all-diploid control embryos. The embryos in the control did not receive the heat-shock and had above 95% survival. The percentage of survival in heat-shocked embryos (from 40-50 minutes post fertilisation), however, decreased from 41% in embryos treated 40 minutes post fertilisation to 1% in embyros which received treatment 50 minutes post fertilisation.



Cont = Control*

Figure 4.1 Survival rates of embryos (in %) after applying heat-shock of 40.5°C at different time periods post fertilisation in *C. gariepinus*.

Good quality fertilised eggs were obtained as measured by the strong adhesive properties thereof within approximately 15-20 seconds after fertilisation (Britz & Hecht, 1988). This is further confirmed by the relatively high mean fertilisation and hatching percentage of control eggs (non-treated), as well as the relatively high survival rate of pre-hatch embryos in all heat shock treatment groups when compared to no survival in poor quality eggs (none or poorly adhesive) used in previous spawning and heat shock attempts (pers.obs). The hatching rate of heat-shocked eggs was gradually reduced between 40 and 50 minutes initiation time post-fertilisation (ITPF) compared to non-heat shocked eggs. This decrease could be attributed to the heat shock treatment when applied on the eggs. Lebeda and Flajshans (2015) reported the same

outcome; that the temperature shock resulted in decreasing hatching rate success of Siberian sturgeon (*Acipenser baerii*) eggs. It is, therefore, evident that good egg equality, as with normal aquaculture production, is important to increase proportional tetraploid larval yield. Growing the broodstock under optimal conditions (good water quality parameters and feeding regimen/conditions) improved the egg quality, thus resulting improved hatching and larval survival for heat-shock treatment groups in the current investigation. Piferrer *et al.* (2009) indicated that an accurate broodstock management programme is a primary requirement for ploidy manipulation in seawater fish so as to attain gametes of the best possible quality. Komen and Thorgaard (2007), however, concluded that egg quality is still a not well understood phenomenon. They also added that other factors in eggs, such as the fatty acid constitution in marine species, can contribute to the improved fertilisation and fry survival, and may also influence the improved survival after heat or pressure shock treatments.

4.2 Ploidy determination method results

4.2.1 Chromosome spread counting

Following many experimental trials, with consistency as in previous studies procedure and preparations, stained metaphase-restricted chromosome clusters could be detected in most slides for all treatments. However, due to incomplete metaphase spread and possible chromosomal loss, the cluster size and chromosome counts were frequently inconsistent and impossible to quantify (). As a result, it was not even possible to distinguish between spreads in control (diploid) and tetraploid individuals. Cell burst occurred as no intact cell membranes could be observed. The staining protocols for both chromosome spreads did not conform to the experimental outcomes as described by the instructions of Karami *et al.* (2015). It is assumed that

the procedures followed may require more intermediate steps than provided by literature citings. Added to this, some of the steps indicated were not clearly defined; for example, “drying of the slide over a flame without destroying nuclear material”(Karami et al., 2015: p204). Chromosome spreads produced in the current investigation were scattered as a result of cell burst. Variations in the preparation method were tested but the result was the same as shown in Figures 4.2.

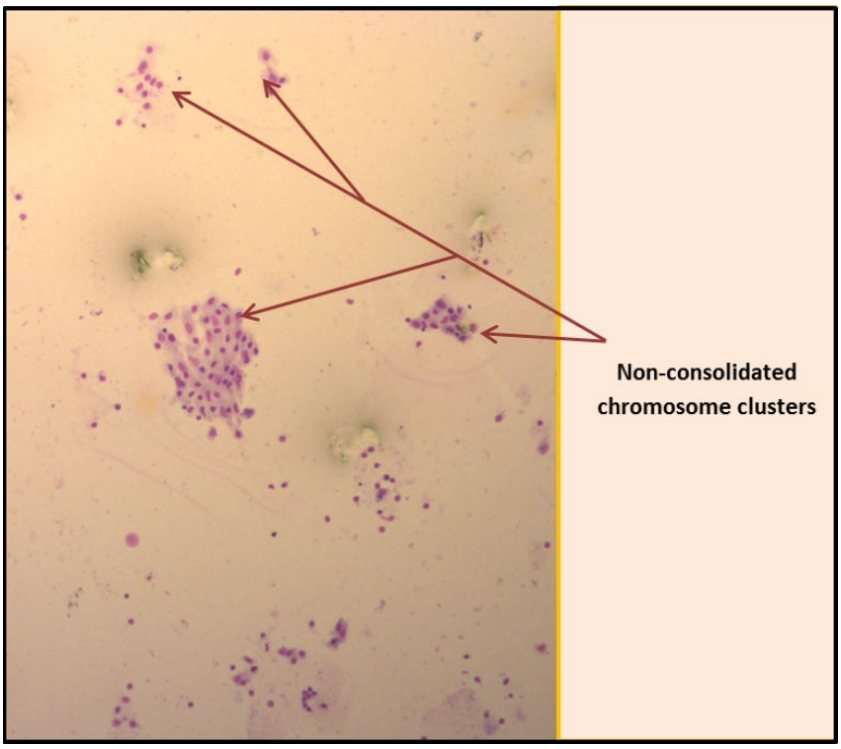


Figure 4.2 Non-consolidated chromosome clusters of stained cells of 2 days post hatched African sharptooth catfish larvae with undistinguished metaphase spread.

4.2.2 Nucleolar organiser regions (Silver staining NORs) identification and count

Intra-nucleus NOR spots could not be detected or identified in all treatments due to excessive background staining. Silver staining in the experiments where NORs must be identified was not possible, since distractive silver deposits occurred within nuclei and the cytoplasm of cells. Experimental variations of the staining technique did not alter the results. Kavalco and Pazza (2004) previously mentioned that silver debris precipitation in the conventional silver nitrate staining procedure is responsible for false positive results, giving difficulties to the viewer when identifying the ploidy of a sample.

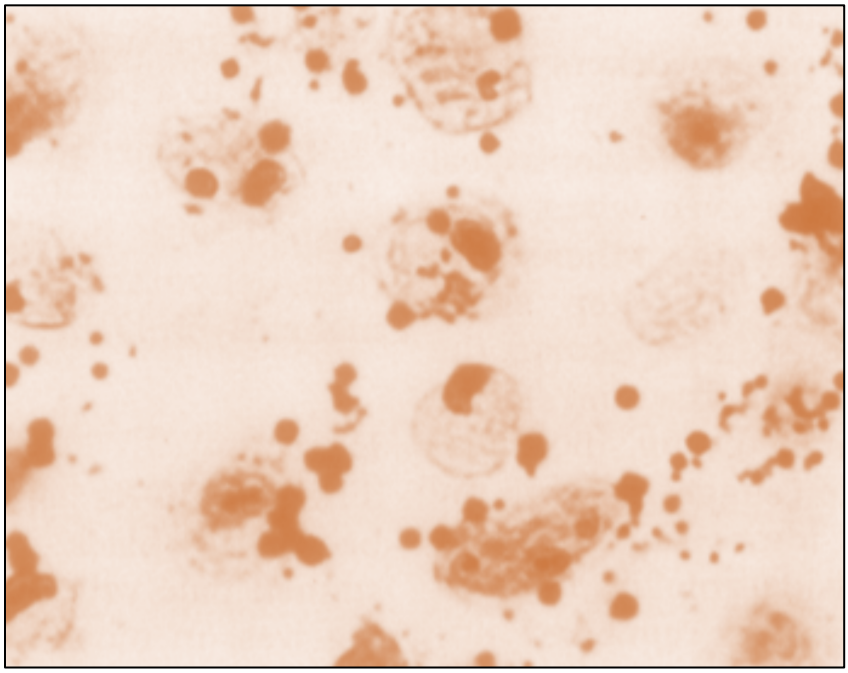


Figure 4.3 Distracting silver deposition or silver debris precipitation was observed in silver stained fin tissue sample obtained from 20-weeks old African sharptooth catfish fingerling (treatment group).

4.2.3 Flow cytometry's interpretation of data

The success of flow cytometry relies on a single cell suspension that is of high quality. Whenever cells with reduced quality are subjected to the machine, the resulting data becomes difficult to analyse. Therefore, sample preparation is the first vital step for any flow cytometry study. Figure 4.4 (a) shows an example of a two-parameter correlated area density plot, which represents the distribution of nuclei in two dimensions as detected by the forward scatter detector and the 90° angle scatter detector. Each dot on the plot represents a nucleus or an individual particle that has passed through the laser. On the graph, the intensity of the 90° angle light scatter (SSC-Area) is on the vertical axis, plotted against the intensity of the forward angle light scatter (FSC-Area) on the horizontal axis. The SSC-Area represents the granularity of the particle while the FSC-Area indicates the size of the particle.

The quality of a flow cytometric histogram of nuclear DNA content is indicated by the width of the peak obtained from cells that are in the G_1 phase of the cell cycle. The width is quantified in terms of the coefficient of variation (CV) across the peak (De Beer, 2004). The CV percentage shows the relative scattering of data by utilising the mean as a unit; therefore CV (%) = SD: MN*100 (Lamatsch *et al*., 2000). Histograms with CV values of less than 5% are generally considered as of acceptable quality (Dressler, 1990).

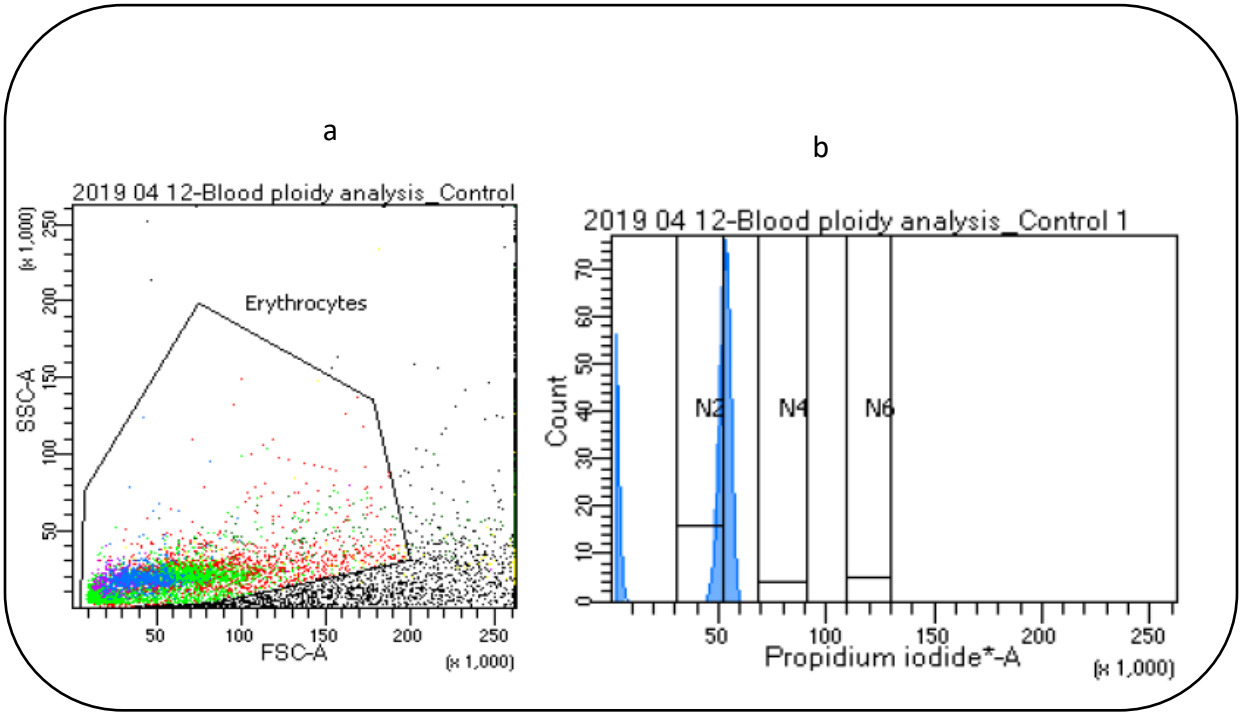


Figure 4.4 (a) Distinguishing the population of cells with single nuclei based on the Forward versus Side Scatter (FSC vs SSC) by gating in a density plot of PI stained African sharptooth catfish erythrocytes nuclei (a) obtained from nuclear extraction by Method 1. (b) A histogram from the flow cytometry analysis showing the number of diploid nuclei obtained from African sharptooth catfish blood cells stained by Propidium Iodide (PI) using sample preparation Method 1 (as shown in section 3.3.4.1).

Figure 4.4 b shows an example of a good quality flow cytometric histogram, representing the number of stained nuclei from *C. gariepinus* blood cells detected by a PI fluorescent intensity detector (Propidium Iodide-Area). The fluorescent intensity is denoted by the channel number on the x-axis (Propidium Iodide-A), which indicates the DNA content in a single nucleus or cell. The nuclear DNA content is, therefore, directly proportional to the fluorescence intensity, which is expressed as channel numbers (Lamatsch *et al.*, 2000). The channel number at which the peak is positioned is used identify the position of the histogram on the x-axis and is located at 51.01 for the erythrocycyte nuclei. There were noticeable amounts of cellular debris, which has relatively little DNA content, found on the left fluorescence side of the histogram. Further flow cytometric analyses on the overall treated samples prepared by Method 1 for nuclear extraction

resulted in histograms of low quality or no results. This is due to most sample having debris and clumps which resulted from blood clotting during sample preparation and processing. Samples with excessive amounts of debris and clumps were difficult to run during flow cytometry analysis and were completely rejected by the machine.

The application of Method 2 for nuclear extraction, which is a modification of Method 1, resulted in histograms of inferior quality and reduced DNA content after analyses. Cellular debris were identified on the left of the G_1 phase of diploid nuclei fluorescence peak in Figure 4.5 obtained from a control blood sample of juvenile African sharptooth catfish. The position of the G_1 peak of the diploid nuclei obtained from the juvenile fish is observed at channel number 50.00. The reduced quality of histograms could be a result of clots which were found in the blood samples even after adding anticoagulant during blood collection. Reapeting sample filtration three times could not assist with removing the cell clumps and debris. However, filtering the samples repeatedly could have resulted in the excessive loss of cells, which reduced the DNA content stained by PI dye. The resulting cellular waste, which was identified by the flow cytometer as debris may have also absorbed some of the propidium fluorescent dye.

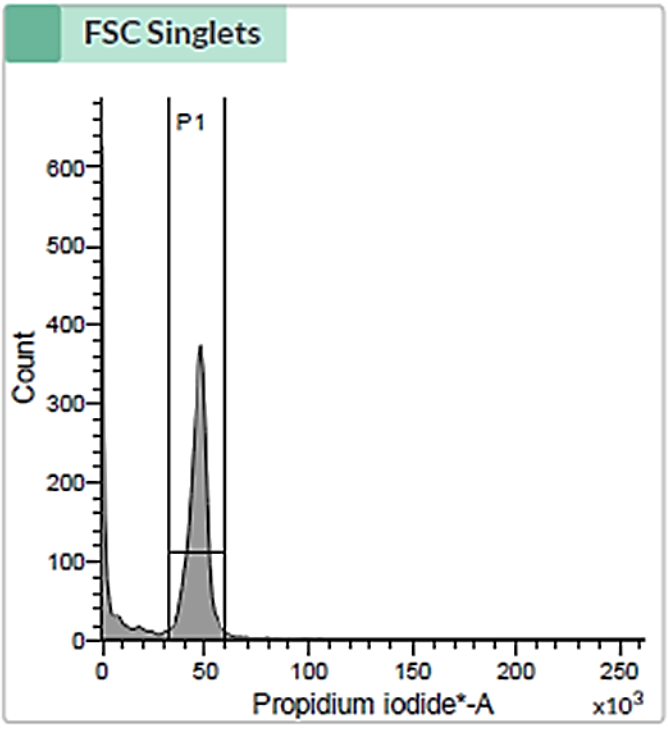


Figure 4.5 A histogram peak showing the number of nuclei stained by Propidium Iodide from control blood sample of diploid juvenile African sharptooth catfish, detected by PI fluorescent intensity detector after nuclear isolation by using Method 2.

An optimised method of sample preparation during nuclear extraction is imperative as the interpretation of the histograms from flow cytometry analyses is highly dependent on proper sample preparation (de Beer, 2004). In preliminary studies of the current investigation, ploidy determination from blood cells was less effective due to the high presence of thrombosis (blood clotting), irrespective of the presence of the anticoagulant (heparin) + EDTA (Method 1 described in section 3.3.4.1) or a combination of EDTA and CPD (Method 2 described in section 3.3.4.1). Subsequently, the method used for nuclear extraction could not be successfully optimised and resulted in blood clots, which were recognised by the flow cytometer as debris or aggregates. The blood clots were unable to pass through the 50-micron filter leading to cell loss during the filtration process, thus resulting in a very slow analysis and finally low nuclei count (value scattering). It is speculated that one of the chemicals used in the Sysmex® procedure may also

have enhanced thrombosis in sample tubes, since samples had to be re-filtered (a second filtration step). The promoting effect of the Sysmex® procedure on blood clotting is possible since non-coagulating blood could be obtained from *Clarias gariepinus* in other studies using heparinised EDTA tubes (Fagbenro *et al.*, 2010; Adeyemi & Zaid, 2016). These results contrast with those of other authors who successfully employed flow cytometry to detect ploidy using red blood cells in other fish species, such as adult rainbow trout (*Oncorynchus mykiss*), juvenile Atlantic salmon (*Salmo salar*) and hybrids of grass carp (*Ctenopharyngodon idella*) X bighead carp (*Hypophthalmichthys nobilis*) (Thorgaard *et al.*, 1982; Allen, 1983; Lamatsch *et al.*, 2000).

Application of Method 3 for extraction and staining of nuclei of African sharptooth larval cells was a success, as it produced high quality histograms (as presented in a, b and c). Figures 4.6 a, b and c, respectively, present typical examples of flow cytometric histograms indicating the diploid control, twin peaks that resemble both intra-population diploid and tetraploid individuals, and predominance of a tetraploid population. Diploid populations were identified by a peak (highest nuclei count) in relative mean fluorescence intensity (MFI) of Propidium Iodide at channel 50 (program selected), while the tetraploid peak displayed a PI MFI channel value of 100.

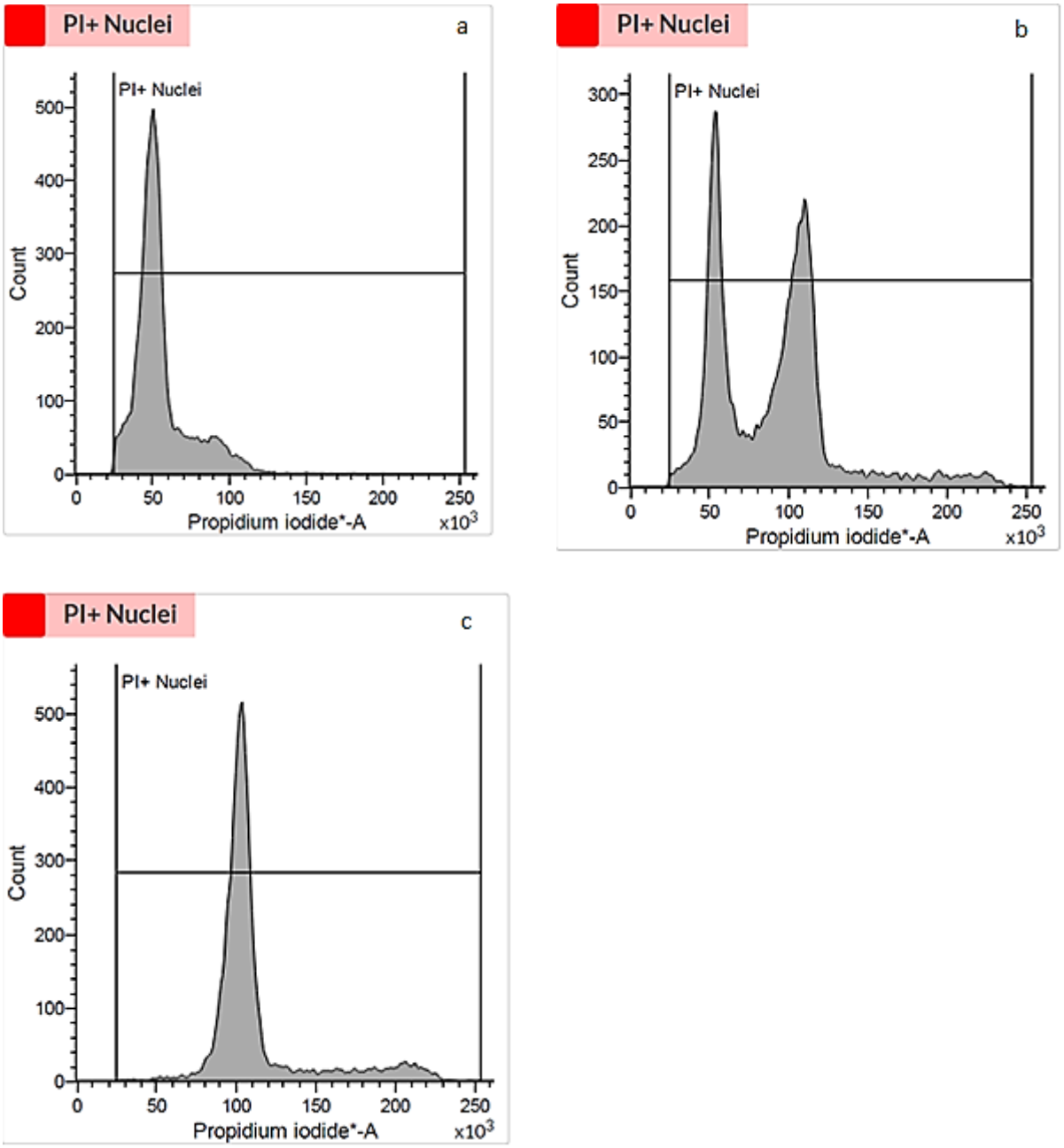


Figure 4.6 A diploid peak (a) configured to be at PI MFI of 50 (program selected channel) ;(b) shows a sample which resulted in two histograms peaks due to having both diploid and tetraploid nuclei (y-axis) while (c) the sample had predominantly tetraploid nuclei (PI MFI of 100).

The flow cytometry application for ploidy analysis using blood cells obtained from juvenile African sharptooth catfish was less effective as confirmed by the current investigation. However, the flow cytometric assessment of African sharptooth catfish larvae (2 days post hatch) demonstrated to be highly efficient, rapid and repeatable. The latter was confirmed by the non-

differentiated duplicate samples tested in the current study. This method is also advantageous since ploidy status detection during embryonic or larval stages can be a beneficial and useful strategy when larger fish are unavailable (Ewing & Scalet, 1991). The current investigation demonstrated that most nuclei of homogenised larvae , processed as per a slightly modified Sysmex® method, stained successfully with PI as reflected in the MFI peaks (Figure 4.6) detected for the respective ploidy groups. With all the advantages of the approach with respect to accuracy, speed and repeatability, a drawback of this approach is that it is destructive. In an aquaculture setting, one would ideally like to identify and retain the tetraploids rather than destroying the organism in the validation of ploidy. However, in a follow up investigation, raising induced larvae to fingerlings, it was determined that a 4mm x 4mm fin clip obtained from fingerlings (20g) was sufficient to determine ploidy status non-destructively by flow cytometry (Fouche, October 2021, pers. comm.). Furthermore, fin clip samples were read fast by flow cytometry and each sample could be karyotyped within less than 2 minutes. The method of using fin clip samples will save individuals for tagging and repetitive use when grown out. Successful induction of tetraploidy postulates that tetraploid fish can be grown to broodstock which will used for mating with diploids to produce functionally sterile triploids. It must still be verified if tetraploid larvae can survive to grow-out stage.

A differential occurred in the proportional ratios of both diploid and tetraploid larvae, between the n = 5 and n = 20 treatment groups. Tetraploid populations predominant in the 42 to 47 minutes post-fertilisation heat shock treatments, with the exception of the 44 minute initiation time post-fertilisation (ITPF) group displaying larger diploid populations for both the selected sample sizes (**Figure 4.7** and **Figure 4.8**). Although more tetraploid individuals were

collectively observed in treatments with five larvae per sample, accuracy will be improved when using increased numbers for sample size (ex. n=20). The current study confirmed that a 47-minute ITPF treatment can effectively be used to produce the maximum percentage of tetraploidy in 2 day post hatch larvae of African sharptooth catfish. A minimum of only 7% diploids was present in the 47-minute ITPF treatment group, which equates to only 1.4 (approximately 2 larvae) of the sample population (n=20).

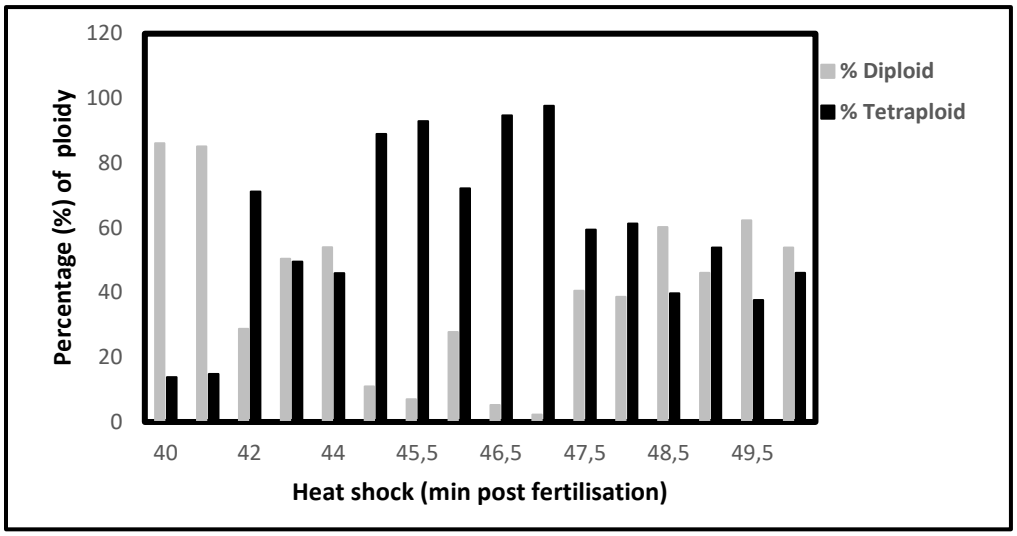


Figure 4.7 Histogram of the proportions of diploid and tetraploid larvae per treatment as determined for the smaller sample population (n=5).

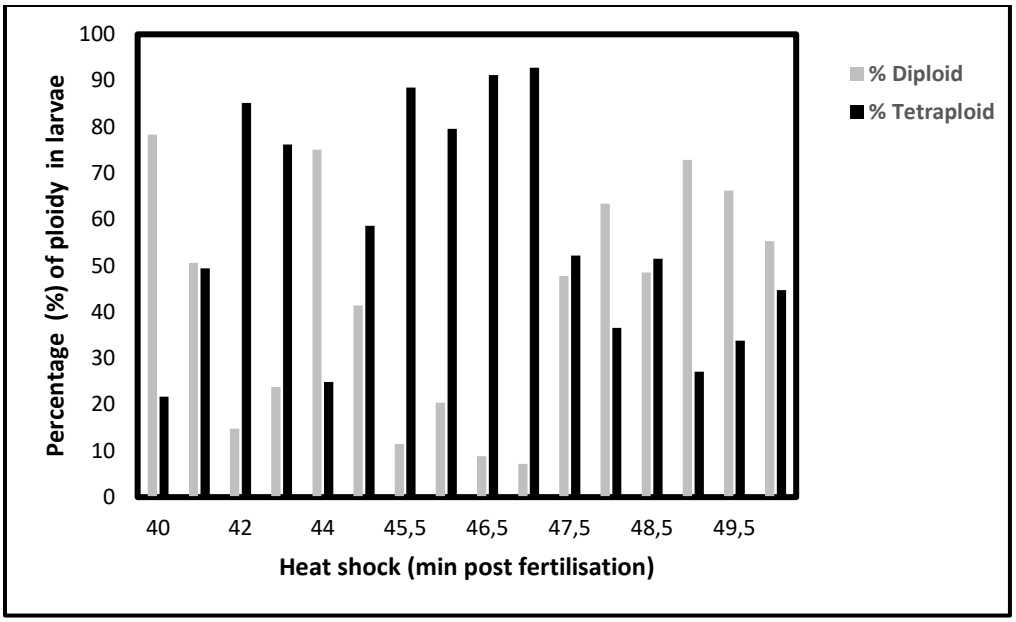


Figure 4.8 Histogram of the proportions of diploid and tetraploid larvae per treatment as determined for the large sample population (n=20).

The histograms in Figures 4.7–4.8 were produced from processed raw data of the flow cytometer computer. The frequency of occurrence of tetraploids and diploids was ratio compared since the nuclei counts were exact for each ploidy determinand. Figure 4.8 clearly indicates the fluctuative decline of diploids from 44 to 47 minutes ITPF, before gradually increasing from 47.5 min ITPF. The peak occurrence (>90%) of tetraploids at 47 minutes ITPF indicative that this intervention time must be used in further research to maximize the production of tetraploid offspring. The results further show that most respective windows of post fertilisation treatment times (42-47 minutes) can be used to induce tetraploidy with predictability (duplicate verification). The heat-shock initiation time range of 45-47 minutes will therefore maximise the occurrence of tetraploid individuals.

It is evident that tetraploidy can be successfully induced in African sharptooth catfish across the indicated broad spectrum of heat shock initiation times (IT) post fertilisation, at a temperature of 40.5 °C and a duration of 2 minutes. Váradi *et al.* (1999) reported that a

temperature of 40.5 °C applied for 2 minutes was 'non-sensitive' or sub lethal and effective for tetraploidy induction, and produced 9.2% tetraploids in *C. gariepinus*. The percentage of tetraploids obtained in the current investigation differed overtly to those produced by Váradi *et al.* (1999). This could be due to the fact that their study focused more on the production of homozygous gynogens over heterozygous tetraploidy induction. Another possible reason could be that their study used a unit called the tau value to express their time of shock initiation. The tau value is said to be equivalent to the duration of the mitotic cycle during synchronous cell divisions at the initial stages of embryogenesis, where in their experiments one tau-value was from the onset to the end of second mitotic cleavage (Váradi *et al.,* 1999). In the present study, a workable procedure was developed determining real time in minutes to initiate tetraploid induction.

A study was done by Sule and Adikwu (2004) which described the embryonic development and time after fertilisation in *C. gariepinus* under laboratory conditions. The study demonstrated that at 40 minutes after fertilisation, embryonic cells were at 2-cell stage of development, where two cells of equal sizes are produced. At 55 minutes post-fertilisation, the cells were at 4- cell stage of development producing 4 cells which were equal in size. This support the current study as the shock initiation times (40 - 50 minutes) studied were within time range (40-55 minutes after fertilisation) described in the previous study's findings. Since the heat shock treatment could induce the highest percentage of tetraploidy between 42 and 47 minutes post fertilisation, it could be suggested that this period corresponds to the first mitotic division. Hence, the mitotic cleavage formation was successfully inhibited, and resulted in tetraploids.

The current investigation confirms ploidy level verification and, especially, tetraploid identification of African sharptooth catfish larvae using flow cytometry. The slightly modified CyStain® PI Absolute P protocol used in the current investigation proved to be a rapid method for processing high numbers of samples compared to the less reliable and cumbersome nuclear staining methods used in this investigation, as well as in other investigators (Váradi *et al.*, 1999; Karami *et al.*, 2015). Although the flow cytometric protocol used in this study focused on ploidy detection in larvae, the method could be applied in future studies where the fish are grown to a larger size, using fin tissue only to test for tetraploidy as proven with in a follow up study conducted on African sharptooth catfish (Fouche, October 2021, pers.comm.). The use of fin clip samples will therefore exclude the destructive nature of the method by sacrificing fish larvae. The latter were specifically used in the current investigation to verify the efficacy of flow cytometry in DNA quantification, and also to prove that tetraploid larvae does survive to 2DPH. The growout survival of tetraploid fish must still be verified in further studies. Such fish will be kept alive as broodstock and be used for future production triploids by crossing tetraploids with diploids. Dorsal fin clips were successfully used to detect triploidy by flow cytometry in *Poecilia formosa* (Lamatsch *et al.*, 2000).

Restricting the second mitotic cycle of the zygote requires the correct time window between fertilisation and thermal shock (Váradi *et al.*, 1999). The current investigation demonstrated the importance of consistency in the timing of egg fertilisation, the egg incubation period and temperature (25 ±0.1 °C), as well as the final 2 minutes heat shock (40.5 ±0.1 °C) induction time window. The work of Varadi *et al*. (1999) did not confirm most of these outcomes determined in the current investigation.

No triploid larvae were detected, which was indicative that second polar body extraction occurred before the heat shock event (startpoint of 40 minutes post-fertilisation). The current method will exclusively mediate detainment of an extra full set of chromosomes during correctly-timed heat shock intervention, preventing the completion of the second mitotic cycle. Fish eggs do not extrude the second polar body until they are fertilised. Because of this, if a newly fertilised egg is shocked, the shock prevents the extraction of the second polar body. Consequently, the fertilised egg will contain three haploid nuclei; one from the egg, one from the sperm, and one from the second polar body (Tave, 1993). These three haploid nuclei will combine to form a triploid zygote nucleus, which creates a triploid. In the African sharptooth catfish, the second polar body can be successfully retained by cold shocking the eggs for 4 minutes after fertilisation at 5°C for 40 minutes. This induction produced 80 – 100 % triploidy success rate (Richter *et al*., 1987).

This investigation further validated the predominant production of African sharptooth catfish tetraploid offspring using a 42 - 47 minutes time window between fertilisation and thermally-induced tetraploidy. The higher diploid population in the 44 minutes TPF group cannot be explained, except if an assumption is made that not all the fertilised eggs were in a similar physiological and mitotic cycle before heat shock intervention.

The outcome of the current work unlocks the opportunity for the production of triploid offspring by crossing identifiable female tetraploids with male diploids. The use of tetraploid males may compare with findings in a study on rainbow trout, which indicated that diploid spermatozoa from tetraploid fish may exhibit reduced fertility, as their enlarged heads have more difficulty in passing through the oocyte micropyle, which makes females more preferred in

tetraploids and diploids crosses (Chourrout *et al.*, 1986; Blanc *et al.*, 1993). The finding could be species-specific as another study by Nam & Kim (2004) found that the mud loach tolerated polyploidy better. In this species about 6% of tetraploid males permanently produced diploid sperm, while the rest released haploid or aneuploid sperm. By crossing normal diploid females with tetraploid males releasing diploid sperm, they achieved 100% triploids.

Flow cytometrically-verified sterile seed production (triploid) could facilitate the regulated production of African sharptooth catfish in prohibited areas in South Africa and other countries where its invasive nature was previously a barrier to aquaculture production. The information obtained from the current investigation contributes the intermediate steps towards the future production of triploid African sharptooth catfish, which could benefit farmers and the development of the catfish industry.

Chapter 5 General conclusions and recommendations

5.1 Conclusions

Artificial tetraploid induction is one of the important techniques of complete fish chromosome manipulation, and it is the intermediate step for achieving mass production of sterile-triploid fish by crossing tetraploids with diploids. The aim of this study was to verify the success of inducing tetraploidy by determining the optimal conditions for heat shock induction, specifically the initiation time of treatment after fertilisation, and to further identify the best method for determining ploidy status in heat shock-induced *Clarias gariepinus*.

The current study proved that good quality fertilised eggs are a pre-requisite to ensure the viable availability of heat-shocked tetraploid induced zygotes. Overall, the study demonstrated that the incubation temperature (25 °C ±0.1) and correctly timing the heat shock induction event (heat shock initiation time at 47 minutes post fertilization) resulted in a high percentage of tetraploidy and therefore, are critical for the quantitative maximisation of tetraploid offspring. Furthermore, the heat shock temperature (40.5 °C ±0.1) exposure for 2 minutes was effective in restricting the second mitotic cycle in tetraploid eggs. Hatching success of heat-shocked eggs was gradually reduced between 40 and 50 minutes ITPF (41-1%) compared to non-heat shocked eggs (>95%).

The flow cytometric method used in the current investigation, and specifically the slightly modified method using the Sysmex CyStain® PI Absolute P protocol, proved to be a rapid and efficient method for processing and determining of karyotype in 2DPH larvae. The chromosome spreads and Silver-NORs staining methods were inconsistent and unreliable to assess ploidy level in treatment samples. Nuclear sizing (ploidy identification), using erythrocytes may be a cheaper and effective method that can replace flow cytometry in situations where remote hatcheries do not have access to flow cytometry facilities. It will still be required to compare flow cytometry results with nuclear sizing assessments to validate the accuracy of the latter method.

5.2 Recommendations

It is recommended that fertilised African sharptoothcatfish eggs are incubated at 25°C ± 0.1, and zygotic tetraploidy induced at 47 min ITPF. Effective induction mediated within a period of two minutes at a consistent induction temperature of 40.5°C ± 0.1. This is because high percentage of tetraploidy was achieved when these parameters where utilised in the heat-shock induction protocol. Flow cytometry is recommended as an effective method for tetraploidy validation in 2 days post hatch heat shock-induced larvae of African sharptooth catfish. By using Method 3 of nuclear extraction, which required the use of roughly minced larvae samples, filtered by a 50 µm polyamide screen filter and stained with PI, nuclei were successfully isolated from African sharptooth catfish larvae. The method resulted in distinctly stained nuclei which were collected from a comparatively pure sample with less clumps and debris. This method was then used for the preparation of the subsequent samples. However, a fluorescence microscopic examination is recommended prior to analysis of samples on the flow cytometer to confirm proper preparation of samples with less debris and clumps. Variations in the percentage of tetraploids with time are

a common occurrence, possibly due to differences in mortality of tetraploids and diploids (Cassani *et al.*, 1990; Malison *et al.*, 1993; Nam & Kim, 2004). Evaluating the percentage of tetraploidy at different developmental stages towards grow out is recommended. The use of fin clips for ploidy determination is recommended since the method is non-destructive and reliable, added that the fin tissue completely repair within a month after sampling for African sharptooth catfish.

5.3 Further Research

The data obtained in this investigation fill the knowledge gap on the optimal conditions or parameters required for tetraploidy induction in *C. gariepinus*. Further studies are recommended to use the observed findings and protocol of the present study so as to narrow down the treatments to optimal ranges which maximise the yield of tetraploids. Due to time limited time resources, this study focused on larval tetraploidy induction and verification. Therefore, the next investigation needs to draw its focus to growing the fish to adults and to verify the ploidy status by sampling fin tissue to minimise injury-inflicted stress in test subjects. Also, successfully raised and identified tetraploid broodstock can be crossed with untreated diploids to produce triploid offspring. Another notable limiting factor was the environmental temperature, which affected our results during experimental trials. It was noted that the heat shock induction experiments were successful when conducted on a day with a mild climate, compared to an extremely hot or cold day. With mild weather (21 – 25 °C), the temperature on the apparatus (the incubation tank, warm baths and the system for keeping the fish) could be maintained at optimal levels; however, these fluctuated with very hot or cold temperatures, influencing results negatively. Further research should avoid this and must be conducted in a temperature controlled room.

Although flow cytometry is a preferred method as it is accurate, effective and fast, it can be quite costly. Therefore, future investigations need to explore cheaper alternative methods, such as nuclear sizing, that can be easy to use and access on farm sites. Results can be verified by testing with flow cytometry as cross reference. In this study, heat shock could effectively induce tetraploidy in African sharptooth catfish. However, it reduces the fertilisation and hatching rate as well as causing egg mortalities. Further research should demonstrate that low level survival of tetraploid females will potentially facilitate a proliferative output in future offspring requirements. These studies must also confirm that acceptable high numbers (hatchery level outputs) of F1 generated tetraploid breeders can be produced by crossing male and female tetraploids. More research is needed to expand understanding and knowledge on the effects of heat shock on tetraploids and to address other possible problems associated with tetraploidy in African sharptooth catfish. The risk however, does not outweigh the benefit of tetraploidy as the survival achieved in the current study is still sufficient to potentially produce high numbers of broodstock fish for future use by crossing tetraploid males with females in order to produce tetraploid offspring. This will proliferatively multiply future breeder availability. However, further studies must confirm survival of tetraploid larvae when grown to broodstock size.

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